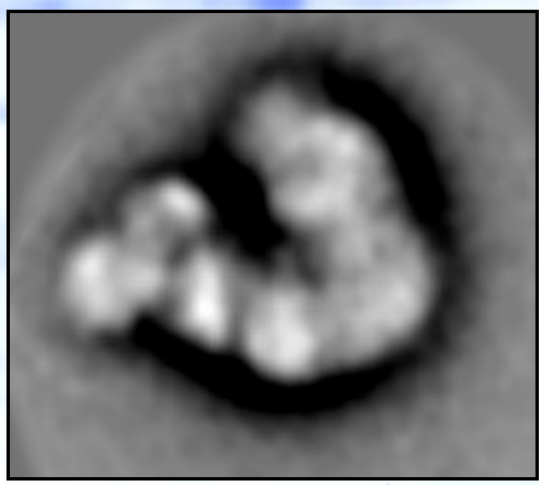
The Supramolecular Structure of the Respiratory Chain of Higher Plants



Vom Fachbereich Biologie der Universität Hannover
zur Erlangung eines Grades des
Doktors der Naturwissenschaften

Dr. rer. nat.

Genehmigte Dissertation
von

Dipl.-Biol. Holger Eubel

geboren am 7. April 1975 in Minden

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Zusammenfassung

Seit kurzem erlaubt eine neuartige experimentelle Strategie die weitergehende Erforschung der supramolekularen Struktur der Atmungskette von Säugern, Hefe und Bakterien. Durch vorsichtige Solubilisierung der mitochondrialen Proteinkomplexe mit dem milden Detergenz Digitonin, gefolgt von einer Auftrennung der Proteinkomplexe durch Blau-native (BN) Polyacrylamid Gelelektrophorese (PAGE), konnte die Existenz von supramolekularen Strukturen gezeigt werden. Die vorliegende Dissertation hat eine Anwendung dieser Strategie zur Untersuchung der supermolekularen Struktur der Atmungskette pflanzlicher Mitochondrien zum Gegenstand. Solubilisierung isolierter Mitochondrien aus verschiedenen höheren Pflanzen mittels Digitonin erlaubt die Visualisierung von zusätzlichen hochmolekularen Banden auf BN-Gelen. Durch die Verwendung von Natrium-Dodecylsulfat- (SDS) oder BN-PAGE als zweite Gel-Dimension konnte die Untereinheitenkomposition der Superkomplexe aufgeklärt werden. In nicht-grünen Geweben von Arabidopsis, Bohne und Gerste wurde ein dominanter Superkomplex gefunden, der aus monomerer NADH Dehydrogenase (Komplex I) und dimerer Cytochrom c Reduktase (Komplex III) besteht. „Substrate Channeling“ innerhalb dieses Superkomplexes könnte den Zugriff der alternativen Oxidase (AOX) auf ihr Substrat Ubiquinol einschränken und *in vivo* die Aktivität dieses Enzyms reduzieren. Elektronenmikroskopische Untersuchungen des Superkomplexes mit anschließender Einzelpartikel-Analyse zeigen eine hoch definierte Struktur. Die Pflanzen-spezifische Untereinheit At1g47260 scheint eine entscheidende Rolle bei der Stabilisierung des I_1III_2 Superkomplexes und des singulären Komplex I zu spielen. Eine Insertions-Mutante des korrespondierenden Gens zeichnet sich durch eine deutlich verringerte Abundanz beider Komplexe aus. Frisch geerntete Kartoffel-Knollen besitzen Superkomplexe, die neben dem I_1III_2-Superkomplex noch ein, zwei oder vier Kopien von Komplex IV zusätzlich besitzen. In ethiolierten Kartoffel-Sprossen finden sich außerdem noch Superkomplexe, die eine kleinere Form von Komplex IV enthalten. *In gel*-Aktivitätsfärbungen zeigen eine höhere Aktivität für die große Form des Komplexes IV. Umwandlung der einen Form in die andere könnte einen Regulationsmechanismus für die Aktivität des Superkomplexes darstellen. Im Vergleich zum Succinat Dehydrogenase Komplex aller bisher untersuchten Organismengruppen enthält dieser Proteinkomplex in Pflanzenmitochondrien mindestens vier Zusatzuntereinheiten. Diese Proteine integrieren wahrscheinlich Nebenaktivitäten in die pflanzliche Succinat Dehydrogenase. Solche Zusatzaktivitäten sind bereits für die Komplexe I und III bekannt und treffen möglicherweise auch für den Komplex IV pflanzlicher Mitochondrien zu.

Schlagwörter: Atmungskette, Superkomplexe, pflanzenspezifische Untereinheiten

Abstract

Recently, a novel experimental procedure for the investigation of the supramolecular structure of the respiratory chain of mammals, yeast and bacteria has been introduced. Gentle solubilization of respiratory protein complexes by the non-ionic detergent digitonin followed by Blue-native (BN) polyacrylamide gel electrophoresis (PAGE) revealed the presence of several supramolecular structures formed by components of the respiratory chain. This thesis is a systematic investigation of the organization of the respiratory chain in mitochondria of higher plants. Solubilization of isolated plant mitochondria with digitonin resulted in several additional bands in the high molecular range on BN gels. Second gel dimensions employing sodium dodecylmaltoside (SDS)-PAGE or BN-PAGE in the presence of n-Dodecylmaltoside (DDM) revealed the subunit compositions of these supercomplexes. In non-green tissue of Arabidopsis, bean and potato, the dominant supercomplex was found to be composed of a single copy of NADH dehydrogenase (complex I) and dimeric cytochrome c reductase (complex III). Substrate channelling within the I_1III_2 supercomplex might reduce access of the alternative oxidase (AOX) to its substrate ubiquinone and therefore limit *in vivo* activity of this oxido-reductase. Investigation of the I_1III_2 supercomplex by electron micrography followed by single particle analysis revealed a defined association of this structure. In Arabidopsis, the plant specific subunit At1g47420 was found to be essential for the stability of this supercomplex and singular complex I. *Knockout* of the corresponding gene leads to a highly decreased abundance of both complexes. In freshly harvested potato tubers, low abundant supercomplexes made of the I_1III_2 supercomplex were found comprising additionally one, two or four copies of complex IV. In etiolated potato stems, also supercomplexes containing a smaller form of complex IV were present. *In gel* activity staining of complex IV revealed a higher activity of the larger version of complex IV. Conversion of one form of complex IV into the other might present a mechanism to regulate electron flow through complex IV containing supercomplexes. Finally, the plant succinate dehydrogenase (complex II) was found to contain twice as many subunits as known for all other organisms investigated so far. These subunits are thought to exhibit side functions not related to electron transport. Such subunits are known to be present in the plant respiratory complexes I and III. Subunits with side activities are also predicted to be existent in complex IV.

Keywords: plant respiratory chain, supercomplexes, plant specific subunits

Contents

Abbreviations

1D	one dimensional
2D	two dimensional
AMP	Adenosin monophosphate
AOX	alternative oxidase
ATP	Adenosin triphosphate
BN	Blue native
CL	Cardiolipin
CN	Colourless native
CO_2	carbon dioxide
Complex I	NADH dehydrogenase
Complex II	succinate dehydrogenase
Complex III	cytochrome c reductase
Complex IV	cytochrome c oxidase
Complex V	mitochondrial ATP-Synthase
DDM	n-dodecylmaltoside
DNA	desoxy ribonucleic acid
ETC	electron transport chain
FAD	Flavin adenine dinucleotide (oxidiced form)
$FADH_2$	Flavin adenine dinucleotide (reduced form)
IMS	inter membrane space
MPP	mitochondrial processing peptidase
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxydized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
Nd_{ex}(NADH)	external NADH dehydrogenase
Nd_{ex}(NADPH)	external NADPH dehydrogenase
Nd_{in}(NADH)	internal NADH dehydrogenase
Nd_{in}(NADPH)	internal NADPH dehydrogenase
O_2	molecular oxygen
OXPHOS	oxidative phosphorylation
P_i	inorganic orthophosphate
PP_i	inorganic pyrophosphate
PAGE	polyacrylamide gel electrophoresis
PUMP	plant uncoupling mitochondrial protein
UQ	ubiquinone
Q_r	reduced ubiquinone
Q_t	total ubiquinone
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecylmaltoside
SHAM	salicylhydroxamic acid
SMP	submitochondrial particle
TIM	translocase of the inner mitochondrial membrae
TOM	translocase of the outer mitochondrial membrae

Introduction

Energy Metabolism in Plant Cells

According to the second law of thermodynamics, a closed system will allways move towards the state of the highest possible entropy. In many respects, a cell can be regarded as such a system. To keep the cell in a steady state of low entropy which is required for the proper execution of all its different functions, the cell needs a continous supply of energy. Apart from this sheer conservation of the status quo, several other processes like growth, movement, transport of ions and organic molecules or the execution of non-spontanous chemical reactions are also driven by energy supplied by catabolic reactions and/or photosynthesis.

The universal carrier and transmitter of energy in the cell is Adenosin triphosphate (ATP). Hydrolytic cleavage of one of the two phosphoanhydride bonds of the molecule is a reaction resulting in the liberation of energy, which can be used for the above mentioned purposes. The products of ATP hydrolysis are Adenosin monophosphate (AMP) and pyrophosphate (PP_i) or Adenosin diphosphate (ADP) and orthophosphate (P_i). AMP is converted into ADP by the transfer of a phosphoric acid residue from ATP, resulting in two molecules of ADP. ADP is then recycled to ATP by phosphorylation, a process which requires energy.

A major site of ATP production in plant cells is the photophosphorylation of ADP in chloroplasts. However, the ATP produced in this process is used up in plastids and is therefore not available to supply the need of the cell. Instead, trioses synthezised in chloroplasts can be transported into the cytoplasm where they can be converted into sucrose. In aerobic respiration, oxidation of sucrose to carbon dioxide and water provides energy to photosynthetic and non-photosynthetic tissues. Aerobic respiration can be divided into three steps. The first step is called glycolysis and takes place in the cytoplasm. Monosaccharides are oxydized to organic acids in a series of reactions in which electrons are transferred to NAD^+, leading to the formation of NADH. Additionally, also ATP is produced by substrate chain phosphorylation. The final products of glycolysis in plants are pyruvate and malate. Both can be imported into mitochondria by the pyruvate transporter and the dicarboxylate transporter, respectively. In the mitochondrial matrix, malate can be directly transformed into pyruvate by the action of the NAD malic enzyme or converted to oxalacetate by the malate dehydrogenase. During the citric acid cycle, the second step of respiration, pyruvate and

malate are oxidized to carbon dioxide and electrons are transferred to the co-enzymes NAD^+ and FAD^+. In contrast to the citric acid cycle of animals, also one molecule of ATP (instead of GTP) is generated by the plant succinyl-CoA synthetase in this process. Besides the production of ATP and reduced coenzymes, intermediates of the cytric acid cycle serve as precursors for the biosynthesis of amino acids, fatty acids, isoprenes, nucleic acids and porphyrins.

The majority of ATP is produced in the last step of respiration, the oxidative phosphorylation, which also takes place in mitochondria. The reduced coenzymes generated in the citric acid cycle are oxidized and electrons are transferred step by step to molecular oxygen, resulting in the formation of water. The free energy of this exergonic reaction is indirectly used to phosphorylate ADP.

Structure, Evolution and Properties of Mitochondria

Mitochondria are double-membrane bound spherical to elipsoid shaped organelles with a size of 1 to 3 µm. Their abundance varies from 1 to 1000 per cell. The two membranes, called inner and outer mitochondrial membrane (Douce 1985), enclose the intermembrane space (IMS). The outer mitochondrial membrane separates the IMS from the cytosol, the inner membrane is highly invaginated forming the so called “cristae” and separates IMS from the mitochondrial matrix. A connection between the cristae and the IMS is provided by narrow, sometimes long tubular structures (Mannella et al. 1997). This led to the assumption that a gradient of ions and molecules exists between these two spaces of the same compartment. Lipid and protein content as well as the permeability for solutes and organic molecules of the inner and outer membrane are different. Pores allow solutes and small molecules up to a size of approximately 10 kDa to pass the outer membrane. The inner membrane restricts the flow of molecules into and out of the organelle. Uncharged small molecules like O_2, CO_2 and several hydrophobic inhibitors of mitochondrial respiration are able to pass the lipid bilayer by diffusion. Ions and other charged, hydrophilic or big molecules have to be transported across the inner mitochondrial membrane by specialized channels or translocases. The lipid cardiolipin is responsible for the impermeability of the inner membrane.

According to the endosymbiont hypothesis, the progenitor of mitochondria was an aerobe prokaryote, taken up by the eukaryotic cell by endocytosis (Sagan 1967). This event is

assumed to have happened only once in the evolution of the eukaryotic cell. The closest today living relatives to this prokaryote are the α-proteobacteria (for review see Gray et al. 2001).
As most of the genetic information of the mitochondria has been transferred to the nucleus during evolution, the majority of the mitochondrial proteins are synthesized in the cytosol and imported into the organelle. In *A. thaliana*, only 57 of the predicted >2000 proteins (The Arabidopsis Genome Initiative 2000) are encoded by the organelle (Unseld et al. 1997). They are subunits of respiratory protein complexes and ribosomes and are also involved in heme and cytochrome assembly. Two specialized transport complexes located in the outer and inner membrane (translocase of the outer/inner mitochondrial membran, "TOM/TIM") and the matrix protein complex HSP70 are involved in the transport process (reviewed in Braun and Schmitz 1999).
Although the genetical system of mitochondria of all organisms derived from the same prokaryotic ancestor, many differences can be found between plants and mammals. The genome of plant mitochondria is several times bigger than that of mammals (Arabidopsis 367kb, mammals 16kb) and contains more genes and non-coding sequences. In addition, introns can be found in genes of plant mitochondria, which are absent in their mammalian counterparts. Several repeats in the plant mitochondrial genome enable it to recombine and to form rings coding only for a subset of the mitochondrial proteins (Unseld et al.1997). On the RNA level, trans-splicing and editing is common in mitochondria of plants (Covello and Gray 1989).

The Respiratory Chain of Plant Mitochondria and the Oxidative Phosphorylation

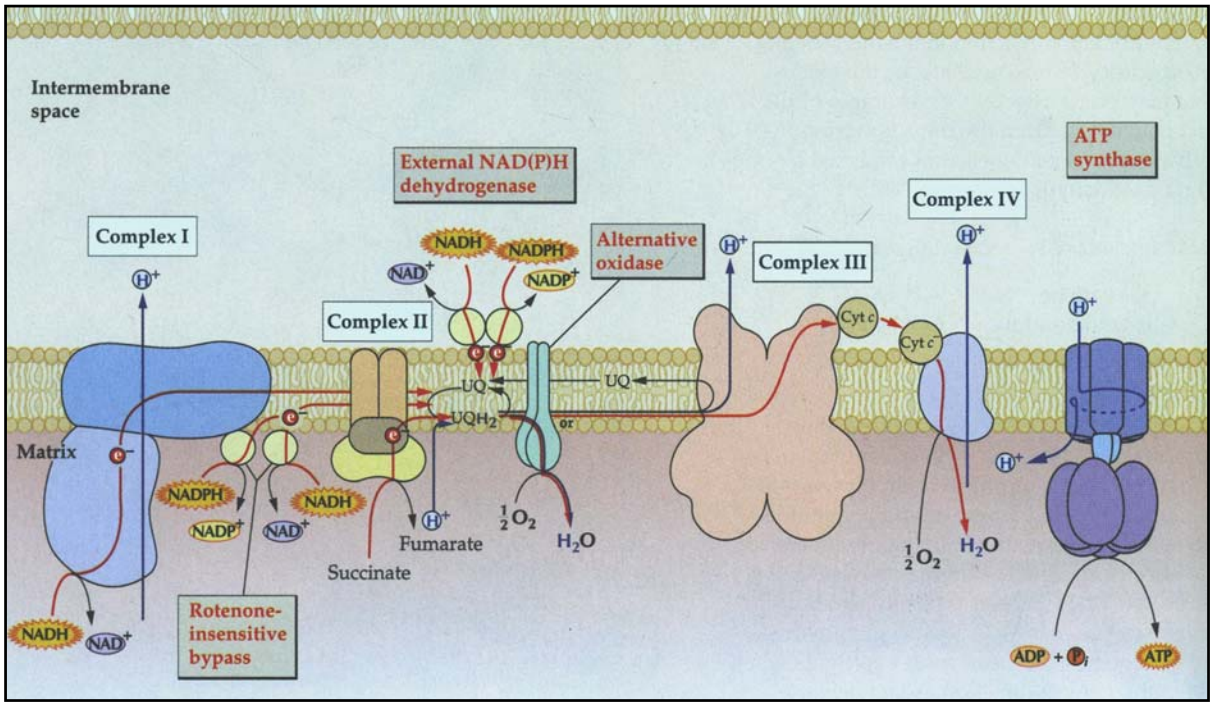
The inner mitochondrial membrane harbors the protein complexes of the oxidative phosphorylation (OXPHOS) system. Five integral protein complexes, four of them displaying oxidoreductive activity, are involved in this process: NADH-Dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III), cytochrome c oxidase (complex IV) and the mitochondrial F_0F_1 ATP synthase, often referred to as complex V (Hatefi 1985). Complex II has a dual function by catalyzing the transformation of succinate to fumarate in the citric acid cycle as well as being a bifurcation of the respiratory chain.
Complexes I to IV form the respiratory chain. Electron transport within the respiratory chain is performed by two carriers: the lipid ubiquinone and the hydrophylic 12.5kDa protein cytochrome c, which is located in the IMS. At complex I, NADH produced in the citric acid

cycle is oxidized and the electrons are transferred to ubiquinone which in turn is oxidized at complex III. Electrons of $FADH_2$ generated by the succinate dehydrogenase (complex II) are also transferred to ubiquinone. At complex III, cytochrome c takes over the electrons and passes them to complex IV via cytochrome c. Molecular oxygen is the final electron acceptor. The redox reactions at the complexes I, III and IV are exergonic and serve to create a proton gradient across the inner mitochondrial membrane. This gradient has an electrochemical nature. The return flow of protons into the matrix is only possible via the ATP synthase and the uncoupling protein (UCP). The plant uncoupling mitochondial protein (PUMP, Vercesi et al. 1995) is suggested to play a role either in thermogenesis during cold stress (Voinikov et al. 2001) or in translocation of certain fatty acids across the inner mitochondrial membrane (Jezek et al. 1997). However, the vast majority of H^+ reflux into the mitochondrial matrix occurs via the ATP synthase complex which is using the energy of the proton gradient for ADP-phosphorylation. The picture of the OXPHOS system as drawn above is true for the mitochondria of nearly all organisms with the exception of the yeast *Saccharomyces cerevisae* which lacks complex I but instead possesses three singular non-proton pumping NADH dehydrogenases associated with the inner mitochondrial membrane (De Vries et al. 1992, Luttnik et al. 1998, Small and McAllister-Henn 1998).

The respiratory chain of plant mitochondria, however, differs from that in many respects. Plant mitochondria possess several alternative oxidoreductases which do not contribute to proton translocation across the inner membrane but allow an electron flow from NADH respectively NADPH to oxygen. These alternative oxidoreductases do not take part in energy conservation by not contributing to the formation of the proton gradient. Complex I is bypassed by alternative NADH dehydrogenases and the cytochrome c-pathway (complexes III and IV) by the alternative oxidase.

For complex I at least four different alternative rotenone-insensitive NAD(P)H dehydrogenases are known (Moller 2001). They are located in the inner mitochondrial membrane and are capable of oxidizing either cytosolic or mitochondrial NADH and NADPH. According to their location on the IMS-side of the inner membrane they are called NDex(NADH) respectively NDex(NADPH). Using mitochondrial generated NADH and NADPH as substrates, the other two rotenone insensitive NAD(P)H dehydrogenases are located on the matrix-side of the inner membrane. Following the designation of their external counterparts, these enzymes are named NDin(NADH) and NDin(NADPH).

The alternative oxidase is insensitive to inhibitors of complex III like antimycin, stigmalletin and myxothiazol or inhibitors of the regular cytochrome c oxidase like cyanide, carbon monoxide or azide but sensitive to salicylhydroxamic acid (SHAM) and *n*-propyl gallate (Siedow 1982). It is also found in some fungi. Together with NDin(NADH) it might protect the organelle from the production of reactive oxygen species (ROS) during stress leading to rather high amounts of reduced components of the electron transport chain (ETC).



The liquid state model of the respiratory chain of plants including the alternative oxidoreductases. Taken from Buchanan BB, Gruissem W, and Russel LJ (2002) Biochemistry and Molecular Biology of Plants. Courier Companies, Inc, USA, page 688.

Another special feature of the respiratory chain of plant mitochondria is the presence of subunits in respiratory complexes which are not involved in electron transfer or proton translocation but fulfill completely different functions. One example of such subunits are the core proteins of complex III. These subunits show mitochondrial processing protease (MPP) activity by cleaving off presequences of nuclear encoded proteins after their import into the organelle (Braun et al. 1992). A second example of such a subunit is the final enzyme of the ascorbate synthesis pathway, the L-galactono-1,4-lacton dehydrogenase, which was found to be part of complex I (Millar et al. 2003). For the complexes II and IV so far no subunits with side activities have been described.

The Liquid and the Solid State Model of the Respiratory Chain

At first the structure of the respiratory chain was described by a solid state model. Hatefi et al. reported stoichiometric associations of the protein complexes of the respiratory chain. Preparation and reconstitution of an active supercomplex of the complexes I and III of beef heart mitochondria and reconstitution of several other supercomplexes containing the complexes I, II and III; complexes I, III and IV; complexes II, III and IV and complexes I, II, III and IV (Fowler and Hatefi 1961, Hatefi et al. 1961, Hatefi et al. 1962) supported this model.

Later, inhibiton of the activity of the I-III supercomplex by antimycin A was found to be re-established nearly completely by the addition of free complex III; a result which questioned the necessity of an association of the two complexes for electron transfer (Fowler and Richardson 1962).

The solid state model of the respiratory chain was again challenged by experiments diluting the inner mitochondrial membrane of rat liver cells with exogenous phospholipids and measuring the electron transfer rate from NADH and succinate to cytochrome c and to oxygen (Schneider et al. 1980). In respect to electron transfer, a decrease in the activity of the complexes I, II and III by this dilution indicated the structural independence and diffusion dependance of this complexes. It was also found that the decreased electron transport rate can be recovered by the addition of ubiquinone (Schneider et al. 1982). Among others, these findings led to the establishment of the liquid state model of the respiratory chain, which became widely accepted. According to this model, the electron carriers and the individual protein complexes of the respiratory chain are randomly distributed within the mitochondrial membrane and free to diffuse laterally. As electron transfer between the carriers and protein complexes only occurs by random contacts, this model is also referred to as the random collision model (reviewed in Hackenbrock et al.1986).

In a different approach, associations of cytochrome c oxidase with the bc1 complex (complex III) were shown for *P. denitrificans* and the Gram positive thermophilic bacterium PS3 (Berry and Trumpower 1985, Sone et al. 1987). In these experiments, purified cytochrome c oxidase by means of gentle solubilization with a non-ionic detergent succeeded by differential centrifugation or immunoprecipitation, also displayed ubiquinol oxydizing activities. In denaturating SDS-PAGE of this so called ubiquinol oxidase unit, subunits of the complexes III and IV were identified.

Investigation of the pool behaviour of ubiquinone and cytochrome of yeast using inhibitor titration showed no pool behaviour of these carriers at all, indicating the presence of a functional unit of electron transfer in yeast mitochondria (Boumans et al. 1998). At the same time, the discovery of a dimeric F_0F_1 ATP synthase in yeast (Arnold et al. 1998) by gentle solubilization with Triton X100 and subsequent Blue-native (BN) polyacrylamide gel electrophoresis (PAGE) was the starting point for further research on the structure of the OXPHOS system employing this technique.

The solid state model recently experienced a renaissance by demonstrating the presence of defined supercomplexes in the respiratory chain of yeast and mammals on the basis of gentle solubilization using the mild detergent digitonin and two dimensional BN-PAGE (reviewed in Schägger 2002). In contrast to the methods applied before in supercomplex research, this solubilization method in combination with the native gel system employing Coomassie dye to attach negative charges on proteins allowed for the first time to directly separate native supercomplexes electrophoretically and investigate their structure by comparison of subunit composition.

In yeast, associations of the complexes III and IV were found (Schägger and Pfeiffer 2000). A yeast knock-out mutant of the Cardiolipin (CL) synthase gene was found to contain nearly only dimeric complex III and IV whereas the wild type clearly displayed the presence of a III_2IV_2 supercomplex. Introduction of an exogenous copy of the CL synthase gene under exogenous regulation restored the supercomplex formation to a certain level (Zhang et al. 2002). A role of Cardiolipin in the stabilization of the III_2IV_{1-2} supercomplex of *S. cerevisae* was confirmed later by a slightly different approach (Pfeiffer et al. 2003) but in contrast to the results reported by Zhang et al. the formation of the supercomplex was not found to be impaired as considerable amounts were visible after CN-PAGE and gel filtration.

In bovine mitochondria, associations of the complexes I, III and IV of different composition were observed: I_1III_2, $I_1III_2IV_{1-4}$, IV_2, I_1IV_1, III_2IV_1 and III_2IV_2 (Schägger and Pfeiffer 2000). Most of complex I has been found to be associated with complex III whereas only a small portion of complex IV was found to be associated with the complexes I or III_2. For complex II, no participation in any comparable supramolecular structure has been reported. Those supercomplexes containing the complexes I, III and IV were suggested to be called “respirasomes”, refering to their ability to autonomously transfer electrons from NADH to oxygen in the presence of ubiquinol and cytochrome c (Schägger and Pfeiffer 2000).

For *P. denitrificans,* supercomplexes of $I_1III_4IV_4$, III_4IV_4 and III_4IV_2 composition were found with the two smaller ones being probably dissociation products of the respirasome (Schägger 2002).

Further evidence supporting the idea of supercomplexes in the respiratory chain was added by investigation of a human cytochrome b mutant. Surprisingly, not only the assembly of complex III was found to be impaired, these mutants also lacked complex I. Metabolic inhibition of complex III resulted in a normal assembly of complex one. These results indicate a dependance of complex I assembly upon the presence of complex III (Acin-Perez et al. 2004) and at least a tight temporary association of these complexes *in vivo*. Functional analysis of bovine submitochondrial particles (SMPs) by flux control measurements seem to confirm a complex I – complex III association but exclude a perpetual participation of complex IV in the supercomplex (Genova et al. 2003).

Until now, the organization of the respiratory chain of plant mitochondria has not been investigated and the question of existence and possible composition of respiratory supercomplexes therefore remains unanswered. Considering the outstanding complexity of the respiratory chain in plant mitochondria as known so far, one might also expect to find plant specific features in this respect.

Chapters 2, 3 and 4 of this thesis will show the presence and describe the composition of respiratory supercomplexes in mitochondria from higher plants, chapter 5 deals with the composition of the respiratory complexes II and IV. Chapter 6 provides evidence for the importance of a plant specific subunit for the stability of complex I and the I_1III_2 supercomplex.

BN-PAGE

The key method for the investigations on the plant respiratory chain presented in this thesis is Blue native (BN) PAGE in combination with gentle solubilization of protein complexes and supercomplexes by digitonin (Schägger 2001). Since stabilization of large protein complexes is achieved by omitting harsh detergents/salts from the sample preparation, it proves to be an ideal tool to investigate the structure and composition of protein complexes and supercomplexes. Compared to the anionic sodium dodecylmaltoside (SDS) commonly used for denaturating sample preparation, in BN-PAGE solubilization of the proteins and the attachment of negative charges to them are two separate steps. First, solubilization of the

proteins is carried out by mild, non-ionic detergents like Triton X100, dodecylmaltoside or digitonin. The dye Coomassie blue is then used to attach negative charges on the proteins. To provide a good resolution capacity over a broad size range, acrylamide gels used for BN-PAGE are favourably gradient gels with the gradient adapted to suit the requirements of the sample. Second dimensions employing SDS-PAGE allow to compare the subunit composition of putative supercomplexes with singular complexes thereby revealing which protein complexes are building the supercomplexes. A second BN dimension in the presence of *n*-dodecyl maltoside (DDM), which destabilizes supercomplexes but not the singular respiratory complexes, directly displays the protein complex composition of supercomplexes.

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New Insights into the Respiratory Chain of Plant Mitochondria. Supercomplexes and a Unique Composition of Complex II[1]

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A project to systematically investigate respiratory supercomplexes in plant mitochondria was initiated. Mitochondrial fractions from Arabidopsis, potato (*Solanum tuberosum*), bean (*Phaseolus vulgaris*), and barley (*Hordeum vulgare*) were carefully treated with various concentrations of the nonionic detergents dodecylmaltoside, Triton X-100, or digitonin, and proteins were subsequently separated by (a) Blue-native polyacrylamide gel electrophoresis (PAGE), (b) two-dimensional Blue-native/sodium dodecyl sulfate-PAGE, and (c) two-dimensional Blue-native/Blue-native PAGE. Three high molecular mass complexes of 1,100, 1,500, and 3,000 kD are visible on one-dimensional Blue native gels, which were identified by separations on second gel dimensions and protein analyses by mass spectrometry. The 1,100-kD complex represents dimeric ATP synthase and is only stable under very low concentrations of detergents. In contrast, the 1,500-kD complex is stable at medium and even high concentrations of detergents and includes the complexes I and III_2. Depending on the investigated organism, 50% to 90% of complex I forms part of this supercomplex if solubilized with digitonin. The 3,000-kD complex, which also includes the complexes I and III, is of low abundance and most likely has a III_4I_2 structure. The complexes IV, II, and the alternative oxidase were not part of supercomplexes under all conditions applied. Digitonin proved to be the ideal detergent for supercomplex stabilization and also allows optimal visualization of the complexes II and IV on Blue-native gels. Complex II unexpectedly was found to be composed of seven subunits, and complex IV is present in two different forms on the Blue-native gels, the larger of which comprises additional subunits including a 32-kD protein resembling COX VIb from other organisms. We speculate that supercomplex formation between the complexes I and III limits access of alternative oxidase to its substrate ubiquinol and possibly regulates alternative respiration. The data of this investigation are available at http://www.gartenbau.uni-hannover.de/genetik/braun/AMPP.

Structural basis for oxidative phosphorylation in mitochondria are five protein complexes termed NADH dehydrogenase (complex I), succinat dehydrogenase (complex II), cytochrome c reductase (complex III, which is a functional dimer), cytochrome c oxidase (complex IV), and ATP synthase (complex V). They were first characterized about 40 years ago by solubilizations of mitochondrial membrane proteins using detergents and differential precipitations or chromatographic separations. According to the popular "liquid state" model, the protein complexes of the respiratory chain are randomly arranged in the membrane and freely diffuse in lateral direction within the inner mitochondrial membrane (for review, see Rich, 1984). However, other results rather indicate an ordered association of these protein complexes forming larger structures. These so-called "supercomplexes" were first described for bacteria (Berry and Trumpower, 1985; Sone et al., 1987; Iwasaki et al., 1995; Niebisch and Bott, 2003). Later the existence of respiratory supercomplexes was also reported for yeast and mammalian mitochondria (Schägger and Pfeiffer, 2000).

In Brewer's yeast (*Saccharomyces cerevisiae*), which does not comprise complex I, three large mitochondrial complexes were identified by Blue-native gel electrophoresis after gentle protein solubilization using nonionic detergents: (a) dimeric ATP synthase, (b) a supercomplex containing dimeric complex III + one copy of complex IV, and (c) a supercomplex containing dimeric complex III + two copies of complex IV (Arnold et al., 1998; 1999; Cruciat et al., 2000; Schägger and Pfeiffer, 2000; Schägger, 2001a, 2002; Zhang et al., 2002). Dimeric ATP synthase from yeast includes three dimer-specific subunits, two of which are directly involved in dimer formation. Supercomplexes containing complexes III and IV were not only prepared by Blue-native gel electrophoresis but also by gel filtrations and co-immunoprecipitations (Cruciat et al., 2000). Their formation depends on the cardiolipin content of the inner mitochondrial membrane and also is influenced by growth conditions. Functional implications of complex III-complex IV associations were shown by ubiquinol-oxidase activity measurements in the presence of mild detergents (Schägger and Pfeiffer, 2000).

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In mammalian mitochondria, five large complexes were found: (a) dimeric ATP synthase, (b) a supercomplex containing dimeric complex III + one copy of complex I, and (c–e) supercomplexes containing dimeric complex III + one copy of complex I + one to three copies of complex IV (Schägger and Pfeiffer, 2000, 2001; Schägger, 2001a, 2002). All of these supercomplexes can be visualized on Blue-native gels after solubilization of mitochondrial proteins using digitonin. Solubilization using Triton X-100 additionally allow detection of a supercomplex consisting of dimeric complex III + monomeric complex I + four copies of complex IV. A high percentage of complex I forms part of supercomplexes, whereas dimeric complex III and monomeric complex IV also exist in singular form, because abundance of these protein complexes is significantly higher in comparison with complex I. NADH-cytochrome c activity measurements in dependence of various mild detergents have revealed functional importance of supercomplex formation between complexes III_2 + I. The term "respirasome" was suggested for supercomplexes containing the complexes I, III_2, and IV, which autonomously can carry out respiration in the presence of cytochrome c and ubiquinone (Schägger and Pfeiffer, 2000).

The supramolecular structure of the respiratory chain of plant mitochondria is unknown. The five protein complexes of oxidative phosphorylation are well characterized and structurally resemble their counterparts in fungi and mammals (Jänsch et al., 1996; Vedel et al., 1999; Heazlewood et al., 2003b; Sabar et al., 2003). Some plant-specific subunits of respiratory chain complexes were described, e.g. the subunits of the mitochondrial processing peptidase, which form an integral part of complex III in plant mitochondria (Braun et al., 1992a; Eriksson et al., 1994). Additionally, the electron transfer chain of plant mitochondria is very much branched due to the presence of several alternative oxidoreductases like a cyanide-insensitve terminal oxidase and rotenone-insensitive NAD(P)H dehydrogenases (for review, see Siedow and Umbach, 1995; Vanlerberghe and McIntosh, 1997; Mackenzie and McIntosh, 1999; Rasmusson et al., 1999).

Here, we describe a systematic investigation of supercomplexes in plant mitochondria. Using gentle protein solubilizations with nonionic detergents and Blue-native gel electrophoresis, three supercomplexes could be visualized: (a) dimeric ATP synthase, (b) a supercomplex formed by dimeric complex III and complex I, and (c) a supercomplex containing two copies of dimeric complex III and two copies of complex I. The complexes II and IV as well as the alternative oxidase (AOX) do not form part of supercomplexes under all conditions applied. Furthermore, a larger and a smaller form of cytochrome c oxidase were found, which differ by at least two protein subunits, and a complex II is described, which has a very unusual subunit composition.

RESULTS

Identification of Respiratory Supercomplexes in Mitochondria from Arabidopsis

Blue-native gel electrophoresis was previously employed for the characterization of the respiratory chain of plant mitochondria (Jänsch et al., 1995, 1996; Brumme et al., 1998; Kügler et al., 1998; Karpowa and Newton, 1999; Ducos et al., 2001; Kruft et al., 2001; Mihr et al., 2001; Rasmusson and Agius, 2001; Werhahn and Braun, 2002; Bykova and Moller, 2003; Heazlewood et al., 2003a, 2003b, 2003c; Sabar et al., 2003). Nevertheless, respiratory supercomplexes were not described, most likely because very similar conditions for protein solubilization were chosen, which seem to have destabilizing effects on labile protein-protein interactions. In an attempt to systematically search for the occurrence of respiratory supercomplexes in plants, mitochondria from Arabidopsis were solubilized using varying concentrations of the nonionic detergents dodecylmaltoside, Triton X-100, and digitonin and analyzed by Blue-native PAGE (Fig. 1, A–C). Protein complexes were identified by their known subunit compositions upon analyses on second gel dimensions and by partial sequence analysis of selected proteins using mass spectrometry (Figs. 1, D and E, and 2; Table I).

Solubilization of Arabidopsis mitochondria with 1 g dodecylmaltoside g^{-1} protein allows resolution of known singular complexes of the oxidative phosphorylation system (Fig. 1, A and D): complex I (1,000 kD), F_0F_1 ATP synthase (580 kD), complex III (480 kD), which always is dimeric for functional reasons, and the F_1 part of ATP synthase (390 kD). Furthermore, the soluble HSP60 (750 kD) and formate dehydrogenase complexes (200 kD) are visible on the gel. Additionally, some low amount of dimeric ATP synthase can be seen at about 1,100 kD, which was overlooked on the Blue-native gels shown before by Kruft et al. (2001). In contrast to yeast and mammals, the amount of dimeric ATP synthase does not increase if mitochondrial proteins are solubilized with lower dodecylmaltoside concentrations (Fig. 1A). Usage of dodecylmaltoside to protein ratios >1 g per g allows visualization of a supercomplex of about 1,500 kD, which is composed of the complexes I and III and probably has the structure III_2I. However, only a small proportion of total complex I forms part of this supercomplex and an even smaller proportion of complex III, which is more abundant than complex I.

Solubilization of Arabidopsis mitochondria with Triton X-100 allows visualization of the same protein complexes and supercomplexes on Blue-native gels (Fig. 1, B and E). The amount of dimeric ATP synthase is highest between 0.25 and 0.5 g Triton X-100 g^{-1} protein, which is in line with observations reported for yeast (Arnold et al., 1998). The ratio of dimeric to monomeric ATP synthase is about 1 upon solubilization using 0.25 g Triton g^{-1} protein but

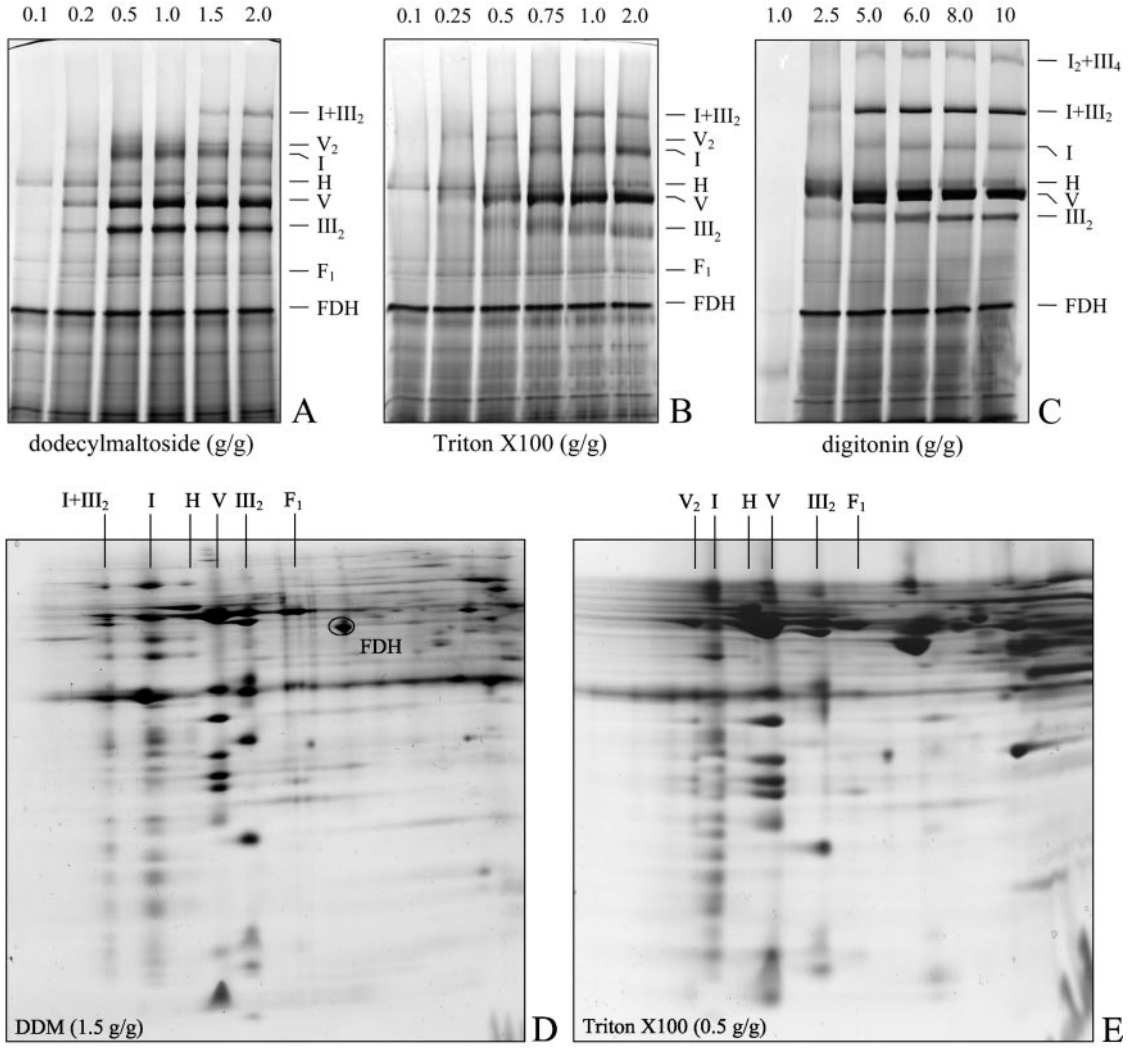

Figure 1. Resolution of mitochondrial protein complexes and supercomplexes by Blue-native PAGE. A through C, Solubilization of mitochondrial protein complexes from Arabidopsis using different detergents. Isolated mitochondria were treated with varying concentrations of dodecylmaltoside (A), Triton X-100 (B), or digitonin (C), and protein complexes were subsequently resolved by one-dimensional Blue-native PAGE. Detergent to protein ratios are given above the gels (in grams of detergent per gram protein), and the identity of protein complexes is given to the right of the gels. D and E, Two-dimensional resolution of mitochondrial protein complexes from Arabidopsis by Blue-native/SDS PAGE after solubilization with dodecylmaltoside (1.5 g per g protein) (D) and Triton X-100 (0.5 g per g protein) (E). Designations of the protein complexes are given above the gels. FDH, Formate dehydrogenase; F_1, F_1-part of the ATP synthase complex; III_2, dimeric cytochrome c reductase; V, ATP synthase; H, HSP60 complex; I, NADH dehydrogenase; $I+III_2$, supercomplex formed by complex I and dimeric complex III; IVa and VIb, large and small form of cytochrome c oxidase; V_2, dimeric ATP synthase.

decreases sharply upon solubilizations using higher amounts of detergent (Fig. 1B). About 50% of complex I forms part of the III_2I supercomplex on Blue-native gels after protein solubilizations using 0.5 to 1.0 g per g Triton X-100 per g protein (Fig. 1B).

In general, higher detergent to protein ratios are necessary for protein solubilizations using digitonin, which is in accordance with results found for yeast and mammals. However, starting with a digitonin to protein ratio of 2.5 g per g, this detergent proved to be very suitable for supercomplex stabilization (Figs. 1C and 2). Under these conditions, about 80% of complex I forms part of the III_2I supercomplex. Furthermore, a supercomplex of about 3,000 kD can be seen on Blue-native gels (Fig. 1C), which also is composed of subunits of the complexes III and I as found by two-dimensional Blue-native/SDS gel electrophoresis and silver staining (data not shown). This supercomplex most likely has a III_4I_2 structure, because the ratio of single complex I and complex III subunits is unchanged if compared with their ratio in the 1,500-kD III_2I complex. Dimeric ATP synthase only is visible at very low digitonin to protein ratios (data not shown). The F_1 part of the ATP synthase complex is not detectable on the Blue-native gels, indicating a stabilizing effect of the detergent on

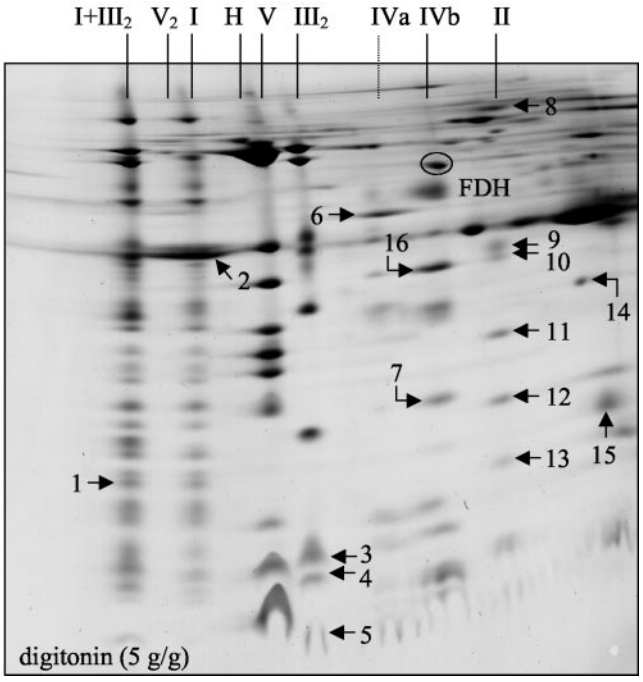

Figure 2. Two-dimensional resolution of digitonin-solubilized mitochondrial protein complexes and supercomplexes by Blue-native/SDS-PAGE. Identities of protein complexes are given above the gel (for designations, see Fig. 1). Arrows indicate proteins identified by mass spectrometry (Table I).

complex V (Fig. 1C). Furthermore, in contrast to dodecylmaltoside and Triton X-100, digitonin allows solubilization of three novel protein complexes of about 150, 220, and 300 kD. These protein complexes do not form visible bands on one-dimensional Blue-native gels, probably because the background on the gels is too high in this molecular mass range. However, these multisubunit complexes nicely are resolved on corresponding two-dimensional gels (Fig. 2). The subunit compositions of the 220- and 300-kD complexes very much resemble the one of cytochrome c oxidase from potato (*Solanum tuberosum*; Jänsch et al., 1996). In contrast, identity of the 150-kD complex was unclear on the basis of subunit composition.

Protein identifications by mass spectrometry allowed unambiguous identification of subunits of complexes I, III, and IV (Fig. 2; Table I). Furthermore the 1,000-kD prohibitin complex was identified, as was the preprotein translocase of the outer mitochondrial membrane, the so-called TOM complex, at 390 kD (Werhahn et al., 2003).

Dissection of Supercomplexes into Protein Complexes by Two-Dimensional Blue-Native/Blue-Native Gel Electrophoresis

A novel two-dimensional Blue-native/Blue native gel electrophoresis method (Schägger and Pfeiffer, 2000) was employed to investigate whether the complexes I and III are the only components of the 1,500- and 3,000-kD supercomplexes. For this procedure, protein complexes and supercomplexes are separated by a first-dimension Blue-native-PAGE in the presence of digitonin. Afterward, protein supercomplexes are destabilized by incubation with dodecylmaltoside, which is followed by a second-dimension Blue-native-PAGE. On the resulting two-dimensional gels, supercomplexes are separated vertically into protein complexes, whereas singular protein complexes are located on a diagonal line. Two-dimensional Blue-native/Blue-native PAGE clearly revealed that the complexes I and III are the only constituents of the 1,500- and 3,000-kD supercomplexes in Arabidopsis (Fig. 3).

Characterization of Mitochondrial Supercomplexes in Potato, Bean (*Phaseolus vulgaris*), and Barley (*Hordeum vulgare*)

To investigate whether occurrence of the III_2I and III_4I_2 supercomplexes and dimeric ATP synthase is a special characteristic of Arabidopsis or a general feature of plant mitochondria, the above described experiments were repeated with isolated organelles from potato, bean, and barley. All protein solubilizations were done with digitonin (5 g per g protein), which proved to be optimal for visualizations of mitochondrial protein complexes and supercomplexes in Arabidopsis on Blue-native gels. The III_2I supercomplex is also present in potato, bean, and barley (Fig. 4). About 50% of complex I forms part of this supercomplex in bean and potato, whereas even 90% of complex I from barley is associated with dimeric complex III. Under the conditions applied, dimeric ATP synthase of all three plants only represents a very minor fraction of total ATP synthase complex. Furthermore, the large and the small form of cytochrome c oxidase are present in all plants investigated. However, the ratio of large to small forms varies considerably: In Arabidopsis and barley, the smaller form is very abundant, whereas in potato, the larger form is present in higher quantities, and in bean, both forms of complex IV are of equal abundance. The newly discovered 150-kD complex is also present in potato and bean but could not be clearly detected in barley under the conditions applied.

The Larger Form of Cytochrome c Oxidase Contains Additional Protein Subunits

Although Blue-native gel electrophoresis is not a suitable procedure for precise molecular mass determinations, the larger 300-kD form of the cytochrome c oxidase complex (IVa) probably cannot be considered to be a dimer of the 220-kD complex (IVb). Careful evaluation of the Blue-native gels in the region of the two forms of cytochrome c oxidase from Arabidopsis and bean revealed the presence of addi-

Table I. *Identified subunits of mitochondrial protein complexes of Arabidopsis and bean*

The numbers of the spots correspond to those given in Figures 2 and 5. Proteins were identified by electrospray tandem mass spectrometry (ESI), matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI), or Edman degradation (ED). Amino acid sequences given in italics represent N-terminal protein sequences determined by Edman degradation. Calculated molecular masses are given for all proteins with known N-terminal sequence, otherwise molecular masses are estimated on the basis of migration during SDS gel electrophoresis (see Figs. 2 and 5). Protein accessions correspond to the code of the Arabidopsis Genome Initiative.

Spot	Identified Peptides	Identification Strategy	Protein	Molecular Mass	Arabidopsis Genome Initiative Accession No.
				kD	
Arabidopsis					
1	WDPQISQVAGR RDPYDDLLEDNYTPPSSSSSSSD	ESI	Subunit of complex I	~10	At1g67350
2	SPNVAYLPGGQSMLFALNR	ESI	Prohibitin	30.4	At5g40770
3	YEDISVLGQRPVEE	ESI	8.0-kD subunit, complex III	8.0	At3g52730
4	AVVYALSPFQQK	ESI	8.2-kD subunit, complex III	8.2	At3g10860 At5g05370
5	TFIDPPPTEEK	ESI	6.7-kD subunit, complex III	6.7	At2g40765
6	IPTAHYEFGANYYDPK IDSNGVASALLEER	ESI	TOM40	34.1	At3g20000
7	RLDDIDFPEGPFGTK	ESI	COX subunit Vb	~16	At3g15640
8	–	MALDI	Flavoprotein subunit, complex II	~65	
9	DLVVDMTNFYNQYK WNPDNPGKPELQDYKIDLK	ESI	Iron sulphur subunit, complex II	~29	At5g40650
10	DLVVDMTNFYNQYK	ESI	Iron sulphur subunit, complex II	~29	At5g40650 At3g27380
11	AAEAVEEFGGILTSIK YAEYLDSFEPEEVYLK *SEDVSHMPEMDSXVLNAFK...*	ESI MALDI ED	Hyp. prot. (complex II)	18.4	At1g47420
12a	FMEWWER LDTMAAQVK	MALDI ESI	Hyp. prot. (complex II)	~15	At1g08480
12b	QGPNLNGLFGR	ESI	Cytochrome c	12.4	At4g10040 At1g22840
13	*STISGDIKTTQEEP*	ED	Subunit 3 of complex II	12	At5g09600
14	LVVDTTANQDPLVTK_ YASEVYEKESN_	ESI	Superoxide dismutase	22.5	At3g10920
15	QYIQEPATVEK LASIPGRYETFWK	ESI	g subunit, complex V	~12	At4g29480
16	LNQISILVQR	ESI	COX subunit II	29.4	y08501
Bean					
17	YLEYHR GDDAPE TPATPEE LETAPVDFR EATSEEAVVEK	ESI	Subunit VIb of cytochrome c oxidase	~32	Highly similar to At1g22450

tional subunits in the larger form, which might explain the size difference between the two forms of this complex (Fig. 5). Data are especially clear for bean, because both forms of complex IV are equally abundant. A 32-kD protein and at least one very small subunit of <6 kD are unique to complex IVa. Proteins of comparable size are also present in Arabidopsis (Fig. 5) but are difficult to detect in potato and barley under the conditions applied. According to our interpretation of the Blue-native gels, the subunit composition of cytochrome c oxidase is as follows (see scheme in Fig. 5): Complex IVa includes 12 separable proteins, and complex IVb is composed of 10 proteins. Furthermore, complex IVb of Arabidopsis can be further subdivided into two complexes of very similar molecular masses that differ with respect to the presence of a 10-kD subunit.

The 32-kD subunit of complex IVa is homologous to the 10-kD COX VIb protein of heterotrophic eukaryotes (see Table I, protein 17), which is known to be easily detached from cytochrome c oxidase in yeast and mammals and which was shown to have regulatory functions on cytochrome c oxidase activity (LaMarche et al., 1992; Weishaupt and Kadenbach, 1992).

Complex II from Plant Mitochondria Contains Seven Subunits

The newly discovered 150-kD complex of Arabidopsis comprises seven subunits of 65, 28, 18, 15, 12, 8, and 6 kD. In bean and potato, this complex has a very comparable subunit composition, except that the molecular masses of the three smallest subunits

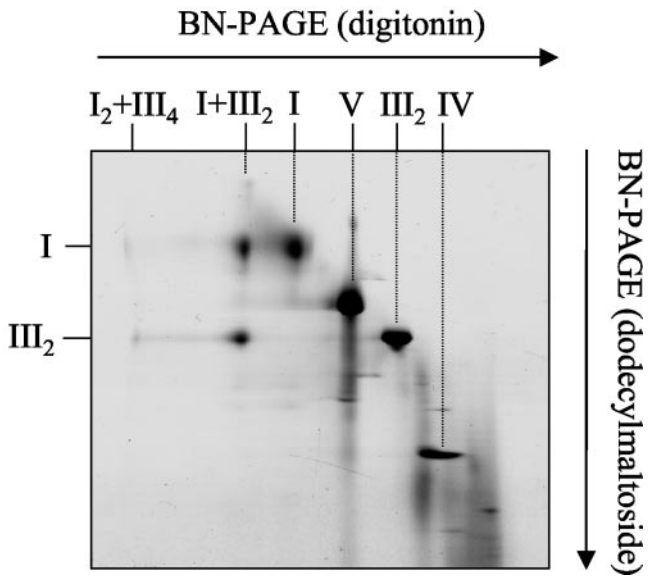

Figure 3. Separation of supercomplexes into protein complexes by two-dimensional Blue-native/Blue-native gel electrophoresis. Identities of protein complexes are given above and to the left of the gel (for designations, see Fig. 1).

slightly vary (Fig. 4). To identify the 150-kD complex, subunits were subjected to analysis by mass spectrometry. Peptide sequences of five of the seven subunits allowed identification of corresponding genes of the Arabidopsis genome (Fig. 6; Table I). Surprisingly the 65-, 28-, and 12-kD proteins could be identified as being subunits of the succinate dehydrogenase complex (complex II) of the respiratory chain: the flavoprotein subunit (SDH1), the iron-sulfur subunit (SDH2), and the so-called subunit III (SDH3). Succinate dehydrogenase is well characterized for several bacteria, fungi, and mammals and is known to be a four-subunit complex comprising the above mentioned subunits and one additional subunit in the size range of 10 to 15 kD termed subunit IV or SDH4 (Lemire and Oyedotun, 2002; Yankovskaya et al., 2003). Although the subunits of this protein complex from plants were never biochemically characterized, counterparts of the SDH1-SDH4 proteins could be identified on the basis of sequence similarities of predicted proteins with known complex II subunits from mammals, fungi, algae, and protozoans (Daignan-Fornier et al., 1994; Burger et al., 1996; Figueroa et al., 2001; Figueroa et al., 2002). However, sequence identities for the SDH3 and SDH4 proteins, which constitute a hydrophobic membrane anchor of this protein complex, are very low (Burger et al., 1996). Most interestingly, complex II from plants seems to contain additional subunits of unknown function. The 18-kD protein corresponds to the putative At1g47420 protein of Arabidopsis, which was identified previously in the course of a proteomic approach to characterize novel mitochondrial proteins of this organisms (Kruft et al., 2001; protein No 4). Furthermore, the 15-kD protein represents the putative At1g08480 protein. However, presence of this protein within complex II is slightly uncertain, because one of the three identified peptides perfectly matches to Arabidopsis cytochrome c. Hence the corresponding protein spot contains more than one protein. The 8- and 6-kD subunits could not be identified by mass spectrometry; one of these subunits might represent the SDH4 protein.

Most likely all seven proteins of plant succinate dehydrogenase are single-copy subunits of the 150-kD complex, because the sum of their apparent molecular masses (153 kD) is very close to the apparent molecular mass of this protein complex on Blue-native gels (150 kD). Furthermore, probably no additional subunits form part of this complex.

The AOX Does Not Form Part of Respiratory Supercomplexes

AOX represents a characteristic oxidoreductase of the respiratory chain of plant mitochondria. Its possible association with the complexes I to V after digitonin-solubilization of mitochondrial protein fractions was investigated by two-dimensional Blue-native/SDS gel electrophoresis and immunoblotting using a monoclonal antibody directed against AOX from *Sauromatum guttatum* (Elthon et al. 1989). On the two-dimensional gels, the antibody reacts with a protein of 32 kD in bean and Arabidopsis, which forms a smear on the first gel dimension between 30 and 300 kD (Fig. 7). Therefore, AOX seems not to form part of any supercomplexes but rather aggregates under the conditions applied. Also the rotenone-insensitive NAD(P)H dehydrogenases are not attached to respiratory protein complexes or supercomplexes as investigated by similar experiments using antibodies directed against these proteins (data not shown).

DISCUSSION

Supercomplexes in Plant Mitochondria

Respiratory protein complexes form supercomplexes in plant mitochondria. In the course of our investigations, V_2, III_2I, and III_4I_2 supercomplexes could be identified. Possibly further supercomplexes exist in vivo that are instable in the presence of detergents and Coomassie Blue. Digitonin proved to be the optimal compound for supercomplex solubilization, which is surprising because it specifically binds sterols, which are believed to be absent in inner mitochondrial membranes. Furthermore, digitonin also allows stable extraction of singular protein complexes that cannot be solubilized by dodecylmaltoside or Triton X-100. It therefore is a very suitable tool for plant mitochondrial research.

Between 50% and 90% of complex I from plants forms part of the III_2I supercomplex on Blue-native gels upon digitonin solubilizations. In contrast, the

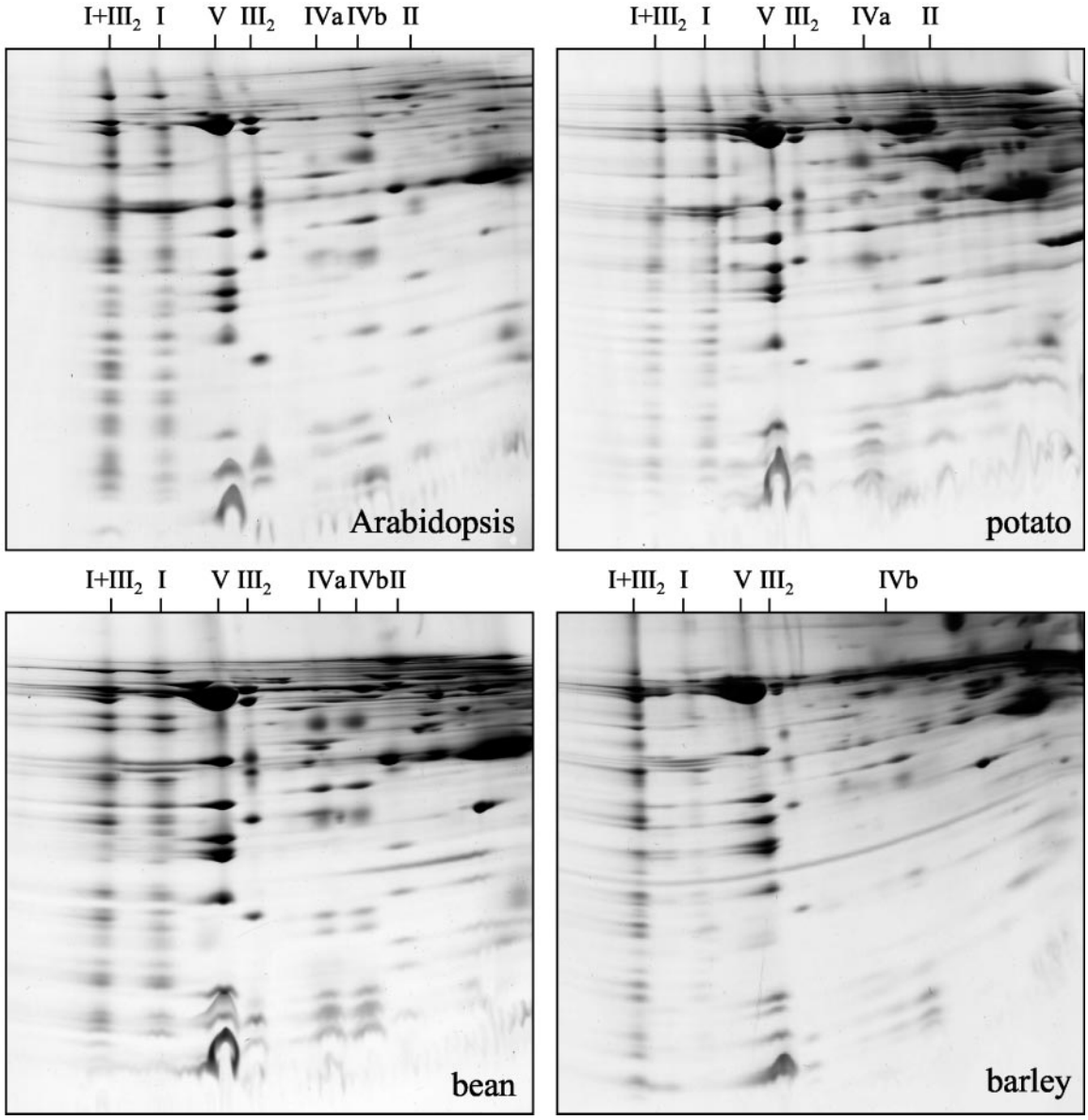

Figure 4. Mitochondrial supercomplexes and protein complexes in Arabidopsis, potato, bean, and barley. Proteins were solubilized by 5 g per g digitonin and separated by two-dimensional Blue-native/SDS PAGE. Identities of protein complexes are given above the gels (for designations, see Fig. 1).

same supercomplex from beef only contains 17% of complex I under identical conditions (Schägger, 2002). However, more than 50% of complex I from beef is part of even larger supercomplexes that include dimeric complex III and additionally one to four copies of complex IV. In Brewer's yeast, complex I-containing supercomplexes are absent due to the general absence of this protein complex, but also in this organism, the complexes IV and III are associated forming III_2IV or III_2IV_2 supercomplexes (Schägger and Pfeiffer, 2000; Cruciat et al., 2000). In contrast, associations of complexes III and IV of plant mitochondria are not detectable on Blue-native gels under all conditions applied. Also the AOX and the rotenone-insensitive NAD(P)H dehydrogenases seem not to be present in supercomplexes. Furthermore, plant complex II only is present in singular form, which is in line with findings for yeast and mammals. Dimeric complex V from plants is present on Blue-native gels after solubilizations using low concentrations of detergents, but its abundance is lower than in other organisms (Arnold et al., 1998, 1999). Recently, a very stable dimeric ATP synthase complex was reported for mitochondria from *Chlamydomonas reinhardtii* (van Lis et al., 2003).

What is the functional role of supercomplexes in plant cells? In yeast, supercomplexes were reported to enhance activity rates of respiratory electron transport (Schägger and Pfeiffer, 2000). Furthermore, it is speculated that supercomplex formation increases

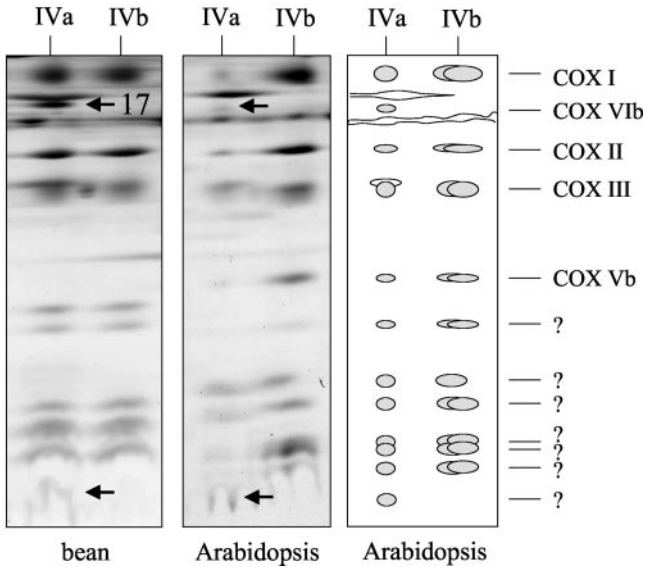

Figure 5. Large and small forms of cytochrome c oxidase in bean and Arabidopsis after two-dimensional resolution by Blue-native/SDS PAGE. Proteins only forming part of the larger form of this protein complex are indicated by arrows. Protein number 17 was identified by mass spectrometry (Table I). A scheme of the two-dimensional Blue-native/SDS gel of cytochrome c oxidase from Arabidopsis is given to the right.

the capacity of the inner mitochondrial membrane for protein insertion (Arnold et al., 1998). The protein content of this mitochondrial membrane, which is estimated to lie at about 70%, can only be realized if proteins are very efficiently packed. In plant mitochondria, the III_2I supercomplex possibly has important consequences for the regulation of alternative respiration, because it might reduce access of AOX to its substrate ubiquinol. Because alternative respiration is known to increase under various stress conditions (Vanlerberghe and McIntosh, 1997), the occurrence of respiratory supercomplexes in Arabidopsis was investigated in mitochondria isolated from suspension cell cultures that were treated with antimycin A, a known inhibitor of complex III. However, our initial data reveal only small differences concerning respiratory supercomplexes in antimycin-treated and untreated cells, which are at the borderline of significance (data not shown). Therefore the role of supercomplexes in plant mitochondria has to be further investigated.

Respiratory Protein Complexes in Plant Mitochondria

Recently, the subunit compositions of protein complexes of the oxidative phosphorylation system of Arabidopsis were studied intensively. Complex I from plants can be resolved into 27 to 30 different subunits on two-dimensional Blue-native / SDS gels (Fig. 8) but possibly comprises more than 40 proteins (Rasmusson et al., 1998). Heazlewood et al. (2003a) identified 30 subunits of Arabidopsis complex I after separation on two-dimensional gels by mass spectrometry. Several of the identified proteins have counterparts in fungi and mammals, but others seem to be unique to plants. Using a similar approach, Heazlewood et al. (2003c) identified 10 subunits of Arabidopsis complex V. Some further subunits remain to be characterized, because up to 13 proteins can be resolved on two-dimensional gels (Fig. 8). All 10 subunits of potato complex III were biochemically characterized (for review, see Braun and Schmitz, 1995) and counterparts for all 10 subunits are present in Arabidopsis protein databases at The Institute for Genomic Research or the Munich Information Center for Protein Sequences (β-MPP subunit, At3g02090; α-MPP subunit, At1g51980 and At3g16480; cytochrome b, Y08501; cytochrome c_1, At5g40810 and At3g27240; 'Rieske FeS' protein, At5g13440 and At5g13430; counterpart to 14-kD subunit from potato, At4g32470 and At5g25450; counterpart to 7.8-kD subunit from potato, At2g01090 and At1g15120; counterpart to potato 8.0-kD subunit, At5g05370 and At3g10860; counterpart to potato 8.2-kD subunit, At3g52730; counterpart to potato 6.7-kD subunit, At2g40765).

The least characterized respiratory protein complexes of plants are the complexes IV and II. Arabidopsis complex IV can be resolved in two different forms on Blue-native gels, which comprise 10 to 12 subunits (Figs. 5 and 8). The identity of the five largest subunits is known, whereas the identity of most smaller subunits remains to be established. The larger form of cytochrome c oxidase includes an additional 32-kD protein, which resembles the 10-kD subunit COX VIb of yeast and beef. This subunit is very hydrophilic, lacks membrane spanning helices, and is localized on the intermembrane-space side of cytochrome c oxidase (Tomizaki et al., 1999). Removal of this protein from complex IV was shown to activate cytochrome c oxidase of beef (Weishaupt and Kadenbach, 1992). Furthermore, COX VIb from beef was shown to be important for dimerization of

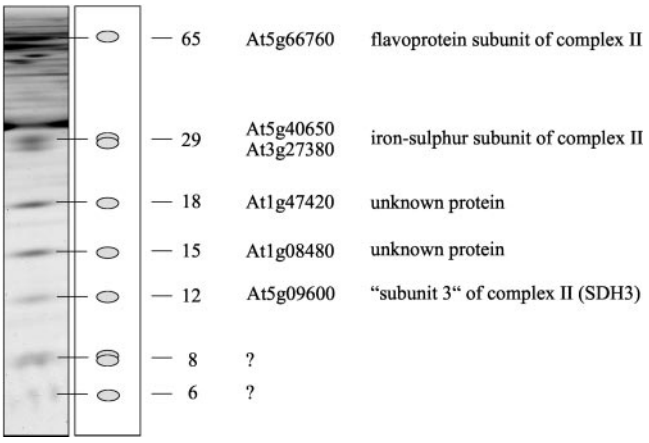

Figure 6. Subunit composition of complex II in Arabidopsis. Apparent molecular masses of the subunits and the accession numbers of the corresponding genes are given to the right of the gel.

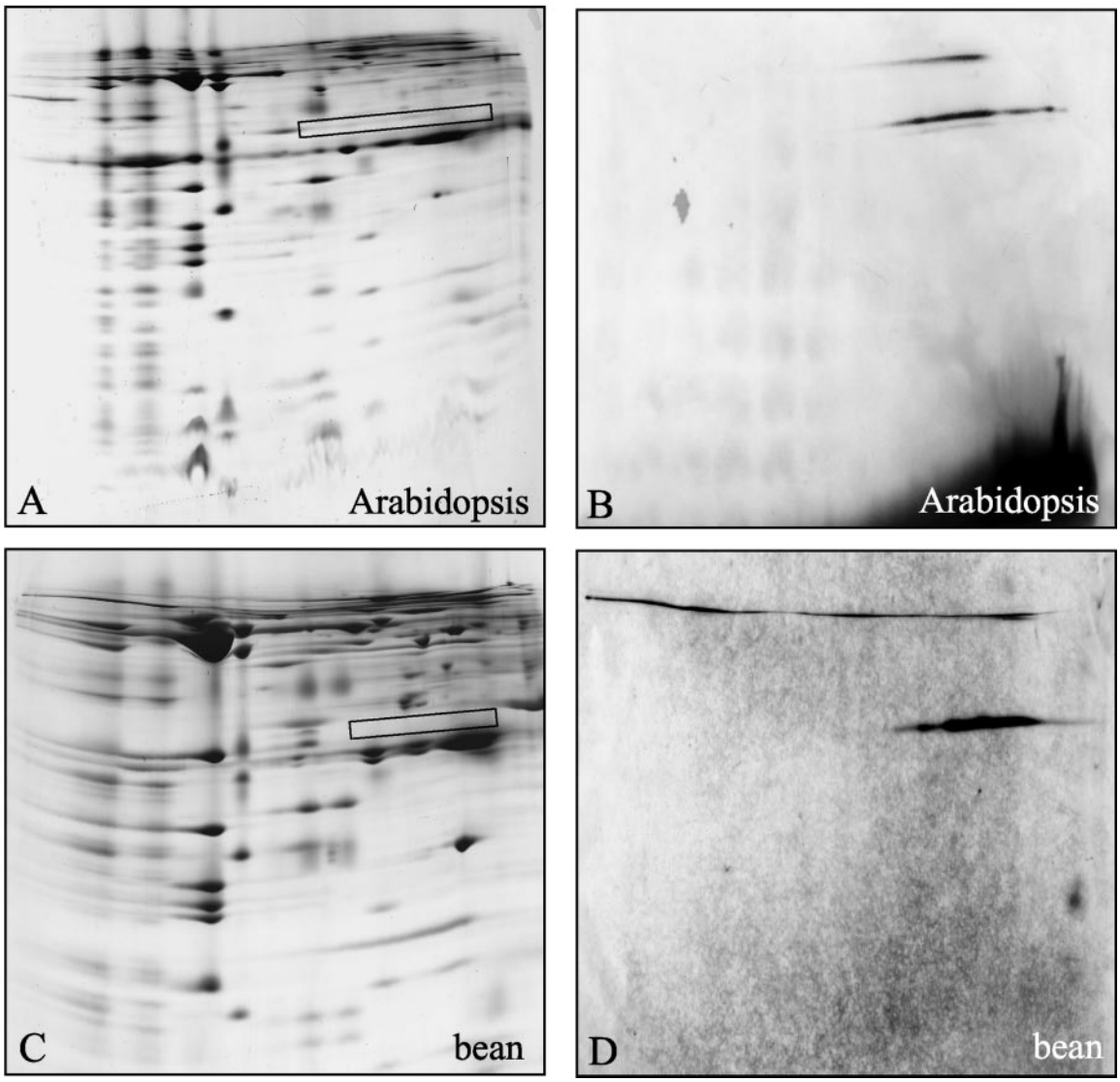

Figure 7. The AOX does not form part of mitochondrial supercomplexes. Mitochondrial proteins from Arabidopsis (A and B) and bean (C and D) were solubilized by 5% (w/v) digitonin and separated by two-dimensional Blue-native/SDS PAGE. Afterward, gels were either directly stained with Coomassie Blue (A and C) or electroblotted onto nitrocellulose membranes and immunostained with an antibody directed against AOX (B and D). The boxes on the Coomassie Blue gels indicate the position of the main immunosignals.

cytochrome c oxidase (Tomizaki et al., 1999; Lee et al., 2001). Genes encoding COX VIb from plants were characterized previously in Arabidopsis and rice (*Oryza sativa*; Ohtsu et al., 1999, 2001). Interestingly, two forms of COX VIb proteins are encoded by these genes, which have molecular masses of 10 or 20 kD. Both forms of this protein very much resemble the 10-kD COX VIb from yeast and beef, but the 20-kD form has a long N-terminal extension. Curiously, both predicted forms of the plant COX VIb protein are much smaller than the 32-kD COX VIb found for Arabidopsis and bean on Blue-native gels. Also in rice, a 32-kD COX VIb protein was recently identified in the course of a mitochondrial proteome project (Heazlewood et al., 2003b). Consequently, the 32-kD COX VIb protein either is made by posttranslational modifications of smaller COX VIb proteins or is encoded by additional cox VIb genes, which so far were not discovered. Also, the functional role of this protein in plant mitochondria remains to be established. Possibly, it is important for dimerization of complex IV like in heterotrophic eukaryotes. However, dimeric cytochrome c oxidase was not detectable on our Blue-native gels under all conditions applied. Further investigations on cytochrome c oxidase of plant mitochondria are under way in our laboratory.

Surprisingly, complex II from Arabidopsis turned out to comprise seven subunits, which is three subunits more than the well-characterized succinat dehydrogenases from fungi, mammals, algae, protozoa,

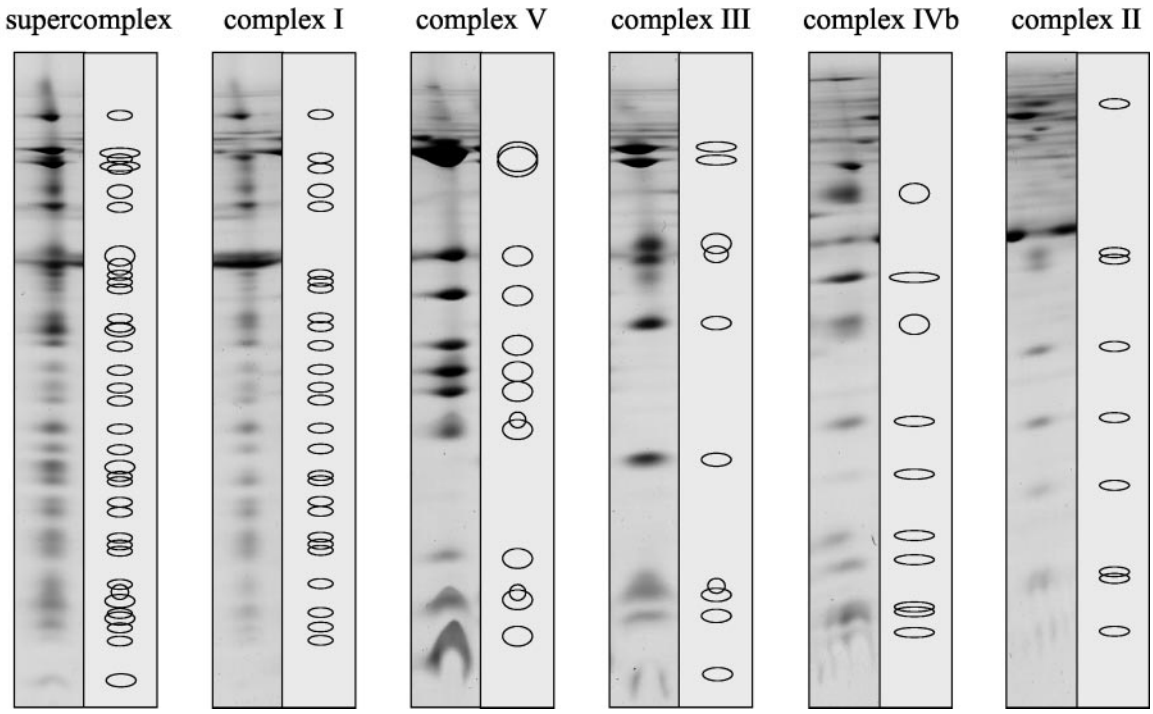

Figure 8. Subunit composition of respiratory protein complexes from Arabidopsis on two-dimensional Blue-native/SDS gels. Proteins were solubilized with digitonin. Sixty-seven different subunits of complexes I through V are separated: 27 subunits of complex I, seven of complex II, 10 of complex III, 10 of complex IV, and 13 subunits of complex V.

and several bacteria. Theoretically, these additional subunits could form a different protein complex, which accidentally runs at an identical position on Blue-native gels as a four-subunit complex II. However, this possibility is highly unlikely, because all seven proteins form an ideal line on Blue-native gels in three different plants: potato, bean, and Arabidopsis. Furthermore, the intensities of the protein spots on the two-dimensional gels is very much in the same range. Finally, the sum of the apparent molecular masses of the seven subunits corresponds to the apparent molecular mass of the protein complex on our native gels. As a general rule, respiratory protein complexes in mitochondria include several additional subunits if compared with their counterparts in prokaryotes. So far, complex II was the only exception, which seems not to be valid for plant mitochondria.

One of the newly described subunits of complex II from plants represents the hydrophilic At1g47420 protein, which was identified previously in the course of an Arabidopsis mitochondrial proteome project and which was reported to be one of the most abundant proteins on two-dimensional isoelectric focusing/SDS gels of mitochondrial fractions (Kruft et al., 2001). On the basis of sequence comparisons, the role of this protein is unclear. Highly similar proteins exist in barley (gi 18652408) and rice (CAD40922). Interestingly, the N-terminal presequence of 89 amino acids has some sequence identity with the N-terminal domain of the putative transcription factor APF1 of Arabidopsis (gi13507025). Also, the newly identified 15-kD subunit of complex II from Arabidopsis (At1g08480) does not exhibit significant sequence identity to previously characterized proteins. For the first time, to our knowledge, the plant SDH3 subunit was biochemically characterized. It comprises an exceptionally long mitochondrial targeting sequence of 105 amino acids as revealed by comparison of its N-terminal sequence and the amino acid sequence deduced from the corresponding gene. In summary, complex II from plants has unique features, which should be characterized by further biochemical and physiological investigations.

Further Protein Complexes in Plant Mitochondria

Besides the V_2, III_2I, and III_4I_2 supercomplexes and the respiratory complexes I to V, several additional protein complexes are visible on our Blue-native gels: the prohibitin complex at 1,000 kD, the HSP60 complex at 750 kD, the TOM complex at 390 kD, and a complex containing formate dehydrogenase at 200 kD (Fig. 2). Further plant mitochondrial protein complexes resolvable on Blue-native gels were not identified in the course of our study but were reported in earlier investigations, e.g. a Glu dehydrogenase complex (Heazlewood et al. 2003b). In contrast, some other protein complexes are known to be present in plant mitochondria but never were detected on Blue-native gels possibly due to their instability in the presence of detergents or Coomassie Blue, e.g. pyru-

vate dehydrogenase or the so-called AAA complexes. Recently, the occurrence of protein complexes comprising mitochondrial dehydrogenases of the citric acid cycle was reported on the basis of diffusion rate measurements of individual enzymes of this metabolic pathway (Haggie and Verkman, 2002). Most likely, these protein complexes are too unstable for biochemical preparations. Protein complexes and supercomplexes offer several physiological advantages in comparison with singular proteins, including substrate channeling, metabolic pathway regulation, and the realization of complicated biochemical reactions with reactive intermediates. Therefore the majority of mitochondrial proteins probably form part of protein complexes, and possibly most protein complexes are involved in the formation of even larger supermolecular structures, which remain to be discovered.

MATERIALS AND METHODS

Isolation of Mitochondria from Arabidopsis, Bean (*Phaseolus vulgaris*), Potato (*Solanum tuberosum*), and Barley (*Hordeum vulgare*)

Starting material for plant mitochondrial preparations were non-green Arabidopsis suspension cell cultures, potato tubers, 6-d-old etiolated barley seedlings, and 18-d-old etiolated bean seedlings. Arabidopsis cell lines were cultivated in the dark at 24°C to 26°C, 30% humidity, and gentle shaking (90 rpm) as described previously (Werhahn et al., 2001), and etiolated seedlings were grown at 24°C. All organelle preparations were carried out on the basis of filtration, differential centrifugation, and Percoll density centrifugation as outlined by Werhahn et al. (2001) for Arabidopsis; Focke et al. (2003) for barley; and Braun et al. (1992b) for potato and bean. Purified organelles were finally resuspended in a buffer containing 0.4 M mannitol, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM Tricine/KOH, pH 7.2, at a protein concentration of 10 mg mL^{-1}, divided into aliquots of 100 μL, and directly used for investigations (the amount of some supercomplexes was significantly reduced if mitochondrial fractions were frozen and stored before analyses).

Solubilization of Mitochondrial Proteins

Mitochondrial aliquots were centrifuged for 10 min at 14,300*g*, and sedimented organelles were resuspended in one of the following buffers (conditions adopted from Arnold et al., 1998; Schägger, 2001): (a) 100 μL of digitonin solubilization buffer (30 mM HEPES pH 7.4, 150 mM potassium acetate, 10% [v/v] glycerol, 2 mM PMSF, and [1–10 g per g protein] digitonin [Fluka, Buchs, Switzerland]); (b) 100 μL of dodecylmaltosid solubilization buffer (750 mM aminocaproic acid, 50 mM BisTris, pH 7.0, 0.5 mM EDTA, 1 mM PMSF, and docedylmaltoside [0.1–2 g per g protein; Roche, Mannheim, Germany]; and (c) 100 μL of Triton solubilization buffer (50 mM NaCl, 2 mM aminocaproic acid, 1 mM EDTA, 50 mM imidazole-HCl, pH 7.4, 10% glycerol, 5 mM PMSF, and Triton X-100 [0.1–2 g per g protein; Amersham-Pharmacia-Biotech Uppsala].

After incubation for 20 min on ice, samples were centrifuged at 18,000*g* for 30 min to remove insoluble material and were subsequently supplemented with 5 μL of Coomassie Blue solution (5% [w/v] Coomassie Blue in 750 mM aminocaproic acid). Dodecylmaltoside-solubilized samples were centrifuged immediately after resuspension of organelles in solubilization buffer and afterward were supplemented with 20 μL of Coomassie Blue solution. Coomassie Blue-treated protein samples were directly loaded onto Blue-native gels.

Two-Dimensional Blue-Native/SDS PAGE

One-dimensional Blue-native PAGE and two-dimensional Blue-native/SDS PAGE were carried out as described by Schägger (2001b). Gradient gels (4.5%–16% [w/v] acrylamide) were used for the Blue-native gel dimensions and two-step Tricine-SDS gels (10% and 16% [w/v] acrylamide) for second gel dimensions. The cathode buffer of Blue-native gel dimensions did not include detergent; only for electrophoresis of dodecylmaltoside-solubilized samples 0.03% of the detergent was added. Gels were either stained with Coomassie-colloidal (Neuhoff et al., 1985, 1990) or with silver (Heukeshoven and Dernick, 1986)

Two-Dimensional Blue-Native/Blue Native Gel Electrophoresis

Two-dimensional Blue-native/Blue-native PAGE was carried out as published by Schägger and Pfeiffer (2000). It proved to be important to stop first-dimension electrophoresis runs after 50% completion to avoid protein complexes and supercomplexes getting stuck in the gels. In contrast, it was important to extend the electrophoresis runs of second gel dimensions by factor two, because protein complexes stuck in gels were best resolved.

Protein Preparations for Mass Spectrometry

For mass spectrometry, gels were colloidal stained with Coomassie Blue (Neuhoff et al., 1990) and single proteins were cut out, transferred into an Eppendorf tube, and incubated with Milli-Q water for 10 min. Rebuffering was carried out by incubating the gel pieces for 15 min in acetonitrile and 0.1 M NH_4HCO_3, respectively. Subsequently, the proteins were dehydrated by acetonitrile and incubated with 20 μL of digestion solution (0.5 μg of trypsin [Promega, Madison, WI] in 20 μL of 50 mM NH_4HCO_3) overnight at 37°C. Peptide extraction was performed at 37°C as follows: Samples were supplemented with 20 μL of 50 mM NH_4HCO_3 and shaken for 15 min, and afterward, supernatants were taken and stored. Gel pieces were then shaken for 15 min in the presence of 20 μL of 5% (v/v) formic acid. Subsequently, the same volume of acetonitrile was added, and samples were shaken for another 15 min. Afterward, all supernatants were pooled and dried down to a volume of about 10 μL. Purification of the generated peptides was achieved using ZipTips (Millipore, Bedford, MA) according to the manufacturer's instructions.

Matrix-Assisted Laser Desorption Ionization/Time of Flight Mass Spectrometry

Determination of the molecular masses of Zip-Tip purified peptides was carried out by positive-ion matrix assisted laser desorption ionization/time of flight mass spectrometry using an Ultraflex instrument (Bruker, Newark, DE) equipped with delayed-extraction and a N_2 laser (337 nm). For each sample, 1 μL of matrix solution (10 mg of α-cyano-4-hydroxycinnamic acid in 1 mL of 60% [v/v] acetonitrile/0.1% [v/v] formic acid) was placed on the Scout ion source and crystallized as a thin layer. One microliter of sample was given directly on the top of the thin matrix layer, and cocrystallization was carried out at room temperature. Spectra were recorded in reflection mode with an acceleration voltage of 25 kV and a reflection voltage of 26.3 kV. Monoisotopic masses from spectra were selected automatically and were used for protein identification with the help of MASCOT (Matrix Science, London).

Electrospray Ionization Tandem Mass Spectrometry

For peptide sequencing, 3 μL of Zip-Tip purified sample was filled into Au/Pd-coated nanospray glass capillaries (Protana, Odense, Denmark). The tip of the capillary was placed orthogonally in front of the entrance hole of a quadropole time-of-flight mass spectrometry instrument (Q-TOF II, Micromass, Watres, Milford, MA) equipped with a nanospray ion source. A capillary voltage between 750 and 1,000 V and a cone voltage of 30 V was applied. Two-fold charged peptides were chosen for collision-induced dissociation experiments, and the corresponding parent ions were selectively transmitted from the quadropole mass analyzer into the collision cell. Argon was used as collision gas, and the kinetic energy was set between 20 and 40 eV. The resulting daughter ions were separated by an orthogonal time-of-flight mass analyzer. Peptide sequencing and protein identification were carried out with the programs PeptideSequencing of the BioLynx software

package (v3.5, Mircomass), Sonar of the Knexus software package (Proteometrics, Manitoba, Canada), and MASCOT (Matrix Science).

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Identification and Characterization of Respirasomes in Potato Mitochondria[1]

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Plant mitochondria were previously shown to comprise respiratory supercomplexes containing cytochrome c reductase (complex III) and NADH dehydrogenase (complex I) of I_1III_2 and I_2III_4 composition. Here we report the discovery of additional supercomplexes in potato (*Solanum tuberosum*) mitochondria, which are of lower abundance and include cytochrome c oxidase (complex IV). Highly active mitochondria were isolated from potato tubers and stems, solubilized by digitonin, and subsequently analyzed by Blue-native (BN) polyacrylamide gel electrophoresis (PAGE). Visualization of supercomplexes by in-gel activity stains for complex IV revealed five novel supercomplexes of 850, 1,200, 1,850, 2,200, and 3,000 kD in potato tuber mitochondria. These supercomplexes have III_2IV_1, III_2IV_2, $I_1III_2IV_1$, $I_1III_2IV_2$, and $I_1III_2IV_4$ compositions as shown by two-dimensional BN/sodium dodecyl sulfate (SDS)-PAGE and BN/BN-PAGE in combination with activity stains for cytochrome c oxidase. Potato stem mitochondria include similar supercomplexes, but complex IV is partially present in a smaller version that lacks the Cox6b protein and possibly other subunits. However, in mitochondria from potato tubers and stems, about 90% of complex IV was present in monomeric form. It was suggested that the $I_1III_2IV_4$ supercomplex represents a basic unit for respiration in mammalian mitochondria termed respirasome. Respirasomes also occur in potato mitochondria but were of low concentrations under all conditions applied. We speculate that respirasomes are more abundant under in vivo conditions.

Prerequisite for oxidative phosphorylation (OXPHOS) in mitochondria are five protein complexes termed NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III), cytochrome c oxidase (complex IV), and ATP synthase (complex V). These protein complexes can be separated by biochemical procedures and are well characterized for several organisms. However, there is mounting evidence that in vivo these protein complexes specifically interact forming supermolecular structures called supercomplexes: (1) purification protocols for individual OXPHOS complexes sometimes lead to the isolation of stoichiometric assemblies of two or more complexes which are functionally active (Hatefi et al., 1961; Hatefi and Rieske, 1967); (2) stable and enzymatically active supercomplexes can be reconstituted upon mixture of complexes I and III (Fowler and Hatefi, 1961; Fowler and Richardson, 1963; Hatefi, 1978; Ragan and Heron, 1978); (3) respiratory protein complexes from several bacteria were found to form specific supermolecular structures (Berry and Trumpower, 1985; Sone et al., 1987; Iwasaki et al., 1995; Niebisch and Bott, 2003); (4) inhibitor titration experiments reveal that the respiratory chain of yeast (*Saccharomyces cerevisiae*) behaves like a single functional unit (Boumans et al., 1998); and (5) flux control experiments indicate specific interactions of respiratory protein complexes (Genova et al., 2003). Several physiological roles were proposed for these respiratory supercomplexes, like substrate channeling, catalytic enhancement, protection of reactive reaction intermediates, and stabilization of individual protein complexes (Schägger and Pfeiffer, 2000; Genova et al., 2003).

Recently, characterization of mitochondrial supercomplexes was very much facilitated by the introduction of a novel experimental strategy which is based on protein solubilizations using mild nonionic detergents and separation of the solubilized protein complexes by Blue-native (BN) gel electrophoresis or gel chromatography (Arnold et al., 1998, 1999; Cruciat et al., 2000; Schägger and Pfeiffer, 2000; Zhang et al., 2002; Pfeiffer et al., 2003). Using this approach, several distinct supercomplexes could be described for mitochondria from different organisms (for review, see Schägger, 2001a, 2002).

In yeast, dimeric complex III (this protein complex always is dimeric for functional reasons) forms supercomplexes with one or two copies of complex IV. Furthermore, complex V was shown to partially occur in a dimeric state, which includes some dimer-specific subunits. In contrast, complex II from yeast does not form part of supermolecular structures under all experimental conditions applied. In beef, the complexes III_2 and I form a supercomplex. Additionally, this supercomplex can include one to four copies of complex IV. The resulting large structures are called respirasomes, because they can autonomously carry out respiration in the presence of cytochrome c and ubiquinone (Schägger and Pfeiffer, 2000). Like in yeast, ATP synthase partially forms dimers, and complex II does not form part of supercomplexes.

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Meanwhile, protein solubilizations using nonionic detergents and separations of solubilized protein complexes by BN-PAGE were used to systematically investigate the structure of the OXPHOS system of plants (Eubel et al., 2003). Three different supercomplexes were found in digitonin-solubilized mitochondrial fractions of Arabidopsis, potato (*Solanum tuberosum*), barley (*Hordeum vulgare*), and bean (*Phaseolus vulgaris*): (1) a 1,500-kD I_1III_2 supercomplex; (2) a 3,000-kD I_2III_4 supercomplex; and (3) a 1,100-kD dimeric ATP synthase complex. Depending on the plant investigated, the percentage of complex I integrated into the I_1III_2 supercomplex varies between 50% and 90%. The I_2III_4 supercomplex is of lower abundance and only becomes visible upon prolonged staining of BN gels. While the I_1III_2 and I_2III_4 supercomplexes are stable at high detergent to protein ratios, dimeric ATP synthase proved to be only stable at very low detergent concentrations. In contrast to yeast and mammals, cytochrome c oxidase (complex IV) of plant mitochondria did not form part of supercomplexes under all conditions applied. Instead, two different forms of monomeric complex IV are visible on BN gels, which are termed complex IVa and IVb (about 300 and 220 kD in Arabidopsis). Complex IVa includes at least one additional subunit, which is homologous to the Cox6b protein from mammals and yeast (Eubel et al., 2003).

Here we report a continuation of our efforts to carefully characterize the supermolecular structure of the OXPHOS system of plant mitochondria. Using highly active mitochondria isolated from freshly harvested potato tubers, five additional supercomplexes of about 850, 1,150, 1,850, 2,200, and 3,000 kD are visible on BN gels. All five protein complexes include complex IVa as shown by one-dimensional (1D) BN-PAGE, two-dimensional (2D) BN/SDS-PAGE, and 2D BN/BN-PAGE in combination with in-gel activity measurements for cytochrome c oxidase. The novel supercomplexes are of comparatively low abundance and have III_2IV_1, III_2IV_2, $I_1III_2IV_1$, $I_1III_2IV_2$, and $I_1III_2IV_4$ compositions. Slightly smaller versions of these protein complexes occur in potato stem mitochondria, which include complex IVb instead of complex IVa. Hence, the OXPHOS complexes of plant mitochondria partially form respirasomes, which most likely have important physiological and/or regulatory functions.

RESULTS

Identification of Novel Supercomplexes in Potato Mitochondrial Fractions

Previous investigations of digitonin-solubilized mitochondrial fractions from Arabidopsis, potato, bean, and barley by BN-PAGE led to the identification of I_1III_2 and I_2III_4 supercomplexes and dimeric ATP synthase (Eubel et al., 2003) but did not reveal hints on complex IV-containing supercomplexes which were described for yeast and mammalian mitochondria (Schägger and Pfeiffer, 2000). However, these findings were based on mitochondrial isolations from etiolated seedlings (bean and barley), aged storage organs (potato), and suspension cell cultures (Arabidopsis), and it so far cannot be ruled out that mitochondrial preparations from other tissues or organs might allow the discovery of further supercomplexes. In an attempt to re-examine our previous findings, freshly harvested potato tubers were used for mitochondrial isolations and subsequent characterizations of digitonin-solubilized protein extracts on 1D BN gels (Fig. 1A). In parallel, mitochondrial preparations from 20-d-old etiolated potato stems were analyzed by this procedure (Fig. 1B).

All molecular masses of protein complexes given in this publication represent apparent molecular masses as deduced from separations on BN gels. These values should be considered with caution, because protein separations on BN gels do not exactly reflect calculated molecular masses. Some values for apparent molecular masses in this publication were corrected in comparison to the values given in Eubel et al. (2003): 600 kD for complex V (previously 550 kD), 350 kD for complex IVa (previously 300 kD), and 270 kD for complex IVb (previously 220 kD).

As expected, all known protein complexes of the OXPHOS system are visible on our gels (Fig. 1): complex I (approximately 1,000 kD), complex V (approximately 600 kD), and dimeric complex III (approximately 500 kD). Complex IVa (approximately 350 kD), complex IVb (approximately 270 kD), and complex II result in diffuse bands on the 1D gels but were clearly identified upon resolution of their subunits on second gel dimensions, which were carried out in the presence of SDS (data not shown). Finally, the I_1III_2 and I_2III_4 supercomplexes are visible. However, the occurrence of the I_2III_4 supercomplex and complex IVb was restricted to potato stem mitochondria. Dimeric ATP synthase could not be detected in both fractions, most likely because digitonin concentrations were too high.

Besides the known mitochondrial protein complexes and supercomplexes, additional complexes of low abundance showed up on our gels at approximately 850 kD and above 1,500 kD in both mitochondrial fractions (Fig. 1). To test if these protein supercomplexes include complex IV, in-gel activity measurements for cytochrome c oxidase were carried out. Indeed, five novel bands of approximately 850, 1,200, 1,850, 2,200, and 3,000 kD specifically were labeled in the potato tuber mitochondrial fraction (Fig. 1A). The 850- and 1,850-kD bands also are present in potato stem mitochondria and additionally two bands at approximately 770 and approximately 1,770 kD (Fig. 1). Identities of the newly discovered protein complexes were analyzed by 2D gel electrophoresis systems and are given below.

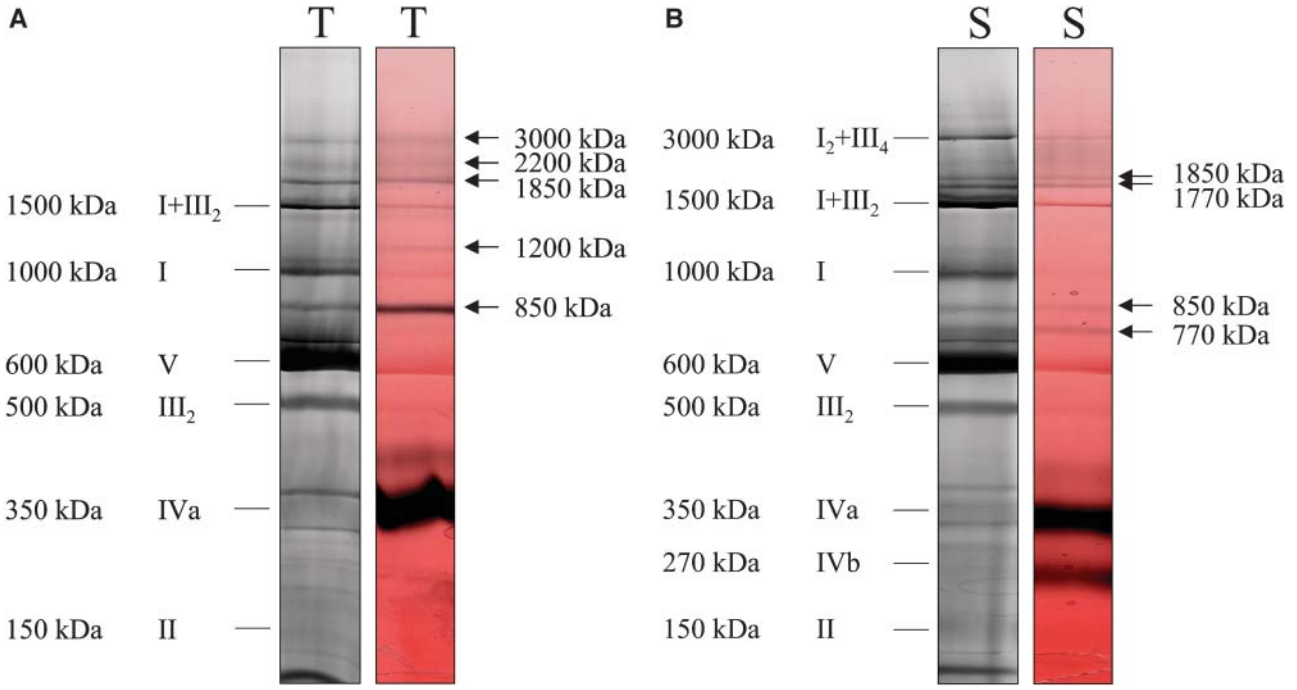


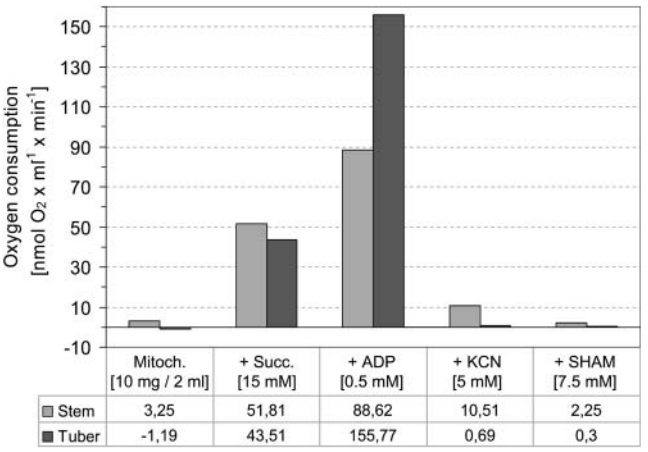
Figure 1. Identification of complex IV-containing supercomplexes in potato tuber (T) and stem (S) mitochondria. Protein complexes were solubilized by 5 g digitonin per g protein, separated by 1D BN-PAGE and either visualized by Coomassie staining (left gel strips) or by in-gel activity staining for cytochrome c oxidase (right gel strips). Activity stains are given in false-color mode to increase color contrast (red, Coomassie; black, enzyme activity). Molecular masses and identities of known protein complexes are indicated on the left side of the gels in Roman numerals (I, NADH dehydrogenase; II, succinate dehydrogenase; III, cytochrome c reductase; IVa and IVb, large and small form of cytochrome c oxidase; V, ATP synthase; I + III_2 and I_2 + III_4, supercomplexes of complexes I and III). Additional supercomplexes exhibiting cytochrome c oxidase activity are indicated by arrows.

Physiological State of Mitochondrial Fractions Used for Supercomplex Characterizations

Oxygen uptake measurements were carried out using a Clark-type oxygen electrode to ensure that mitochondria used for the characterization of the novel supercomplexes are intact and physiologically active (Fig. 2). Organelles prepared from freshly harvested potato tubers exhibited high oxygen consumption rates (on average 155 nmol O_2 min^{-1} mg^{-1} mitochondrial protein under state III conditions). In contrast, activity of potato stem mitochondria reproducibly was 40% to 50% lower under the same conditions. Mitochondria prepared from both organs had comparable state II respiration. Alternative respiration was low in mitochondrial isolations from potato stems and even lower in tuber mitochondria. We conclude that all mitochondrial fractions contained highly active organelles, but that mitochondria prepared from freshly harvested potato tubers exhibited highest state III respiration.

Optimization of Protein Solubilizations for Supercomplex Characterizations

To allow optimal visualization of the novel mitochondrial supercomplexes, isolated mitochondria from potato tubers and stems were solubilized by varying concentrations of digitonin (Fig. 3). As previously reported (Eubel et al., 2003), 1 g digitonin per g mitochondrial protein only partially allowed solubilization of membrane-bound protein complexes as shown by resolutions on 1D BN gels. In contrast,



	Mitoch. [10 mg / 2 ml]	+ Succ. [15 mM]	+ ADP [0.5 mM]	+ KCN [5 mM]	+ SHAM [7.5 mM]
Stem	3,25	51,81	88,62	10,51	2,25
Tuber	-1,19	43,51	155,77	0,69	0,3

Figure 2. Oxygen consumption of isolated mitochondria from potato tubers and stems. Values are based on three independent mitochondrial preparations.

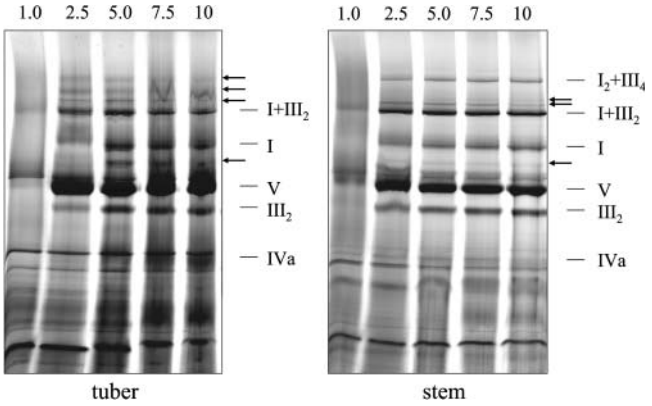


Figure 3. Resolution of mitochondrial protein complexes from potato mitochondria after solubilization using varying digitonin to protein ratios. Protein complexes were separated by 1D BN-PAGE and visualized by Coomassie staining. Detergent to protein ratios are given in g detergent per g mitochondrial protein. The OXPHOS complexes are designated by Roman numerals (see legend of Fig. 1). Unknown protein complexes are indicated by arrows.

solubilization of protein complexes and supercomplexes was very efficient between 2.5 and 10 g digitonin per g mitochondrial protein. Under these conditions, all known protein complexes and the newly discovered complexes of low abundance could be resolved. However, abundance of some supercomplexes decreased slightly in the presence of higher detergent to protein ratios. All further experiments were carried out with digitonin:protein ratios of 5 g/g.

Compositions of Newly Discovered Mitochondrial Supercomplexes

Two-dimensional BN/SDS-PAGE was carried out to characterize the subunit compositions of the novel mitochondrial protein supercomplexes (Fig. 4). High protein amounts had to be loaded onto the gels to overcome their low abundance and to obtain information on subunits of these supercomplexes. The 850-kD complex of potato tuber mitochondria contains subunits of complexes III and IV and most likely has III_2IV composition (Fig. 4A). The 1,200-kD complex could not be detected on our 2D gels. The 1,850-, 2,200-, and 3,000-kD complexes of potato tuber mitochondria all contain the subunits of the I_1III_2 supercomplex and additionally the Cox2 protein, which is the most dominant subunit of complex IV on BN gels (Fig. 4A). Further subunits of complex IV probably are present but could not be detected because they overlap with subunits of the complexes I and III on our gels. Due to low abundance, densitometric measurements of individual protein spots did not allow resolution of the stoichiometry of the protein complexes within these supercomplexes. However, based on the apparent molecular masses on the BN gels, the 1,850-, 2,200-, and 3,000-kD supercomplexes probably have $I_1III_2IV_1$, $I_1III_2IV_2$, and $I_1III_2IV_4$ compositions, which would be in accordance with findings on respiratory supercomplexes in mammalian mitochondria (Schägger and Pfeiffer, 2000). We conclude that complex IV forms part of supercomplexes in potato tuber mitochondria. However, about 90% of complex IV was in monomeric state under the conditions applied (Fig. 4A).

Slightly different results were obtained upon resolution of mitochondrial protein complexes from potato stems by 2D BN/SDS-PAGE (Fig. 4B). First of all, about 50% of monomeric complex IV was not in the larger IVa (350 kD) but in the IVb form (270 kD), which could not be detected in the potato tuber mitochondrial fraction. Since the mitochondrial fractions from tubers and stems were treated equally, artificial generation of this smaller version of monomeric complex IV during mitochondrial isolations and/or BN-PAGE seems unlikely. As reported previously for Arabidopsis and bean, complex IVb lacks at least one 30-kD subunit, which was identified as being homologous to Cox6b proteins from yeast and mammals (Eubel et al., 2003). The 850- and 1,850-kD supercomplexes containing complex IV are also present in potato stem mitochondria and additionally two slightly smaller supercomplexes of 770 and 1,770 kD, which probably include complex IVb instead of complex IVa. The complex IV-containing 2,200- and 3,000-kD supercomplexes could not be found in mitochondrial isolations from potato stems. Instead, the 3,000-kD I_2III_4 supercomplex is present, which previously was described for Arabidopsis (Eubel et al., 2003).

Analysis of the Newly Discovered Supercomplexes by 2D BN/BN-PAGE

To further investigate the structure of the newly discovered complex IV-containing supercomplexes from potato, 2D gel electrophoreses were repeated using 2D BN/BN-PAGE (Schägger and Pfeiffer, 2000). This procedure is based on the separation of digitonin-solubilized protein complexes and supercomplexes on a first dimension BN-PAGE and subsequently a resolution of the separated supercomplexes on a second dimension BN-PAGE in the presence of dodecylmaltoside. Dodecylmaltoside is known to destabilize supercomplexes. Protein complexes and supercomplexes likewise stable in the presence of digitonin and dodecylmaltoside form a diagonal line on the resulting 2D gels, whereas supercomplexes destabilized by dodecylmaltoside dissociate into protein complexes of higher electrophoretic mobility.

2D BN/BN-PAGE of mitochondrial fractions from potato tuber (Fig. 5A) confirmed all results obtained by 2D BN/SDS-PAGE: the 850-kD supercomplex consists of complexes III and IV and the 1,850-, 2,200-, and 3,000-kD supercomplexes of complexes I, III, and IV. Separation of all these supercomplexes not only revealed occurrence of complex IVa but also presence of the smaller complex IVb. However, since monomeric complex IVb is absent in potato tuber mitochondrial fractions after digitonin solubilizations (Figs. 1A and

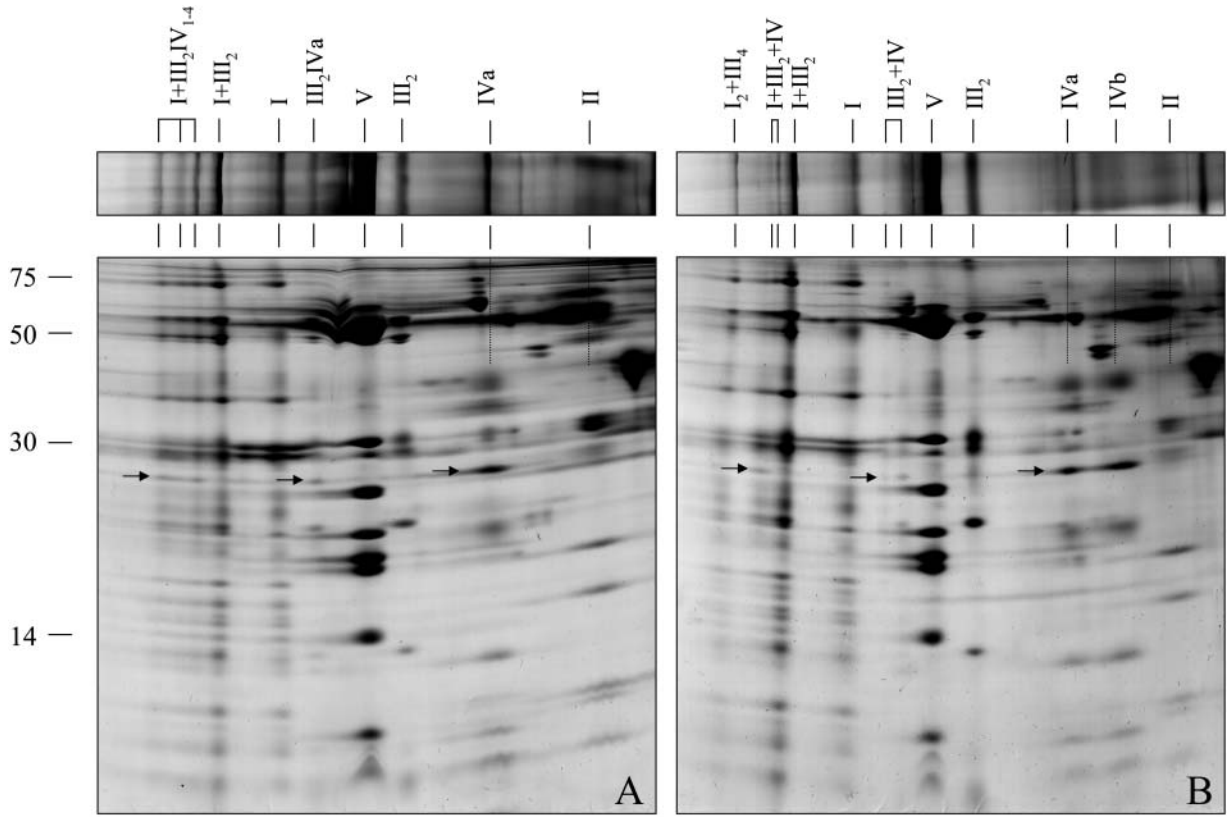


Figure 4. Two-dimensional resolution of mitochondrial protein complexes from potato tubers (A) and potato stems (B) by BN/SDS-PAGE. Mitochondrial proteins were solubilized by 5 g digitonin per g protein. Gels were Coomassie stained. Strips of corresponding 1D BN gels and identities of protein complexes and supercomplexes are given above the 2D gels. The numbers on the left indicate the molecular masses of standard proteins. Subunit II of cytochrome c oxidase is marked by arrows.

4A) but present after additional dodecylmaltoside treatment (Fig. 5A), this version of complex IV most likely is artificially generated during BN/BN-PAGE under the conditions applied. In contrast to 2D BN/SDS-PAGE, 2D BN/BN-PAGE allowed the analysis of the 1,200-kD supercomplex present in potato tuber mitochondria. Like the 850-kD complex, this supercomplex only contains the complexes III and IV and probably has a III_2IV_2 composition.

Analysis of mitochondrial fractions from potato stems by BN/BN-PAGE (Fig. 5B) also confirmed the findings obtained by 2D BN/SDS-PAGE: the 850- and 1,850-kD complexes include complexes III + IVa and I + III + IVa (complex IVa is partially converted into complex IVb as reported for mitochondria from potato tubers). The 770- and 1,770-kD complexes seem to have the same composition but most likely include complex IVb instead of complex IVa. The complex IV-containing 1,200-, 2,200-, and 3,000-kD supercomplexes of potato tuber mitochondria are absent, but a 3,000-kD I_2III_4 complex is present.

Interestingly, destabilization of the large complex IV-containing supercomplexes partially results in generation of the I_1III_2 but not of the III_2IV_{1-2} supercomplexes (Fig. 5, A and B). We conclude that interactions between the complexes I and III are stronger than interactions between complexes III and IV.

In-Gel Activity Measurements for Cytochrome c Oxidase in 2D BN/BN Gels

To increase sensitivity, a 2D BN-BN gel for potato stem mitochondria was repeated and stained by in-gel activity measurements for cytochrome c oxidase. This measurement was not possible after polymerization of the 1D BN gel stripe into the sample gel of a second gel dimension, most likely because *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) diffused into the gel stripe and destroyed enzymatic activities. However, fixation of the first gel dimension with agarose onto the second gel dimension proved to be compatible with this experimental approach. As shown in Figure 6, all previously made conclusions on complex IV-containing supercomplexes could be confirmed. Indeed, the 850- and 1,850-kD complexes include complex IVa, which partially dissociates into complex IVb in the presence of dodecylmaltoside. In contrast, the 770- and 1,770-kD

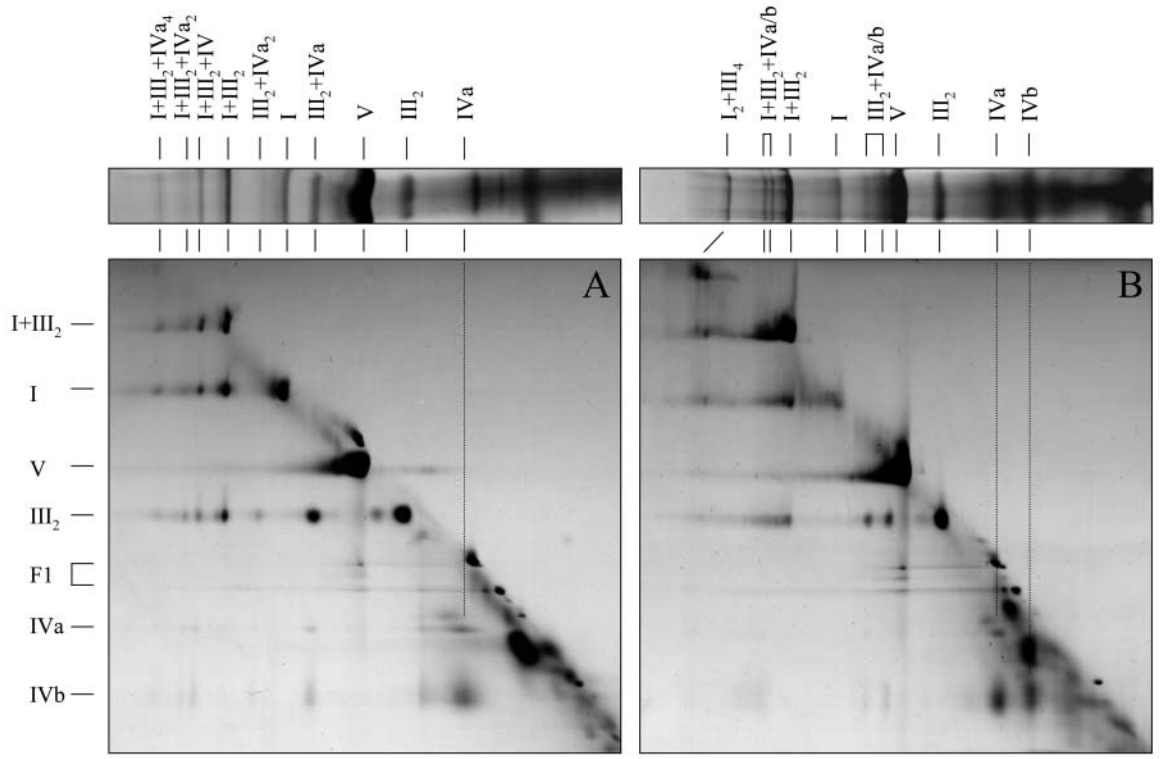


Figure 5. Two-dimensional resolution of mitochondrial protein complexes from potato tubers (A) and stems (B) by BN/BN-PAGE. Mitochondrial proteins were solubilized by 5 g digitonin per g protein. Corresponding strips of 1D BN gels are shown above the 2D gels. Identities of the resolved protein complexes and supercomplexes are given above and to the left of the gels in Roman numerals.

complexes only contain the smaller IVb version of the cytochrome c oxidase complex (Fig. 6).

DISCUSSION

Structure of Respiratory Supercomplexes in Plants, Animals, and Fungi

Besides the previously described I_1III_2 and I_2III_4 supercomplexes and dimeric ATP synthase, potato tuber mitochondria contain five additional respiratory supercomplexes of about 850-, 1,200-, 1,850-, 2,200-, and 3,000-kD, which include complex IV. The 850- and 1,200-kD complexes only contain complexes III and IV and probably have III_2IV_1 and III_2IV_2 compositions; the other three complex IV-containing supercomplexes additionally include complex I and most likely have $I_1III_2IV_1$, $I_1III_2IV_2$, and $I_1III_2IV_4$ structures (Table I; Fig. 7). Similar supercomplexes were found in potato stem mitochondria. However, all newly described supercomplexes are of rather low abundance, because they only contain about 10% of total complex IV upon digitonin solubilizations and analysis on BN gels. Using comparable conditions, nearly 100% of yeast complex IV is associated with dimeric complex III (Cruciat et al., 2000; Schägger and Pfeiffer, 2000). In mammalian mitochondria—which include similar respiratory supercomplexes than potato (Schägger and Pfeiffer, 2000)—most complex IV also is present in the monomeric form. However, there are some striking differences between mammalian and plant mitochondria with respect to respiratory supercomplexes: most complex I of bovine mitochondria forms part of the $I_1III_2IV_1$ complex, whereas in plants the I_1III_2 complex is of highest abundance. In fact the I_1III_2 complex seems to be of special stability in mitochondria from potato and other plants. Furthermore, a larger I_2III_4 supercomplex is present in plant mitochondria, which could not be described for mammalian mitochondria.

The $I_1III_2IV_4$ supercomplex was suggested to represent a basic unit for respiration in mammalian mitochondria termed respirasome (Schägger and Pfeiffer, 2000). Respirasomes are also present in plant mitochondria (Fig. 8). However, only very minor amounts of complex IV form part of respirasomes in mammals and plants (<5%). On the other hand, these structures might be much more abundant in vivo and only destabilized under the experimental conditions used for their characterization. Indeed, low digitonin to protein ratios seem to allow solubilization of higher quantities of respirasomes in potato (Fig. 3). Possibly in vivo even larger structures than respirasomes are formed by oligomerization of supercomplexes. In fact, some very weak protein bands can be seen above 3,000 kD on the gels shown in Figure 7. The I_2III_4 supercomplex of

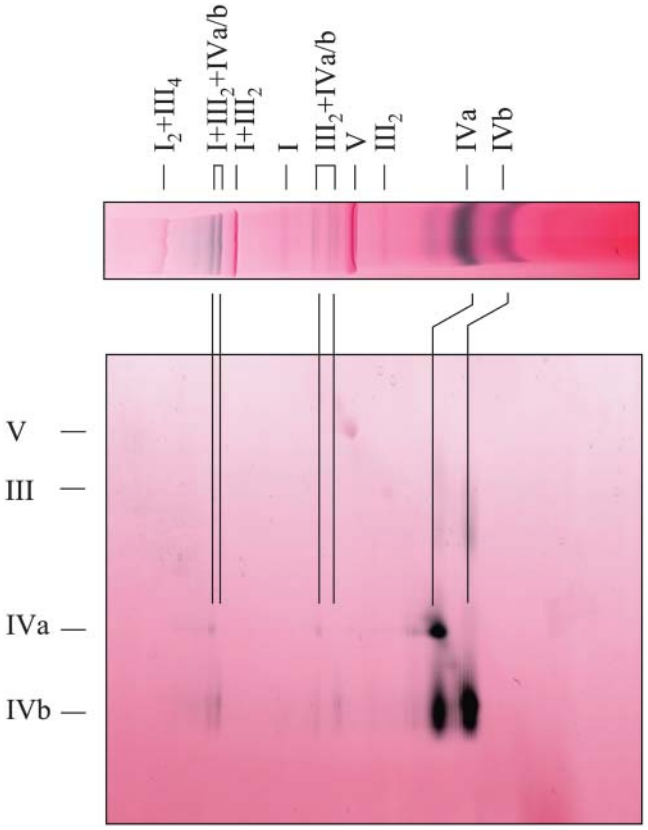


Figure 6. Identification of cytochrome c oxidase-containing supercomplexes of potato stem mitochondria by in-gel activity staining on 2D BN/BN gels. Mitochondrial proteins were solubilized by 5 g digitonin per g protein. A corresponding stripe of an activity-stained 1D BN gel is shown above the 2D gel. Identities of protein complexes and supercomplexes of the OXPHOS system from potato are given in Roman numerals. The activity stain is given in false-color mode to increase color-contrast (red, Coomassie; black, enzyme activity).

plant mitochondria could be a building block of these proposed oligomeric structures.

Are Some Supercomplexes Artificially Formed during Protein Solubilizations?

So far, formation of specific respiratory supercomplexes by artificial aggregation cannot be completely excluded but is highly unlikely for several reasons: (1) all complex IV-containing supercomplexes proved to be active by in-gel activity measurements for cytochrome c oxidase; (2) higher abundance of complex IV-containing supercomplexes in potato tuber mitochondria in comparison to potato stem mitochondria correlated with higher state III respiration; (3) the five OXPHOS complexes could theoretically form 10 different heterodimeric supercomplexes (composed of two different monomeric complexes); however, only heterodimeric I-III and III-IV complexes were observed, which represent the only meaningful associations with respect to the physiology of the mitochondrial respiratory chain (besides II-III associations, which were not observed); and (4) several physiological data reviewed in the introduction section support specific supercomplex formations, like reconstitution, inhibitor titration, and flux control experiments (Hatefi and Rieske, 1967; Ragan and Heron, 1978; Boumans et al., 1998; Genova et al., 2003).

Assembly of Mitochondrial Supercomplexes

Currently the mechanisms for supercomplex formation in mitochondria are only poorly understood. In yeast cardiolipin proved to be essential for supercomplex stability. Based on studies with yeast mutants deficient in individual subunits of OXPHOS complexes, some proteins possibly forming part of supercomplex interphases could be defined (Pfeiffer et al., 2003). In potato the $I_1III_2IV_{1-4}$ complexes partly dissociate into the I_1III_2 supercomplex and monomeric complex VI, indicating that the complex I-III association is much stronger than the interaction between these complexes and complex IV. This disassembly order might represent reverse assembly stages.

Experimental Conditions for Supercomplex Characterizations in Plants

Digitonin solubilization and BN-PAGE proved to be a powerful tool for the investigation of mitochondrial supercomplexes from plants. However, visualization of individual supercomplexes in mitochondrial fractions of plants very much depends on various factors:

1. The digitonin to protein ratio. Five grams detergent per g protein proved to be optimal for the quantitative solubilization of most supercomplexes (Fig. 3). However, lower detergent to protein ratios significantly increase the amounts of some supercomplexes on BN gels. In fact, solubilization using 1 g digitonin per g protein seems to mainly

Table I. *Protein complexes and supercomplexes of the OXPHOS system in potato tuber and stem mitochondria*

Molecular Mass [kD]	Components	Proposed Composition	Occurrence	
			Tuber	Stem
3,000	I, III	$I_2 + III_4$	—	x
3,000	I, III, IVa	$I_1 + III_2 + IVa_4$	x	—
2,200	I, III, IVa	$I_1 + III_2 + IVa_2$	x	—
1,850	I, III, IVa	$I_1 + III_2 + IVa_1$	x	x
1,770	I, III, IVb	$I_1 + III_2 + IVb_1$	—	x
1,500	I, III	$I_1 + III_2$	x	x
1,200	III, IVa	$III_2 + IVa_2$	x	—
1,000	I	I_1	x	x
850	III, IVa	$III_2 + IVa_1$	x	x
770	III, IVb	$III_2 + IVb_1$	—	x
600	V	V_1	x	x
500	III	III_2	x	x
350	IVa	IVa_1	x	x
270	IVb	IVb_1	—	x

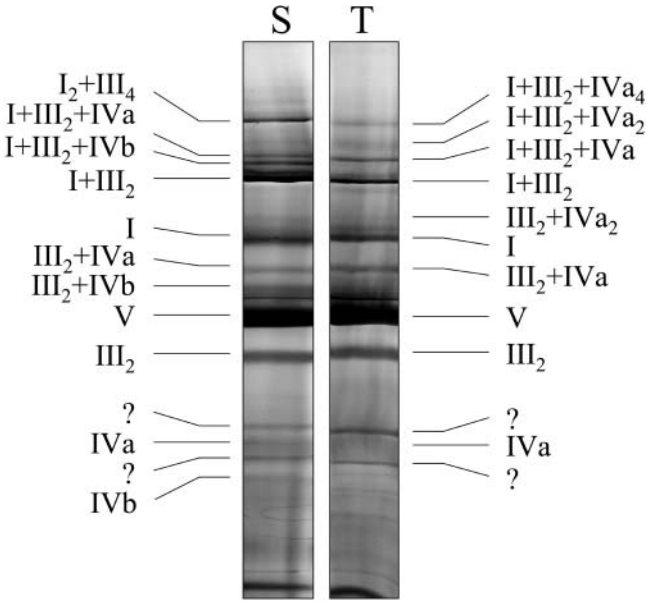


Figure 7. Identities of protein complexes and supercomplexes of the OXPHOS system in potato tubers (T) and stems (S) after separation by 1D BN-PAGE. Proteins were solubilized by 5 g digitonin per g protein. The gels were Coomassie stained. Identities of the protein complexes and supercomplexes are given by Roman numerals.

solubilize I_1III_2 and $I_1III_2IV_4$ supercomplexes (lane 1 of the gels in Fig. 3).

2. The physiological state of the starting material for mitochondrial isolations. Freshly harvested potato tubers gave much better results concerning supercomplex visualization on BN gels than potato tubers stored for some weeks (data not shown). This most likely explains the absence of complex IV-containing supercomplexes of potato tubers in our previous investigations (however, some low amounts of the 850-kD III_2IV_1 supercomplex of potato mitochondria were overseen on the gel in Figure 4 in Eubel et al., 2003).
3. The plant organs selected for mitochondrial preparations. Potato tubers and stems slightly differ with respect to occurrence of individual supercomplexes. Overall, stem mitochondria contained less complex IV-containing respiratory supercomplexes. Furthermore, complex IV partially is present in the smaller IVb form in stem mitochondria. So far it cannot be distinguished whether these differences reflect tissue-specific variations or rather represent differences in physiological states of the organelles of these two tissues. Possibly etiolated seedlings or suspension cell cultures are not optimal as starting material for the characterization of labile interactions of mitochondrial protein complexes.

We speculate that complex IV-containing supercomplexes are present in other plants depending on the physiological state of the organs used for mitochondrial isolations but might be of low abundance. Indeed, mitochondria prepared from Arabidopsis leaves revealed some very small amounts of complex IV-containing supercomplexes (H. Eubel and H.-P. Braun, data not shown).

Functional Relevance of the Monomeric Cytochrome c Oxidase Complexes IVa and IVb of Plant Mitochondria

Monomeric complex IV is represented by two different forms in plants (Jänsch et al., 1996; Eubel et al., 2003; Sabar et al., 2003), the larger of which includes at least one additional protein subunit homologous to the Cox6b protein of fungi and mammals. The smaller complex IVb is generated by dissociation of the larger complex IVa in the presence of dodecylmaltoside. However, digitonin-solubilized mitochondrial fractions from potato tubers and stems differ considerably with respect to complex IVb, which is absent in digitonin extracts of potato tuber mitochondria (Fig. 4A) but represents about 50% of monomeric complex IV of stem mitochondria (Fig. 4B). Furthermore, supercomplexes of potato stem mitochondria seem to partially include the smaller IVb form of cytochrome c oxidase. Complex IVb is enzymatically active, but specific activity is significantly reduced in comparison to complex IVa (compare Figs. 1 and 4/5). At the same time, state III respiration of stem mitochondria is reduced as shown by oxygen consumption measurements of isolated mitochondria (Fig. 2). We therefore speculate that there might be distinct physiological roles of the two forms of cytochrome c oxidase in plants. Possibly plant mitochondria contain a pool of partially inactivated complex IV which rapidly can be activated upon association with the Cox6b protein.

An even larger probably monomeric form of complex IV can be seen by activity stainings of BN gels in the 400-kD range (Fig. 1). This version of complex IV is not visible on Coomassie-stained BN gels (Fig. 4), and its identity so far remains a mystery. Possibly this form of complex IV is a chaperone-bound assembly intermediate of cytochrome c oxidase. Similarly, a slightly larger form of complex III (550 instead of 500 kD) can be seen on the 2D BN/BN gel in Figure 5, which is invisible on the corresponding first gel dimension and

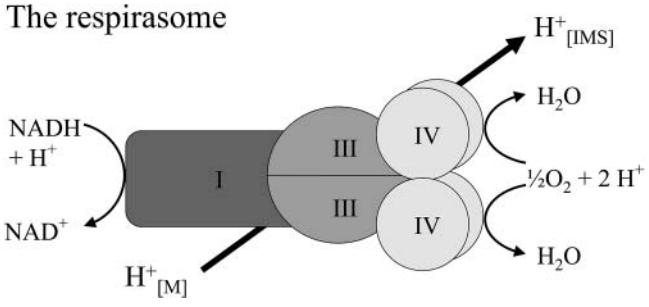


Figure 8. Structure and function of the respirasome in mitochondria. [M], Matrix; [IMS], mitochondrial intermembrane space.

might also represent a chaperone-bound form of this respiratory complex. Further experiments have to be carried out to explain these observations.

Outlook

Plant mitochondria exhibit several special features in comparison to mitochondria from heterotrophic eukaryotes. Due to the presence of numerous alternative oxidoreductases, the respiratory chain of plant mitochondria is very much branched (Vanlerberghe and McIntosh, 1997; Rasmusson et al., 1999; Moller, 2001; Moore et al., 2003). Furthermore, the protein complexes of the respiratory chain include plant-specific protein subunits (Braun and Schmitz, 1995; Eubel et al., 2003; Heazlewood et al., 2003a, 2003b; Millar et al., 2003). For instance, the two subunits of the mitochondrial processing peptidase form an integral part of complex III in plants (Braun et al., 1992b; Eriksson et al., 1994). As a consequence, respiratory supercomplexes most likely have special roles in plant mitochondria, e.g. in regulating access of alternative respiratory oxidoreductases to their substrates during respiration. Experiments to address these questions are under way in our laboratory.

MATERIALS AND METHODS

Isolation of Mitochondria from Potato Tubers and Stems

Freshly harvested potato (*Solanum tuberosum* var. *cilena*) tubers were purchased directly from a local farmer. Half of them were stored in the cold (4°C); the other half were planted into soil and grown in the dark at 20°C. Mitochondria were prepared from stored tubers and from etiolated potato stems after 20 d. Plant material (200 g) was homogenized at 4°C using a Waring blender for 3 × 5 s, filtrated through four layers of muslin, and subsequently organelles were purified by differential centrifugations and Percoll density gradient centrifugation as outlined previously (Braun et al., 1992a). Isolated mitochondria were either directly analyzed by gel electrophoresis or stored at −80°C.

Sample Preparation for Gel Electrophoresis

Mitochondrial samples of 500 μg (50 μg mitochondrial protein) were sedimented by centrifugation for 10 min at 14,000*g*, resuspended in 50 μL of digitonin solution (1%–10% digitonin/30 mM HEPES/150 mM potassium acetate/10% glycerol), and incubated for 20 min at 0°C. Afterwards samples were centrifuged for 10 min at 18,000g. Finally supernatants were supplemented with 5 μL of a Coomassie Blue solution (5% Coomassie Blue/750 mM aminocaproic acid) and directly loaded onto BN gels.

Gel Electrophoresis

BN-PAGE was carried out as described previously (Schägger, 2001b). Gels were destained by incubation in fixing solution (40% [v/v] methanol, 10% [v/v] acetic acid) overnight and subsequently stained with Coomassie colloidal (Neuhoff et al., 1985, 1990). Alternatively, strips of BN gels were transferred horizontally onto second gel dimensions. 2D BN/SDS-PAGE was carried out according to Schägger (2001b) and 2D BN/BN-PAGE according to Schägger and Pfeiffer et al. (2000). However, 1D gel strips for BN/BN-PAGE were fixed by 1.5% agarose onto the second gel dimension and not by direct polymerization into the stacking gel. This modification proved to be essential for subsequent in-gel activity measurements.

In-Gel Activity Stains for Cytochrome c Oxidase

In-gel activity of cytochrome c oxidase was measured according to Zerbetto et al. (1997) and Jung et al. (2000): 1D BN or 2D BN/BN gels were incubated in 20 mM phosphate buffer (pH 7.4), 1.0 mg/mL DAB (3,3′-diaminobenzidine), 24 units/mL catalase, 1 mg/mL cytochrome c, and 75 mg/mL sucrose. Reactions were carried out at room temperature for 1 h (1D gels) or overnight (2D gels). Staining was stopped by fixing the gels in 45% methanol/10% acetic acid. Finally, gels were scanned. To increase color contrast images were false-colored for Coomassie (red) and catalase activity (black) by Photoshop software (Adobe Systems, Mountain View, CA).

Oxygen Electrode Measurements

Oxidative phosphorylation of all mitochondrial preparations was analyzed using a Clark-type oxygen electrode with a reaction chamber of 2 mL (Oxygraph, Hansatech, Norfolk, England). Oxygen consumption of 10 mg mitochondria (1 mg mitochondrial protein) in reaction buffer (0.3 M mannitol, 10 mM K_2HPO_4 (pH 7.2), 10 mM KCl, 5 mM $MgCl_2$) was measured after supplementation of succinate (15 mM), ADP (5 mM), KCN (5 mM), and salicylhydroxamic acid (SHAM; 7.5 mM). Mitochondrial oxygen consumption was calculated in nmol ΔO_2 min^{-1} mg $protein^{-1}$.

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Respiratory chain supercomplexes in plant mitochondria

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Abstract

Supercomplexes are defined associations of protein complexes which are important for several cellular functions. This "quintenary" organization level of protein structure recently was also described for the respiratory chain of plant mitochondria. Except succinate dehydrogenase (complex II), all complexes of the oxidative phosphorylation system (complexes I, III, IV and V) were found to form part of supercomplexes. Compositions of these supramolecular structures were systematically investigated using digitonin solubilizations of mitochondrial fractions and two-dimensional Blue-native polyacrylamide gel electrophoresis. The most abundant supercomplex of plant mitochondria includes complexes I and III at a 1:2 ratio (I_1+III_2 supercomplex). Furthermore, some supercomplexes of lower abundance could be described which have I_2+III_4, V_2, III_2+IV_{1-2}, and I_1+III_2+IV_{1-4} compositions. Supercomplexes consisting of complexes I plus III plus IV were proposed to be called "respirasome", because they autonomously can carry out respiration in the presence of ubiquinone and cytochrome c. Plant specific alternative oxidoreductases of the respiratory chain were not associated with supercomplexes under all experimental conditions tested. However, formation of supercomplexes possibly indirectly regulates alternative respiratory pathways in plant mitochondria on the basis of electron channelling. In this review, procedures to characterize the supermolecular organisation of the plant respiratory chain and results concerning supercomplex structure and function are summarized and discussed.

Keywords: mitochondria, respiratory chain, supercomplexes, blue-native polyacrylamide gel electrophoresis, Arabidopsis

Introduction

Respiration has special features in plant cells. While mitochondrial electron transport in most animals is based on a linear succession of redox reactions, the respiratory chain of plant mitochondria is branched at several points. Besides the "classical" complexes I (NADH dehydrogenase), II (succinate dehydrogenase), III (cytochrome c redutase) and IV (cytochrome c oxidase), plant mitochondria contain at least five additional so-called "alternative" oxidoreductases which participate in respiratory electron transport [41]. Four of these enzymes catalyse electron transfer from NADH or NADPH to ubiquinone and are termed "rotenone insensitive NADH dehydrogenases" because their activities are not affected by the complex I inhibitor rotenone [33, 28, 29, 26]. The fifth enzyme is a terminal oxidase called "alternative oxidase" [44, 35, 45]. It catalyses direct electron transfer from ubiquinol to molecular oxygen. All these alternative oxidoreductases do not couple electron transport to proton translocation across the inner mitochondrial membrane and therefore seem to catalyse energy wasting reactions. However, it is believed that these reactions are important, possibly because they are the basis for overflow-protection mechanisms of the respiratory chain in plant cells under certain physiological conditions.

Regulation of electron transfer between the "classical" oxidoreductases and regulation of electron partitioning between "classical" and "alternative" respiratory pathways in plant mitochondria is not quite understood so far. One possible level of regulation are dynamic associations between enzymes of the respiratory chain. However, for quite a while, the involved components were considered to be separate structures which independently move within the inner mitochondrial membrane by lateral diffusion [42, 9]. Meanwhile there is mounting evidence that *in vivo* respiratory protein complexes specifically interact forming supermolecular structures called "supercomplexes". Evidence is based on biochemical purification and reconstitution experiments [15,16], inhibitor titration experiments [5, 17, 4] as well as characterizations of respiratory mutants [19, 1, 43]. Some years ago, Schägger and co-workers established a new experimental strategy to characterize mitochondrial supercomplexes, which is based on protein solubilizations using the non-ionic detergents digitonin or Triton X100 and protein separations by two-dimensional Blue-native (BN) / SDS-PAGE or two-dimensional BN / BN-PAGE [40, 10, 36, 38, 31, 24]. Using these procedures, stable supercomplexes containing complexes III and IV were described for yeast and even larger supercomplexes that additionally include complex I for mitochondria from mammals. Meanwhile this experimental strategy was also used to characterize the supermolecular organization of

the respiratory chain in plant mitochondria [12, 13]. In this review, we summarize protocols for investigating supercomplexes of plant mitochondria and give an overview on results on the higher-order structure of the plant mitochondrial respiratory chain.

Methods for the characterization of respiratory supercomplexes in plant mitochondria

First step for biochemical characterizations of membrane proteins usually is membrane solubilization (Fig. 1). This step is critical and should be carried out as mild and careful as possible. Concerning respiratory protein complexes and supercomplexes of plants, incubation of isolated mitochondria with 5 g digitonin per g protein gives optimal results [12]. In combination with 2D Blue-native (BN) / SDS-PAGE, digitonin solubilizations of plant mitochondrial fractions allow visualization of all five “classical" protein complexes of the oxidative phosphorylation (OXPOS) system. Supercomplexes proved to be stable under these conditions. In contrast, protein solubilizations using dodecylmaltoside do not allow to monitor complexes II and IV on BN gels for most plants investigated. Furthermore, supercomplexes are less stable in the presence of dedocylmaltoside and some OXPHOS complexes are cleaved into subcomplexes. For instance, mitochondrial F_0F_1 ATP synthase is partially dissected into the F_0 and F_1 parts, which does not take place in the presence of digitonin. Triton X100 is slightly more advantageous than dodecylmaltoside for characterizations of plant mitochondrial respiratory protein complexes in combination with BN-PAGE, but less suitable than digitonin. However, low concentrations of Triton X100 are optimal to visualize dimeric complex V [12].

BN-PAGE represents an ideal tool for the investigation of protein complexes in mitochondria [39, 22, 37]. Negative charges are introduced into protein complexes and supercomplexes prior to electrophoretic separations by incubation of protein mixtures with Coomassie-blue. In contrast to SDS, Coomassie does not denature proteins. Subsequently, protein complexes and supercomplexes can be resolved on low-percentage polyacrylamide gels and their subunits on a second gel dimension, which is carried out in the presence of SDS in high percentage polyacrylamide gels. Since Coomassie-treated proteins all become negatively charged, artificial associations of proteins are very unlikely due to electric repulsion. Visualization of the OXPHOS complexes by 2D BN / SDS-PAGE only requires small amounts of starting material (0.1 – 1 mg mitochondrial protein).

One-dimensional BN gels can be used for in-gel activity stains (Fig. 2). Protocols for in-gel enzyme assays were published for all protein complexes of the OXPHOS system except for complex III [47, 23, 34]. Furthermore, peroxidase activity could be monitored using 1D BN gels [14] and the activity of the mitochondrial processing peptidase was measured after electroelution of the enzyme from a BN gel [8]. In general, treatment of protein fractions with Coomassie-blue seems not to interfere with enzyme activities.

A novel two-dimensional gel system was recently suggested for investigations of protein complex compositions of supercomplexes, which is based on BN-PAGE for both gel dimensions [40]. First dimension BN-PAGE is carried out in the presence of a very mild detergent like digitonin, whereas the second gel dimension takes place in the presence of a less gentle detergent like dodecylmaltoside. All protein complexes and supercomplexes likewise stable in the presence of both detergents form a diagonal line on this 2D gel system, whereas supercomplexes specifically destabilized in the presence of the second detergent are dissected into protein complexes of higher electrophoretic mobility. 2D BN / BN gels also can be used for in-gel activity stains [13].

If usage of Coomassie-dyes and polyacrylamide gel electrophoreses shall be avoided during protein separations, proteins and protein complexes of detergent-treated mitochondrial fractions also can be resolved by gel filtration [10] or sucrose gradient ultracentrifugation (Eubel and Braun, unpublished results). Interestingly, separated protein complexes and supercomplexes exactly correspond to those visible on 2D BN / SDS gels, which supports the reliability of this experimental system.

The supermolecular structure of the respiratory chain in plant mitochondria

Using separations of digitonin-treated mitochondrial fractions by two-dimensional BN / SDS and BN / BN-PAGE, several respiratory supercomplexes could be characterized for plant mitochondria [12, 13]. Plant mitochondria contain a very stable I_1+III_2 supercomplex of 1500 kDa, which is of high abundance (Fig. 3). Depending on the plant investigated, between 50 and 90% of complex I forms part of this supercomplex in Arabidopsis, potato, bean and barley. In contrast, this supercomplex is of low abundance in mammalian mitochondria. Formation of the I_1+III_2 supercomplex possibly has an influence on the rate of alternative respiration by the alternative oxidase in plant mitochondria due to direct electron

channelling between complexes I and III. An even larger supercomplex of 3000 kDa, which also includes the complexes I and III_2 and which probably has I_2+III_4 composition, was discovered in mitochondria from Arabidopsis and potato [12, 13]. However, this supercomplex is of low abundance and only becomes visible on BN / SDS gels upon silver staining (Fig. 3C).

Yeast mitochondria were found to contain a dimeric ATP synthase complex [2, 3, 32, 18]. Interestingly, this dimer includes some dimer-specific subunits which are essential for dimer formation. Also the mitochondrial ATP synthase complex from Chlamydomonas recently was reported to be mainly in a dimeric state [46]. In contrast, dimeric ATP synthase is of low abundance in plant mitochondria. It best can be visualized on 2D BN / SDS gels after membrane solubilization by low concentrations of Triton X100 (Fig. 4). Under these conditions, most of the complexes I-IV are not solubilized and the ATP synthase is visible in monomeric and dimeric state at comparable quantities. Increase of Triton-X100 concentration during protein solubilization leads to a sharp reduction of dimeric ATP synthase on the 2D gels and at the same time to improved visibility of all other protein complexes of the OXPHOS system (Fig. 4).

Complex IV containing supercomplexes, which are of high abundance in mitochondria from mammals and yeast, could not be detected in Arabidopsis mitochondria isolated from suspension cell cultures. However, some low amounts of complex IV form part of respiratory supercomplexes in mitochondria isolated from freshly harvested potato tubers or from potato stems grown for 20 days in the dark [13]. Analysis by 2D BN / SDS-PAGE and 2D BN / BN-PAGE in combination with in-gel activity stains revealed III_2+IV_{1-2} and I_1+III_2+IV_{1-4} compositions of these supercomplexes. Interestingly, occurrence of the supercomplexes slightly differed between mitochondria isolated from potato tubers and stems. Furthermore, the complex IV containing supercomplexes isolated from stem mitochondria partially included a smaller version of cytochrome c oxidase (termed complex IVb), which seems to lack one or two subunits in comparison to the larger version of this complex (IVa). The physiological significance of these findings has to be further investigated.

The term “respirasome” was suggested for I_1+III_2+IV_{1-4} supercomplexes, because these structures can autonomously carry out respiration in the presence of ubiquinone and cytochrome c. Respirasomes were shown to be of high abundance in mammalian mitochondria. In contrast, at least 90% of complex IV was in a monomeric state in potato mitochondria and more or less 100% in mitochondria isolated from

etiolated bean and barley seedlings or Arabidopsis suspension cell cultures under the experimental conditions applied [12, 13]. However, further plants and plant organs should be investigated with respect to respirasomes to understand the importance of these structures in plant respiration.

Alternative respiratory enzymes were not found to associate with respiratory supercomplexes in plants as shown by immunoblotting experiments[12]. Alternative oxidase is present in monomeric and dimeric forms in Arabidopsis and bean. In potato, an oligomeric AOX complex of about 600 kDa was immunologically identified but seems not to be associated with any complex of the OXPHOS system (Eubel and Braun, unpublished results). Similarily, alternative NADH / NADPH dehydrogenases were immunologically localized on 2D BN/ SDS gels and found to be either represented by monomeric proteins or oligomeric structures which are not associated to the "classical" protein complexes of the respiratory chain. Based on our findings we conclude that supercomplex formation rather indirectly influences alternative electron transport pathways in plant mitochondria.

Conclusion

Blue-native gel electrophoresis is a very powerful tool for the characterisation of the supermolecular structure of the respiratory chain in plant mitochondria and recently also was successfully used to investigate supercomplexes in plastids [21]. However, further experimental approaches have to be used to get deeper insights into the structure and function of plant mitochondrial supercomplexes, like analyses of detergent-solubilized mitochondrial fractions by electron microscopy in combination with computer image analysis. Recently, flux control experiments revealed physiological evidence for interaction between complexes I and III [17]. In the future, non-invasive methods should be applied to understand the supermolecular structure of the respiratory chain *in vivo*, e.g. by the usage of fluorescent fusion proteins as subunits of individual respiratory protein complexes. At the moment experimental results concerning defined associations of respiratory protein complexes are partially contradictory, which possibly reflects that these associations are dynamic and that assembled and unassembled supercomplexes co-exist in the inner mitochondrial membrane [25, 17]. In yeast, dynamics of supercomplex formation was recently shown to depend on the cardiolipin concentration of the inner mitochondrial membrane [48, 31].

Characterization of the supermelecular structure of the respiratory chain using highly similar procedures - like solubilization of mitochondrial proteins using 5g digitonin / g mitochondrial protein and subsequently separation of the solubilized proteins by 2D BN / SDS-PAGE – indicated some clear differences concerning abundance and stability of individual supercomplexes between animals, fungi and plants. Plant mitochondria have an especially stable I_1+III_2 complex, suggesting channelling of electrons between these two complexes. Consequences for alternative oxidase, which competes with complex III for electrones of ubiquinol, have to be further investigated. Differences in stability of the I_1+III_2 supercomplex also might reflect differing architecture of complexes I and III in animals, fungi and plants. Besides several highly conserved subunits, individual subunits of these protein complexes seem to be specific to certain groups of organisms. For instance, plant complex I was reported to include a group of five related proteins which most likely have carbonic anhydrase activity [20, 30]. Furthermore, the terminal enzyme of the mitochondrial ascorbic acid biosynthesis pathway, the L-galactono-1,4-lactone dehydrogenase, was recently found to form part of complex I in plant mitochondria [27]. And finally, the core subunits of complex III have unique features in plant mitochondria, because they represent the two subunits of the mitochondrial processing peptidase [7, 11, 6]. Further experiments have to be carried out to get deeper insights into the function of the respiratory protein complexes in plant mitochondria and their supermolecular interactions.

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Figure legends:

Figure1: Procedures to analyse respiratory supercomplexes

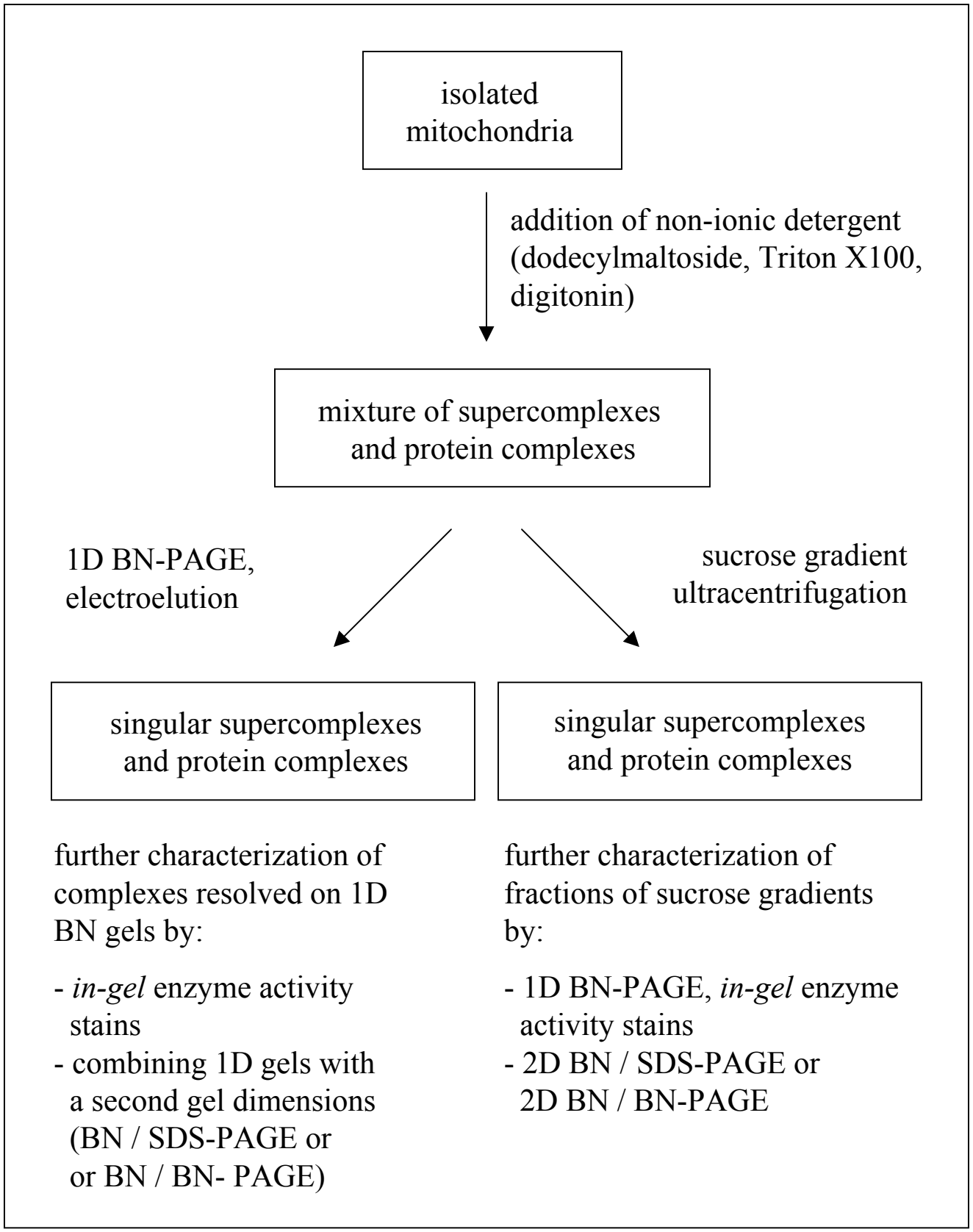
Figure 2: Identification of respiratory protein complexes from Arabidopsis by in-gel activity stains. Mitochondrial proteins were solubilized by 5 g digitonin per g protein and protein complexes were resolved by 1D BN-PAGE. C: Control gel strip, CC: Coomassie-stained control gel strip, I, II, IV: gel strips after in-gel stains for complexes I, II and IV. Identities of the separated protein complexes and supercomplexes are given to the left: I_2+III_4 – supercomplex consisting of two copies of complex I and two copies of dimeric complex III; I_1+III_2 – supercomplex consisting of one copy of complex I and one copy of dimeric complex III; I – complex I; V – ATP synthase; III_2 – dimeric complex III; IV – complex IV; II – complex II.

Figure 3: Separation of mitochondrial protein complexes and supercomplexes from Arabidopsis by 2D Blue-native / SDS-PAGE. Mitochondrial proteins were solubilized with 5 g digitonin per g protein. A: Coomassie-stained 1D Blue-native gel, B: Coomassie-stained 2D Blue-native / SDS gel, C: Silver-stained 2D Blue-native / SDS gel corresponding to the boxed region on the gel in B. Identities of the separated protein complexes are given above the gel (see legend of Figure 2; IVa - larger form of complex IV; IVb – smaller form of complex IV)

Figure 4: Separation of mitochondrial protein complexes from Arabidopsis by 2D Blue-native / SDS gel electrophoresis. A: Proteins were solubilized with 0.25 g Triton X100 per g protein, B: Proteins were

solubilized with 2.0 g Triton X100 per g protein. Both gels were Coomassie-stained. Identities of protein complexes are given above the gels (see legend of Figure 2; V_2 – dimeric ATP synthase; F1 – F1 part of ATP synthase)

Figure 1


isolated mitochondria
addition of non-ionic detergent (dodecylmaltoside, Triton X100, digitonin)
mixture of supercomplexes and protein complexes
1D BN-PAGE, electroelution
sucrose gradient ultracentrifugation
singular supercomplexes and protein complexes
singular supercomplexes and protein complexes
further characterization of complexes resolved on 1D BN gels by:
- *in-gel* enzyme activity stains
- combining 1D gels with a second gel dimensions (BN / SDS-PAGE or or BN / BN- PAGE)
further characterization of fractions of sucrose gradients by:
- 1D BN-PAGE, *in-gel* enzyme activity stains
- 2D BN / SDS-PAGE or 2D BN / BN-PAGE

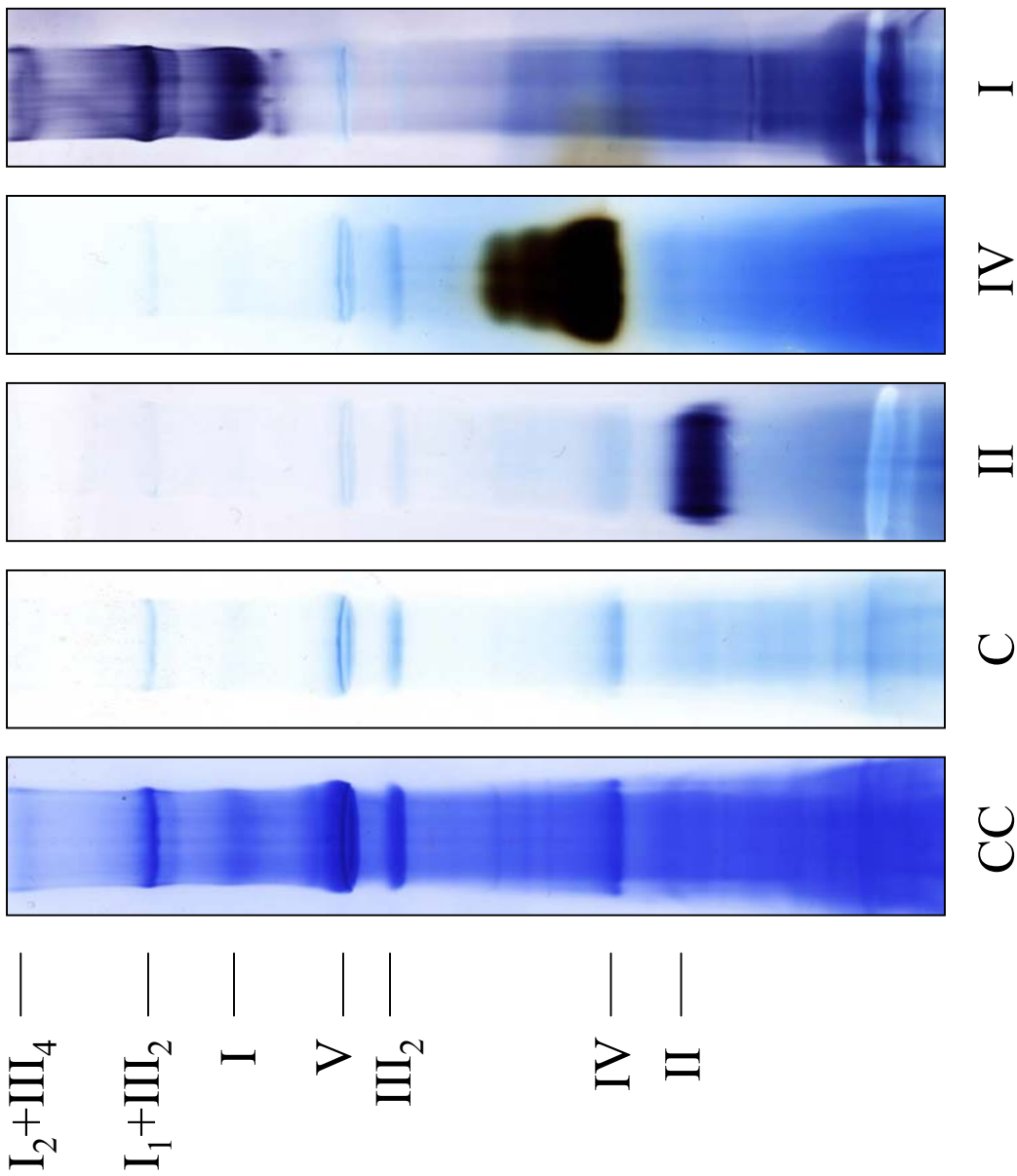


Figure 2

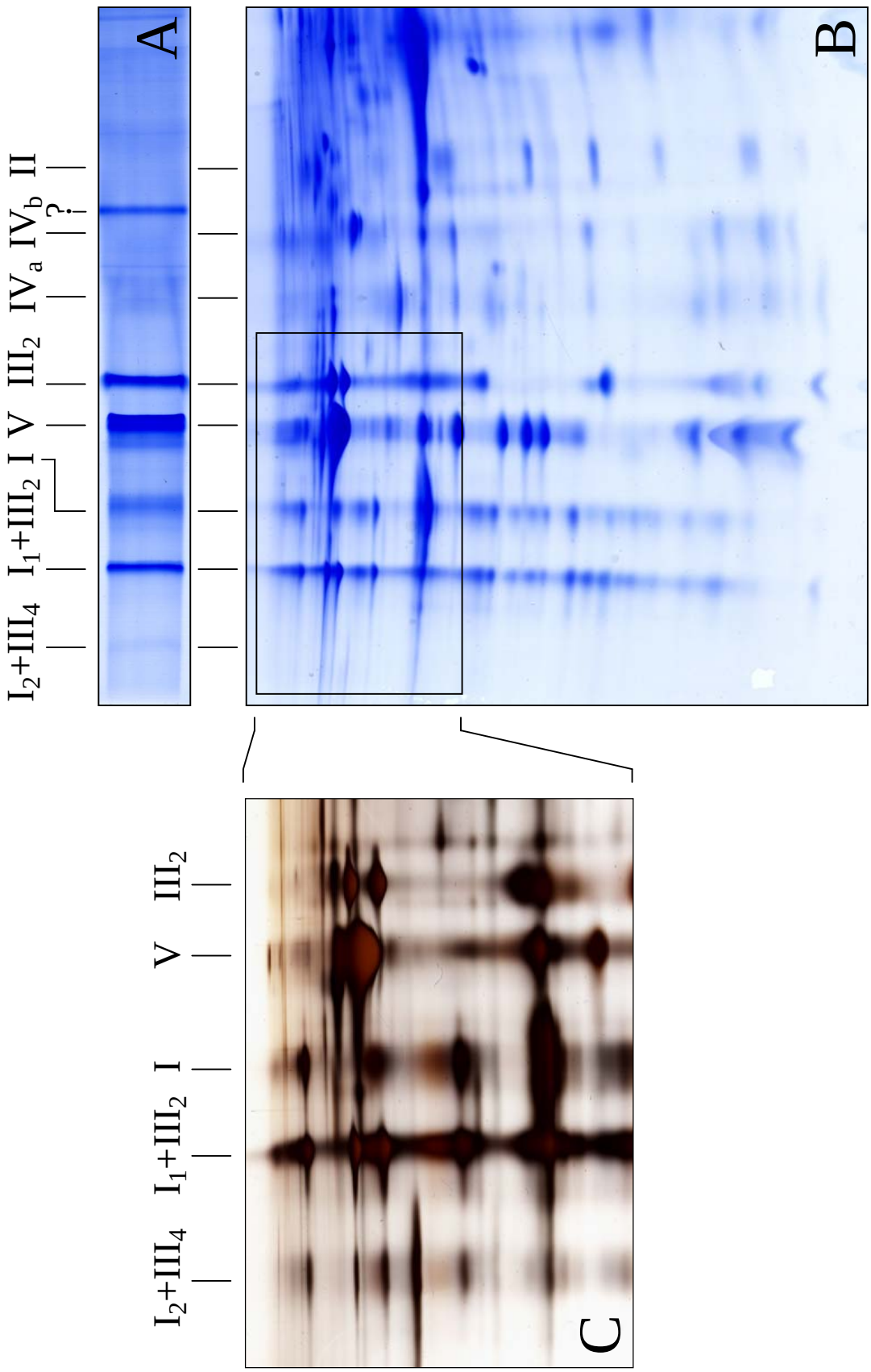


Figure 3

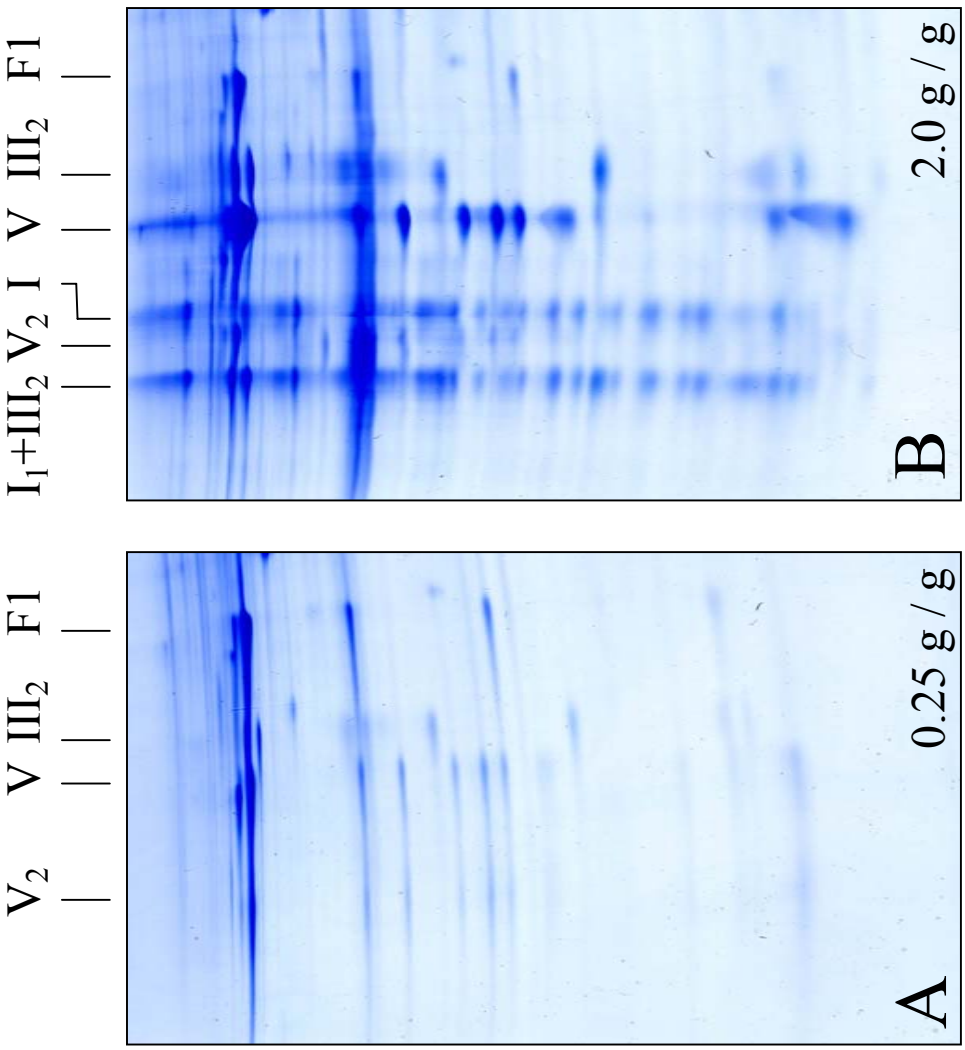


Figure 4

Plant Molecular Biology **00:** 1–13, 2004.

Mitochondrial cytochrome *c* oxidase and succinate dehydrogenase complexes contain plant specific subunits

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Key words: Arabidopsis, cytochrome *c* oxidase, mitochondria, respiratory chain, succinate dehydrogenase

Abstract

Respiratory oxidative phosphorylation represents a central functionality in plant metabolism, but the subunit composition of the respiratory complexes in plants is still being defined. Most notably, complexes II (succinate dehydrogenase) and complex IV (cytochrome *c* oxidase) are the least defined in plant mitochondria. Using *Arabidopsis* mitochondrial samples and 2D Blue-native/SDS-PAGE, we have separated complex II and IV from each other and displayed their individual subunits for analysis by tandem mass spectrometry and Edman sequencing. Complex II can be discretely separated from other complexes on BN gels and consists of eight protein bands. It contains the four classical SDH subunits as well as four subunits unknown in mitochondria from other eukaryotes. Five of these proteins have previously been identified, while three are newly identified in this study. Complex IV consists of 9–10 protein bands, however, it is more diffuse in BN gels and co-migrates in part with the translocase of the outer membrane (TOM) complex. Differential analysis of TOM and complex IV reveals that complex IV probably contains eight subunits with similarity to known complex IV subunits from other eukaryotes and a further six putative subunits which all represent proteins of unknown function in *Arabidopsis*. Comparison of the *Arabidopsis* data with Blue-native/SDS-PAGE separation of potato and bean mitochondria confirmed the protein band complexity of these two respiratory complexes in plants. Two-dimensional Blue-native/Blue-native PAGE, using digitonin followed by dodecylmaltoside in successive dimensions, separated a diffusely staining complex containing both TOM and complex IV. This suggests that the very similar mass of these complexes will likely prevent high purity separations based on size. The documented roles of several of the putative complex IV subunits in hypoxia response and ozone stress, and similarity between new complex II subunits and recently identified plant specific subunits of complex I, suggest novel biological insights can be gained from respiratory complex composition analysis.

Introduction

Mitochondrial oxidative phosphorylation (OXPHOS) in most eukaryotes is based on the sequential operation of five protein complexes termed complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome *c* reductase), complex IV (cytochrome *c* oxidase) and complex V (ATP synthase complex). These protein complexes are all present

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in mitochondria from plants, and in addition, plant mitochondria contain special features that further influence the respiratory chain. Firstly, respiratory electron transport in plant mitochondria is branched due to the presence of rotenone-insensitive NAD(P)H dehydrogenases and a cyanide-insensitive terminal oxidase (Vanlerberghe and McIntosh, 1997; Rasmusson *et al.*, 1999; Moller, 2001; Moore *et al.*, 2003; Michalecka *et al.*, 2003). Secondly, the mitochondrial genomes of plants code for more subunits of respiratory protein complexes than those of heterotrophic eukaryotes (Unseld *et al.*, 1997), which requires special assembly pathways for protein complexes. Lastly, some respiratory protein complexes in plant mitochondria contain additional subunits that allow them to catalyse secondary or modified functions. For example, the two subunits of the mitochondrial processing peptidase form part of complex III in plant mitochondria (Braun *et al.*, 1992a; Eriksson *et al.*, 1994) and complex I contains a L-galactonol-1,4-lactone dehydrogenase, which catalyses the terminal step of ascorbic acid biosynthesis in plant mitochondria (Millar *et al.*, 2003). Additional plant specific subunits are present in complex I and V, but are currently of unknown function (Heazlewood *et al.*, 2003a, b; Parisi *et al.*, 2004).

The subunit compositions of complexes I, III and V of plant mitochondria have been extensively studied. Complex III from potato was purified by cytochrome *c* affinity chromatography and shown to comprise 10 different subunits (Braun and Schmitz, 1992). All 10 potato proteins were partially sequenced by Edman degradation and the genes encoding these proteins were characterized (reviewed in Braun and Schmitz, 1995). Similarly, complex I was chromatographically purified for different plants and resolved into about 30 different subunits (Leterme and Boutry, 1993; Herz *et al.*, 1994; Rasmusson *et al.*, 1994; Jänsch *et al.*, 1996; Combettes and Grienenberger, 1999). Recently, 30 complex I subunits of *Arabidopsis* were identified by mass spectrometry (Heazlewood *et al.*, 2003a). Purified complex V of plant mitochondria can be resolved into about 10–15 different subunits by electrophoresis (Hamasur and Glaser, 1992; Jänsch *et al.*, 1996). Eleven subunits of this complex could be identified by mass spectrometry in *Arabidopsis* (Heazlewood *et al.*, 2003b). Four of these are mitochondrial-encoded, including *orfb* that encodes the plant ATP8 and *orf25* that encodes the plant ATP4. The product of *orfb* has also been shown to be a component of complex V in sunflower (Sabar *et al.*, 2003) and the product of *orf25* a complex V component in protists (Burger *et al.*, 2003). One complex V component appears to be plant specific, the nuclear-encoded F_Ad subunit (Smith *et al.*, 1994; Jänsch *et al.*, 1996, Heazlewood *et al.*, 2003b).

In contrast, subunit compositions of complexes II and IV are less defined for plant mitochondria and our presumptions about these protein complexes in plants are heavily based on studies carried out in beef and yeast mitochondria (for reviews see Capaldi, 1990; Richter and Ludwig, 2003). Complex IV from beef comprises 13 different subunits termed Cox I, II, III, IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc and VIII. This nomenclature reflects the successive discovery of these subunits on SDS-PAGE gels with increasing resolution capacity (Kadenbach and Merle 1981). The core of the complex is formed by the three largest subunits (Cox I, Cox II and Cox III), which are very hydrophobic and typically are encoded by the mitochondrial genome. The remaining 10 subunits are nuclear-encoded and much smaller in molecular mass. Besides Cox IV, which has a molecular mass of 17 kDa, all other nine nuclear encoded subunits have masses of 4.9–12.5 kDa. An 11-subunit cytochrome *c* oxidase complex has been described for yeast. Nine of the 11 subunits are similar in sequence to subunits of cytochrome *c* oxidase from beef while the remaining two subunits are very small and possibly yeast specific.

There has been considerable research on the genes encoding the Cox I, Cox II and Cox III subunits in plants. They are almost always localized on the mitochondrial genome and were each first sequenced about 20 years ago (Fox and Leaver, 1981; Isaac *et al.*, 1985; Hiesel *et al.*, 1987). Careful investigation of these genes and their transcription led to the discovery of introns in plant mitochondrial genes (Fox and Leaver, 1981), editing of transcripts in plant mitochondria (Covello and Gray, 1989; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989) and polyadenylation of mitochondrial transcripts (Lupold *et al.*, 1999). Earlier electrophoretic analyses of chromatographically purified cytochrome *c* oxidase from sweet potato had revealed five protein bands of about 39 kDa (band I), 33 kDa (II), 26 kDa (III), 20 kDa (IV),

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and 6 kDa (V) (Maeshima and Asahi, 1978). Bands I–III correspond to the mitochondrial-encoded Cox I, Cox II and Cox III proteins. The 6 kDa band V was later separated into three different proteins on gels with enhanced resolution capacity, and then termed subunits Va, Vb and Vc (Nakagawa, *et al.*, 1987). Direct amino acid sequencing of protein Vc and isolation of the corresponding gene revealed sequence similarity to the Cox VIII subunit from beef (Nakagawa *et al.*, 1990). Further chromatographic purifications of cytochrome *c* oxidase complexes from pea, maize and wheat revealed similar banding patterns on denaturing polyacrylamide gels. In these organisms, cytochrome *c* oxidase was composed of the three mitochondrial-encoded Cox I, Cox II and Cox III subunits, one further subunit in the 18 kDa range, and additional proteins below 11 kDa which could not be resolved by gel electrophoresis (Matsuoka *et al.*, 1981; Hawkesford *et al.*, 1989; Pfeiffer *et al.*, 1990). The nucleotide sequence of a nuclear gene encoding a protein resembling the CoxVb subunit from beef was characterized in rice (Kadowaki *et al.*, 1996). In summary, besides the three mitochondrial-encoded Cox I, Cox II and Cox III subunits, cytochrome *c* oxidase of plant mitochondria appears to contain quite a number of additional subunits, most are smaller than 10 kDa and probably all are nuclear-encoded. The sequences of only two nuclear-encoded subunits, similar to the Cox Vb and Cox VIII subunits from beef, have been characterized to date in plants.

Better resolution of the small subunits of cytochrome *c* oxidase has been achieved by using 2D Blue-native/SDS-PAGE, which reveals 10 subunits in potato and *Arabidopsis thaliana* (Jänsch *et al.*, 1996; Eubel *et al.*, 2003). For unknown reasons, complex IV from a variety of plant species is visible in two forms on native gels, a larger form termed complex IVa with a native mass of approximately 350 kDa, and a smaller form termed complex IVb of approximately 280 kDa (Eubel *et al.*, 2003; Sabar *et al.*, 2003; Eubel *et al.*, 2004). The larger form includes an additional protein subunit that was identified by mass spectrometry from the bean cytochrome *c* oxidase and resembles the Cox VIb subunit from beef (Eubel *et al.*, 2003). Very recently, 2D Blue-native/SDS-PAGE of digitonin-solubilized mitochondrial fractions from potato revealed the presence of complexes IVa and IVb in respiratory supercomplexes (Eubel *et al.*, 2004).

Complex II has been characterized for bacteria, protozoan, fungi and animals and nearly always is composed of four subunits: SDH1, a hydrophilic protein that carries a FAD group; SDH2, which is also hydrophilic and carries FeS clusters; and SDH3 and SDH4, two small hydrophobic proteins, which anchor the two hydrophilic subunits to the inner mitochondrial membrane and together carry a heme b group (reviewed in Lemire and Oyedotun, 2002). Recently, the structure of complex II from *E. coli* was resolved by X-ray crystallography (Yankovskaya *et al.*, 2003).

Complex II from plant mitochondria has been isolated from tobacco leaves, beet leaves, batatas roots, maize scutulum, bean endosperm and pea cotyledons (Hiatt, 1961; Hattori and Asahi, 1982; Burke *et al.*, 1982; Igamberdiev and Falaleeva, 1994). Only the two soluble SDH1 and SDH2 subunits could be resolved by gel electrophoresis, but genes encoding putative SDH3 and SDH4 were identified in the genome of *Arabidopsis* (Figueroa *et al.*, 2001; 2002). Surprisingly, very recent characterizations of plant mitochondria by 2D Blue-native/SDS PAGE revealed three additional subunits (Eubel *et al.*, 2003). Two of these proteins could be identified by mass spectrometry and do not resemble any known proteins in public databases.

In order to further clarify the protein compositions of complexes II and IV in plant mitochondria, we have attempted to systematically identify subunits of these complexes after separations by 2D Blue-native/SDS-PAGE. Furthermore, peptides of the trypsinated holo-complex purified by Blue-native/Blue-native PAGE were used for subunit identifications by mass spectrometry. Both, cytochrome *c* oxidase and succinate dehydrogenase were found to be unexpectedly complex. Complex IV is composed of more than 10 different protein subunits and complex II is an eight-subunit enzyme. The identity of several new subunits was elucidated, most of which seem to be plant specific components of the two respiratory protein complexes.

Results

Separation of cytochrome c *oxidase and succinate dehydrogenase of* Arabidopsis *by 2D Blue-native/SDS-PAGE*

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Below a native mass of 400 kDa, a range of protein complexes can be resolved by 2D Blue-native/SDS-PAGE of plant mitochondrial membranes. In *Arabidopsis*, this region contains the TOM complex of the outer membrane, closely followed by the cytochrome c oxidase (complex IV) and succinate dehydrogenase (complex II). Figure 1 shows three typical gels highlighting slight variations in the separation of these complexes on the first native dimension and the subsequent separation of these complexes by SDS-PAGE. The primary variations between gels are the abundance of the larger form of cytochrome *c* oxidase (IVa), and the degree of overlap with TOM complex subunits. In the final panel a composite cartoon is depicted, showing the nine protein bands of cytochrome *c* oxidase (1–9) and the eight protein bands of succinate dehydrogenase (15–22), along with the overlapping five protein bands from the TOM complex (10–14).

Identification of subunits of cytochrome c oxidase and succinate dehydrogenase from Arabidopsis

This set of 22 protein bands were excised, trypsinated in-gel and analysed by tandem mass spectrometry to further identify the protein subunits contained by each protein band. Furthermore, selected proteins were also analysed by N-terminal Edman sequencing. The three high molecular mass protein bands from the IVb complex were the products of the mitochondrial-encoded *cox I*, *cox II* and *cox III* genes (Table 1). The further six protein bands (4–9) contained peptides matching to 13 nuclear-encoded proteins. These included six proteins with clear sequence similarity to Cox subunits Vb, Vc and VIa. The additional seven proteins (six different protein types, because one protein is present in two similar forms) are of unknown function and have not previously been associated with cytochrome *c* oxidase in plants. The overlapping putative TOM complex protein bands (10–14) were confirmed by mass spectrometry as TOM40, TOM20, TOM9, TOM7 and TOM5/6.

The eight succinate dehydrogenase protein bands (15–22) contained peptides, each matching to one of eight nuclear-encoded proteins, four have clear sequence similarity to the known SDH1–SDH4 protein products from other organisms. We have previously identified SDH1–3 (Eubel *et al.*, 2003), but the identification of the SDH4 is novel. The remaining four identified proteins appear to be plant specific subunits. The identification of SDH5 (At1g47420) and SDH6

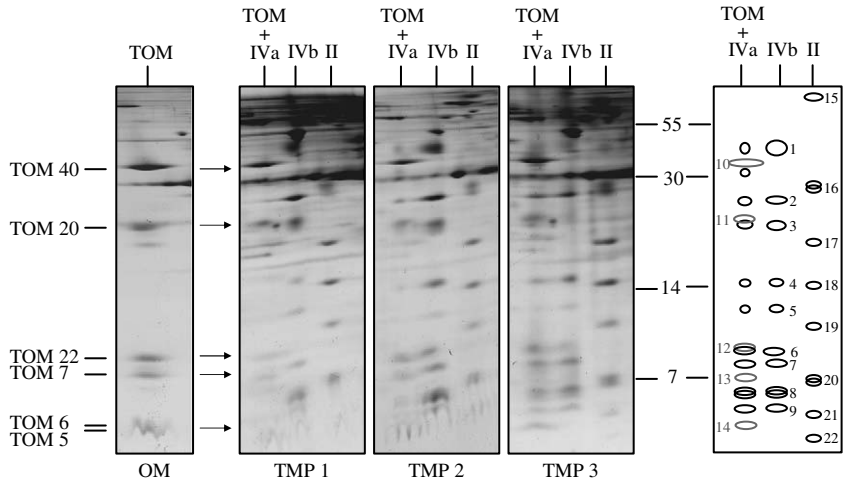

Figure 1. Characterization of protein complexes of Arabidopsis mitochondria in the molecular mass range between 150 and 400 kDa by 2D Blue-native/SDS PAGE. Protein samples represent three different preparations of total mitochondrial protein (TMP1, TMP2, TMP3) and one preparation of outer membrane protein (OM). Molecular masses of standard proteins are given to the right (in kDa) and identities of protein complexes on the top (TOM, translocase of the outer mitochondrial membrane; IVa, large form of cytochrome c oxidase; IVb, small form of cytochrome c oxidase; II, succinate dehydrogenase). Final panel is a cartoon of spots cut out of gels for further analyses. The numbers indicate proteins (results in Table 1).

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Table 1. Identified subunits of complexes II and IV and the TOM complex of *Arabidopsis*

Spot[a]	Gene[b]	Strategy of identification[c]	Protein	MP[d]	Cov[e]	MOWSE score[f]	N-terminal sequence or *De novo* MS seq
1	AtMg01360	ESI	complex IV, subunit 1 (COX I)	1	3	ND	(299.2)FSTNHK
2	AtMg00160	ESI, ED	complex IV, subunit 2 (COX II)	4	11	166	DAAEPWQLGFQDATP
3	AtMg00730	ESI, ED	complex IV, subunit 3 (COX III)	1	3	27	XIESQXXXY
4	At3g15640	ESI	complex IV, subunit 5b (COX Vb)	3	22	73	
5	At1g80230	ESI	complex IV, subunit 5b (COX Vb)	6	32	259	
6	At5g27760	ESI	complex IV, subunit X1	4	44	55	
7a	At4g37830	ESI	complex IV, subunit 6a (COX VIa)	2	14	67	
7b	At3g43410	–	complex IV, subunit X5	3	75	43	
8a	At2g47380	ESI, ED	Complex IV, subunit 5c (COX Vc)	5	64	102	XXXKVAXATLK
	At5g62400						
	At5g61310						
8b	At4g00860	ESI	complex IV, subunit X2b	7	51	247	
8c	At1g01170	ESI	complex IV, subunit X2a	3	38	189	
8d	At4g21105	ESI	Complex IV, subunit X4	3	39	95	
8e	At1g72020	ESI	complex IV, subunit X3	2	14	74	
8f	At2g16460	ESI	complex IV, subunit X6	4	16	112	
9		–	not determined				
10	At3g20000	ESI	TOM40	22	62	607	
11	At3g27080	ESI	TOM20				
	At5g40930	ESI					
12	At5g43970	ESI	TOM22/TOM9	3	46	162	
13	At5g41685	–	TOM7[g]				
14	At1g49410	ESI	TOM5/6	1	24	60	
15	At5g66760	ESI	complex II, subunit 1 (SDH1)	1	3	57	
16	At5g40650	ESI	complex II, subunit 2 (SDH2)	16	40	541	
17	At1g47420	ESI, ED	complex II, subunit 5 (SDH5)	14	43	370	SEDVSHMPEMDSXVL NFK
18	At1g08480	ESI	complex II, subunit 6 (SDH6)	4	28	82	
19	At5g09600	ED	complex II, subunit 3 (SDH3)	1	3	42	STISGDIKTTQEEP
	At5g32210						
20	At3g47833	ESI, ED	Complex II, subunit 7 (SDH7)	5	13	225	FH(I/V)E(P/L)G(T/A)
	At5g62575	–					REKALLAED
21	At2g46505	ESI	complex II, subunit 4 (SDH4)	1	5	32	
22	At2g46390	ED	complex II, subunit 8 (SDH8)	–	–	–	MIYXKXSLLSXPXV

[a] The spot numbers correspond to those given in Figure 1.
[b] Gene nomenclature according to MIPS and TAIR (http://mips.gsf.de/proj/thal/db/index.html, http://www.arabidopsis.org/index.jsp).
[c] Identification strategy: ESI, electrospray ionisation tandem mass spectrometry; ED, Edman degradation.
[d] MP – matching peptides.
[e] coverage (in percent).
[f] MOWSE score from the MASCOT software package (http://www.matrixscience.com/).
[g] Identification of TOM7 was based on gel comparisons (Figure 1) and a previous identification of this protein by mass spectrometry (Werhahn *et al.,* 2001).

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(At1g08480) confirms our previous reports (Eubel *et al.,* 2003), while SDH7 and SDH8 are new identifications.

Comparison of cytochrome c *oxidase and succinate dehydrogenase subunit composition between plant species*

To address whether the apparent subunit complexity of these respiratory chain complexes occurs widely in plants, we considered this less than 400 kDa region on Blue-native/SDS-PAGE gels from a variety of plants compared to *Arabidopsis* (Figure 2). In separations of potato tuber mitochondria protein complexes, a complex IVa was present containing 9–10 protein bands and a complex II consisting of eight protein bands. In potato stem, both complex IVa and IVb were present along with the complex II, again with 9–10 and 8 protein bands, respectively. In bean cotyledon mitochondria, the same pattern emerged, with an 8 band complex II and an 8–10 band complex IVa/IVb.

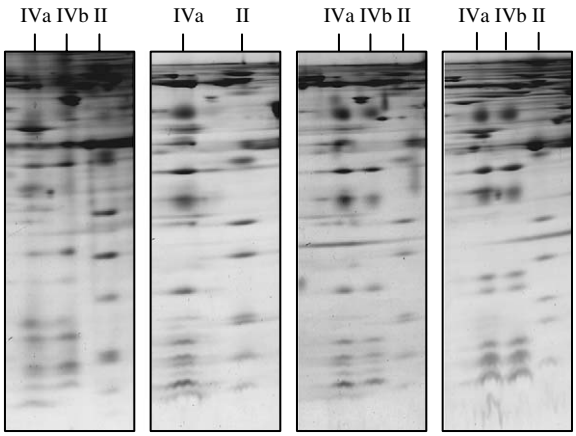

Figure 2. Comparison of protein complexes from Arabidopsis, potato and bean mitochondria by 2D Blue-native/SDS PAGE in the molecular mass range between 150 and 400 kDa. Identities of protein complexes are given above the gels (TOM, translocase of the outer mitochondrial membrane; IVa, large form of cytochrome c oxidase; IVb, small form of cytochrome c oxidase; II, succinate dehydrogenase).

Purification of cytochrome c *oxidase from Arabidopsis by 2D BN/BN-PAGE*

To further characterize complex IV in plants, we performed 2D Blue-native/Blue-native PAGE to move IV complexes away from potential contaminants that can occur due to horizontal streaking of bands in Blue-native/SDS-PAGE separations (Figure 3). First dimension Blue-native PAGE was carried out in the presence of digitonin and 2D Blue-native PAGE in the presence of dodecylmaltoside. Using this experimental system, supercomplexes and singular protein complexes specifically destabilized by dodecylmaltoside treatment are dissected into smaller protein complexes, which are localized below the diagonal line of protein complexes on the 2D gels. Complexes I, III_2 and V proved to be largely stable in the presence of both detergents during 2D Blue-native PAGE and were represented by clear spots on the diagonal. Some low amount of complex V was dissected into defined subcomplexes, one of which represents the F_1 part. Furthermore, the I + III_2 supercomplex was dissected into complexes I and III_2 as reported previously (Eubel *et al*., 2003; Eubel *et al*., 2004). Complexes IVa and IVb both migrated below the diagonal line indicating that subunits are detached in the presence of dodecylmaltoside. The spots representing the two complexes had identical apparent molecular masses and a very diffuse shape when compared to most other mitochondrial protein complexes (Figure 3). Direct in-gel trypsination of the complexes and analysis of peptides by ESI–MS/MS did allow the identification of several of the previously identified subunits of cytochrome *c* oxidase, but also led to the identification of subunits of the TOM complex (not shown). Therefore, although the two forms of complex IV were localized below the diagonal line on Blue-native/Blue-native gels, this gel system did not allow their complete purification for analysis.

Discussion

Purification of complexes II and IV from plants

Besides considerable progress in the understanding of the structure and function of the respiratory chain in plant mitochondria, the molecular composition of complexes II and IV has remained

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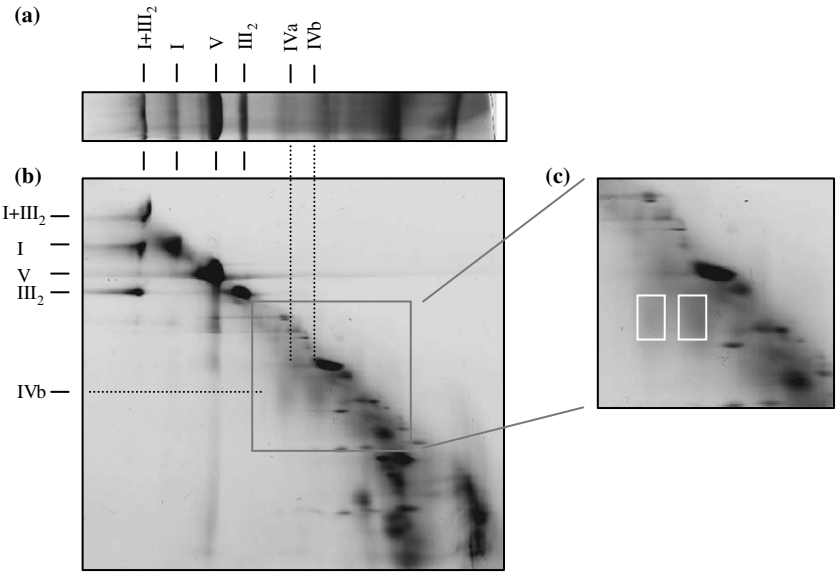

Figure 3. Separation of mitochondrial protein complexes of Arabidopsis by 2D Blue-native/Blue-native PAGE. First dimension gel electrophoresis was carried out in the presence of digitonin, second dimension gel electrophoresis in the presence of dodecylmaltoside. a: 1D Blue-native gel in the presence of digitonin, b: 2D Blue-native / Blue-native gel in the presence of digitonin (first gel dimension) and dodecylmaltoside (second gel dimension), c: enlargement of the inlet indicated in B. Identities of protein complexes and super-complexes are given to the left and on top of the gels.

relatively undefined. For reasons not fully understood, chromatographic procedures carried out by a variety of research groups have failed to substantially purify these two complexes in intact form. Biochemical studies on complex II from plants only led to identification of the two larger SDH1 and SDH2 subunits, which are rather hydrophilic (Hattori and Asahi, 1982; Burke *et al*., 1982). Also, analysis of chromatographically purified complex IV from plants did not allow the identification of more than five subunits besides the three mitochondrial-encoded proteins Cox I, Cox II and Cox III (Matsuoka *et al*., 1981; Nakagawa *et al*., 1987; Hawkesford *et al*., 1989; Pfeiffer *et al*., 1990). Since complex IV from heterotrophic eukaryotes is known to include more than 10 subunits, it can be speculated that additional subunits were lost during purification or not resolved during gel electrophoretic analyses. Analyses of dodecylmaltoside-solubilized mitochondrial fractions from potato by 2D Blue-native PAGE showed 10–12 different subunits in complex IV (Jänsch *et al*., 1996). However, even using this approach, resolution of complex IV was difficult, because it does not form a sharp band on the blue native dimension. Complex IV from *Arabidopsis* is absent on 2D Blue-native/SDS gels using the same experimental system (Kruft *et al*., 2001). Significantly improved resolution has been possible by combining digitonin-based protein solubilizations and 2D Blue-native/SDS-PAGE (Eubel *et al*., 2003). Using this procedure, complex IV from *Arabidopsis* was shown to include 10–12 different subunits and complex II was surprisingly found to have more than the four well-known SDH1–4 components described for this complex in bacteria and heterotrophic eukaryotes. Two-dimensional Blue-native/SDS PAGE of digitonin-treated mitochondrial fractions is currently the most gentle procedure for investigating complexes II and IV in plants. However, even this procedure has clear problems, because a larger the so-called IVa form of the complex is easily broken into a smaller IVb form and because the subunits of the two complex IV forms are less sharply focused on the second gel dimension than the subunits of other respiratory complexes.

We therefore tested a novel purification strategy for cytochrome *c* oxidase, which is based on 2D Blue-native/Blue-native PAGE in the presence of digitonin for the first gel dimension and dodecylmaltoside in the second dimension. However,

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even using this 2D gel system, cytochrome *c* oxidase from plants proved to be fragile and also was represented by especially diffuse spots. Most likely the holo-complex has larger hydrophobic interfaces than the other OXPHOS complexes upon solubilizing mitochondrial membranes.

Newly identified subunits of complexes II and IV

Complex II of *Arabidopsis* was found to be composed of eight different subunits, seven of which were previously detected on Blue-native/SDS gels (Eubel *et al*., 2003). The novel protein has an apparent molecular mass of about 5 kDa and only is weakly stained by Coomassie-blue. Evidence for the occurrence of this eight subunit complex II is very strong, because all subunits are localized exactly in one vertical row on 2D Blue-native/SDS gels in all plants investigated. Furthermore, stoichiometry of all subunits seems to be very similar, with the exception of the very smallest subunit, which might be sub-stoichiometric. However, staining efficiency is known to be low for very small proteins.

The SDH1, SDH2 and SDH3 subunits as well as two plant specific subunits encoded by the At1g47420 and At1g08480 genes were identified previously, whereas the SDH4 protein and the two remaining plant specific subunits encoded by At3g47833/At5g62575 and At2g46390 were first identified in the course of our present study (Table 1). We suggest a nomenclature for the four plant specific complex II subunits to follow on from SDH1–4, namely SDH5, SDH6, SDH7 and SDH8 (Table 1). Apparent molecular masses of the subunits are 65 kDa (SDH1), 29 kDa (SDH2), 18 kDa (SDH5), 15 kDa (SDH6), 12 kDa (SDH3), 7 kDa (SDH7), 6 kDa (SDH4) and 5 kDa (SDH8). All eight subunits are nuclear-encoded and have to be imported into plant mitochondria. Therefore, complex II from plants contains no mitochondrial-encoded subunits, just like complex II from other heterotrophic eukaryotes. This makes complex II the exception in the electron transport chain, because all other OXPHOS complexes include at least one mitochondrial-encoded subunit in all organisms investigated. SDH3 and SDH5 comprise very long mitochondrial targeting sequences of 105 and 89 amino acids (Eubel *et al*., 2003). Also, the SDH4 protein probably has a very long presequence, because the calculated molecular mass of the precursor protein is 10 kDa larger than the apparent molecular mass of this protein on our gels. Based on the same considerations, the SDH1 and SDH2 proteins can be predicted to have presequences of intermediate length (2–5 kDa). Presequences of this length are also found in the SDH6 and SDH7 precursor protein sequences, as determined by N-terminal sequencing of the mature proteins (Table 1). In contrast, the newly discovered SDH8 protein does not have a presequence (Table 1), which was previously reported for several other small nuclear-encoded subunits of respiratory protein complexes (Braun and Schmitz, 1995). In fact the SDH8 protein, which has a calculated molecular mass of 4.9 kDa, is the smallest component of any plant respiratory chain complex described today. Several of the complex II subunits are encoded by more than one gene in *Arabidopsis*. For SDH7, two isoforms could be identified by N-terminal Edman degradation (Table 1).

Analyses of complex IV by mass spectrometry allowed identification of six of the known Cox subunits of other organisms (Cox I, Cox II, Cox III, Cox Vb, Cox Vc and Cox VIa). Furthermore, the Cox VIb subunit was shown to form part of the larger IVa complex in *Arabidopsis* and bean (Eubel *et al*., 2003), which was not identified by MS in the current study. Sequence similarity searches using all sequences of complex IV subunits from yeast and beef identified only one further *Arabidopsis* gene that most likely encodes a Cox subunit (Cox VIc, At3g22210). This protein was not identified in the course of our study and possibly only forms part of the larger IVa complex. Overall it appears that eight of the complex IV subunits of *Arabidopsis* have counterparts in heterotrophic eukaryotes. Unfortunately nomenclature for cytochrome *c* oxidase subunits is very complicated because similar subunits are named differently in yeast and beef (Table 2). Based on the assignments shown in the table, counterparts to the Cox IV (beef)/Cox Va–b (yeast) protein and to the Cox Va (beef)/Cox VI (yeast) protein seem to be absent in plants.

Our study newly identified six further protein types in the 5–10 kDa range, which putatively form part of complex IV in *Arabidopsis*. However, since complex IV is not absolutely pure on 2D BN/SDS gels, but co-migrates with the TOM complex

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Table 2. Subunits of cytochrome c oxidase in beef, yeast and *Arabidopsis*

Beef		Yeast		Arabudopsis		
Cox I	P00396	Cox 1	P00401	Cox I	AtMg01360	
Cox II	P00404	Cox 2	P00410	Cox II	AtMg00160	
Cox III	P00415	Cox 3	P00420	Cox III	AtMg00730,	At2g07687
Cox IV	P00423	Cox 5A	P00424			
		Cox 5B	P00425			
Cox Va	P00426	Cox 6	P00427			
Cox Vb	P00428	Cox 4	P04037	Cox Vb	At3g15640,	At1g80230
Cox VIa	P13182	Cox 13	P32799	Cox Via	At4g37830	
Cox VIb	P00429	Cox 12	Q01519	Cox Vib	At5g57815,	At4g28060
					At1g22450,	At1g32710
Cox VIc	P04038	–		Cox VIc	At3g22210	
Cox VIIa	P07470	–		–	–	
Cox VIIb	P13183	–		–	–	
Cox VIIc	P00430	Cox 8	P04039	–		
Cox VIII	P10175	–		Cox Vc	At2g47380,	At5g62400,
					At5g61310	
–		Cox 7	P10174	–		
–		Cox 9	P07255	–		
–				Cox X1	At5g27760,	At3g05550
–				Cox X2	At4g00860,	At1g01170
–				Cox X3	At1g72020	
–				Cox X4	At4g21105	
–				Cox X5	At3g43410	
–				Cox X6	At2g16460	
13 subunits		11–12 subunits		8 subunits + 6 subunits		?

Designations on the right of the three columns correspond to protein names and designations on the left to protein accession numbers as given in http://www.expasy.org/sport/. Subunits of Arabidopsis were either identified by mass spectrometry (Table 1) or represent isoforms of these subunits, which were identified by searching Arabidopsis protein databases. Cox VIc from Arabidopsis was identified being a homologue of Cox VIc from beef by sequence comparison.

and possibly unknown protein complexes of lower abundance, the assignment of these proteins being subunits of complex IV is, in our view, not definitive and awaits independent confirmatory information. Until further clarification, we suggest these proteins be treated as candidates of complex IV subunits and therefore designated them Cox X1 to Cox X6. As a result, cytochrome *c* oxidase of plants can be assumed to have about 12–14 different subunits and therefore is of comparable complexity to the corresponding complex in yeast (11 subunits) and beef (13 subunits).

Possible functions of plant specific subunits of complexes II and IV

Respiratory protein complexes I and III are known to include plant specific subunits, which integrate side activities into these complexes. Complex III includes the mitochondrial processing peptidase in plants (Braun *et al.*, 1992a; Eriksson *et al.*, 1994), and complex I the terminal enzyme of the mitochondrial ascorbic acid biosynthesis pathway as well as putative carbonic anhydrases (Millar *et al.*, 2003; Heazlewood *et al.*, 2003a; Parisi *et al.*, 2004). The four additional subunits present in succinate dehydrogenase of plant mitochondria may also present a secondary or peripheral activity of this complex. However, sequence comparisons between the SDH5, SDH6, SDH7 and SDH8 subunits with protein databases did not allow identification of similar proteins of known functions. Similar sequences exist in several higher plants, but none have been functionally characterized to date. Therefore, these subunits currently have to be considered to be plant specific proteins of unknown function.

The presequence of the SDH5 protein has very significant sequence similarity to the presequence of one of the putative carbonic anhydrase

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subunits of complex I (At1g47260). Sequence identity in the region of the SDH5 presequence is 66%, whereas identity in the region of the mature SDH5 protein is below 15% (Figure 4). We assume that DNA duplication events took place during mitochondrial presequence acquisition in evolution between the genes encoding these two proteins.

Some of the newly identified candidates for plant specific complex IV subunits exhibit significant sequence similarity to previously characterized proteins. At4g00860 (Cox X2) was previously identified in the course of a study aiming to identify ozone and pathogenesis inducible genes (Sharma and Davis, 1995). The protein was termed AtOZI1 and shown to be induced three to five fold at the transcript level in leaves within 6 h of ozone treatment and sixfold following *Pseudomonas* infection of *Arabidopsis*. The authors noted an N-terminal signal sequence on the predicted protein and putative phosphorylation sites. A similar protein in rice is predicted from genome sequencing (Genbank AAF69008), but no sequences with significant similarity are apparent in non-plant organism. Sharma and Davis (1995) noted that AtOZI1 represented a novel stress protein that accumulates in response to the production of active oxygen species. Our finding of this protein as a putatively novel subunit of cytochrome *c* oxidase opens up new opportunities to interpret these earlier results. Thus AtOZI1 could potentially represent a retrograde signal to COX of electron transport chain damage and/or a signal associated with the ROS activated programmed cell death that requires mitochondria involvement through cytochrome *c* release. Notably, a recently identified complex I subunit in both mammals and plants, GRIM-19, is also a component implicated in ROS activated programmed cell death (Fearnley *et al.*, 2001).

The At5g27760 product (Cox X1) belongs to a family of the so-called 'hypoxia response proteins' found widely among eukaryotic organisms and even in some eubacteria. Hypoxia responses of family members have been documented in fish, human and mouse (Gracey *et al.,* 2001). These data lead to the development of a Pfam domain family (PF04588) that currently contains 33 members, three of which are *Arabidopsis* proteins (At5g27760, At3g05550 and At3g48030). The domain is based on an N-terminal transmembrane region of each protein. Clearly the role of cytochrome *c* oxidase as an oxygen consumption step could be aided or adapted by hypoxia detection. Evidence is already available of hypoxia response in the expression of COX genes in mammals (Dagsgaard *et al.*, 2001). However, no specific roles have yet been defined for any of the PF04588 domain containing proteins to our knowledge.

Overall, we have concluded the identification of complex II subunits and have significantly extended our understanding of complex IV composition. The apparent difficulties with complex IV

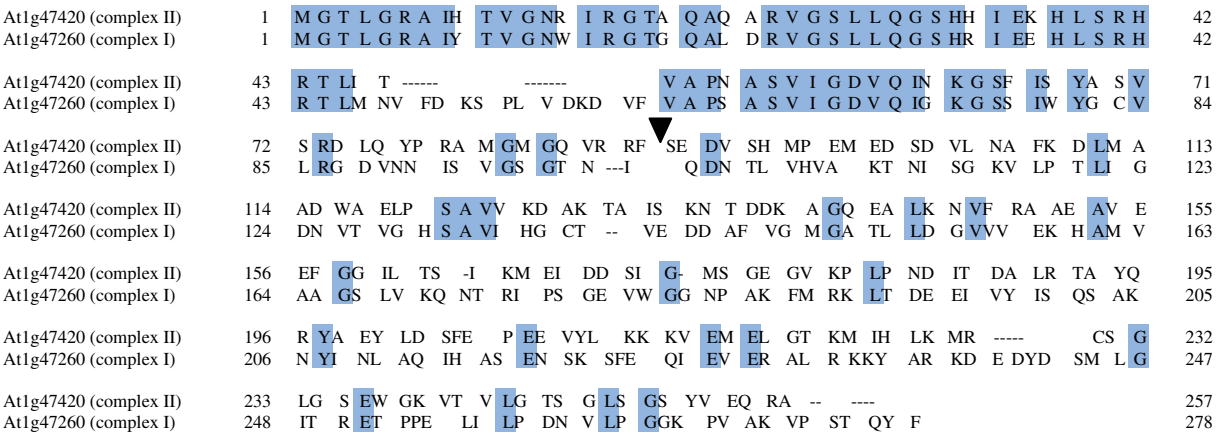

Figure 4. Alignment of the SDH5 subunit (At1g47420) of complex II from Arabidopsis with a putative carbonic anhydrase subunit (At1g47260) of complex I from Arabidopsis. Identical amino acids are indicated in blue. The arrowhead indicates the site for proteolytic processing of SDH5 as determined by cyclic Edman degradation (Table 1).

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purification may well mean that final confirmation of its subunits will need to be obtained by alternative means, such as knock-out removal of putative subunits in *Arabidopsis* and study of the impact of these genetic manipulations on complex IV size, composition and function.

Materials and methods

Isolation of mitochondria from Arabidopsis*, potato and bean*

Mitochondria from *Arabidopsis* were isolated from suspension cell cultures by differential centrifugation and Percoll density gradient centrifugation as described by Werhahn *et al*. (2001) or Millar *et al*. (2001). Using a starting material of about 300 g cells, a typical organelle preparation yields about 300 mg mitochondria, which corresponds to approximately 30 mg mitochondrial protein. Aliquots of 1 mg mitochondrial protein were stored at −80 °C. Mitochondria from potato tuber and stem and bean cotyledons were purified as outlined in Braun *et al*. (1992b).

Two - dimensional BN/SDS-PAGE and BN/BN-PAGE

For gel electrophoresis, aliquots of organelles corresponding to 1 mg mitochondrial protein were directly dissolved in 100 μl digitonin solubilization solution (30 mM HEPES pH 7.4, 150 mM potassium acetate, 10% [v/v] glycerol, 2 mM PMSF, and 5 mg digitonin [Fluka, Buchs, Switzerland]). Proteins were incubated for 20 min on ice and subsequently centrifuged at 18,000 *g* for 30 min to remove insoluble material. Finally supernatants were carefully discarded, supplemented with 20 μl Coomassie-blue solution (5% [w/v] Coomassie-blue in 750 mM aminocaproic acid) and directly loaded into the wells of a blue-native gel (Jänsch *et al*., 1996).

Two-dimensional Blue-native/SDS-PAGE was carried out as recommended by Schägger (2001). Gradient gels of 4.5–16% polyacrylamide were used for the first, and two-step Tricine–SDS gels of 10% and 16% polyacrylamide for the second gel dimension. After completion of electrophoresis runs, gels were stained with Coomassie-colloidal according to Neuhoff *et al*. (1985, 1990). Two-dimensional Blue-native/Blue-native gel electrophoresis was carried as reported by Schägger and Pfeiffer (2000) with the following modifications: (i) time for electrophoresis runs of the first dimension was reduced by 50% to avoid protein complexes remaining within this gel during the second dimension, while the time for electrophoresis run of the second dimension was doubled to obtain optimal resolution of protein complexes and supercomplexes, (ii) fixing of strips of Blue native gels onto second dimension gels was performed using agarose and not by direct polymerisation of strips into the stacking gel.

Protein identifications by mass spectrometry

Proteins of interest were directly cut out from 2D Blue-native/SDS gels, trypsinated and prepared for analyses by Electrospray ionisation tandem mass spectrometry (ESI–MS/MS) as outlined in Eubel *et al*., (2003) or by Heazlewood *et al*. (2003a). Alternatively, protein spots representing entire protein complexes were cut out from BN/BN gels, trypsinated and analysed by liquid chromatography (LC-) ESI–MS/MS as outlined in Heinemeyer *et al*. (2004) and Heazlewood *et al*. (2004). Primary data of MS analyses were analysed using the Mascot server at http://www.matrixscience.com/ searching against the NCBI *Arabidopsis thaliana* database, or the translated TAIR *Arabidopsis* protein set (Ath4 release) analysed in-house using Mascot version 2.0.

Protein identifications by Edman degradation

For N-terminal amino acid determination, proteins separated on 2D BN/SDS gels were electroblotted onto PVDF membranes, cut out and directly analysed by Edman degradation as outlined in Werhahn *et al*., (2001).

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Knockout of a gene encoding a plant specific subunit of complex I (At1g47260) dramatically affects mitochondrial respiration and physiology

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Abstract

Mitochondrial NADH dehydrogenase (complex I) of plants includes quite a number of plant-specific subunits, some of which exhibit sequence similarity to bacterial γ-carbonic anhydrases. A homozygous Arabidopsis *knockout* mutant carrying a T-DNA insertion in a gene encoding one of these subunits (At1g47260) was generated to investigate its physiological role. Isolation of mitochondria and separation of mitochondrial protein complexes by Blue-native polyacrylamide gel electrophoresis or sucrose gradient ultracentrifugation revealed drastically reduced complex I levels in cell suspension cultures. Remaining complex I had normal molecular mass, indicating substitution of the At1g47260 protein by one or several of the structurally related subunits of this respiratory protein complex. Immuno-blotting experiments using polyclonal antibodies directed against the At1g47260 protein indicated its presence within complex I, the $I+III_2$ supercomplex and smaller protein complexes, which most likely represent subcomplexes of complex I. Changes within the mitochondrial proteome of mutant cells were systematically monitored by fluorescence difference gel electrophoresis (DIGE) using 2D Blue-native / SDS and 2D IEF / SDS polyacrylamide gel electrophoresis. Complex I subunits are largely absent within the mitochondrial proteome. Further mitochondrial proteins are reduced in mutant plants, like mitochondrial ferredoxin, others are increased, like formate dehydrogenase. Development of mutant plants was normal under standard growth conditions. However, a suspension cell culture generated from mutant plants exhibited clearly reduced growth rates and respiration. In summary, At1g47260 is important for complex I assembly in plant mitochondria and respiration. A role of At1g47260 in mitochondrial one-carbon metabolism is supported by micro-array analyses.

Key words:

mitochondria, respiratory chain, NADH dehydrogenase complex (complex I), carbonic anhydrase, Arabidopsis

Introduction

The respiratory chain of plant mitochondria has unique features. Electron transport is very much branched due to the presence of "alternative oxidoreductases" which are functional equivalents to cytochrome c oxidase (complex IV) and the Rotenone-sensitive NADH dehydrogenase (complex I) (Siedow and Umbach 1995, Vanlerberghe and McIntosh 1997, Rasmusson et al. 1999, Moller 2002, Moore et al. 2003, Michalecka et al. 2003). Most likely the presence of these enzymes represents the basis for some kind of "overflow protection mechanism" of the oxidative phosphorylation (OXPHOS) system under certain physiological conditions in plant cells. Furthermore, the respiratory protein complexes of plant mitochondria contain a comparatively large number of mitochondrial-encoded subunits (Unseld et al. 1997). As a consequence, assembly of these protein complexes follows special routes in plants. However, the most mysterious features of the respiratory chain in plants are the numerous extra subunits which form part of its electron transfer complexes and which partially introduce side activities into them. For example, the two subunits of the mitochondrial processing peptidase, which cleaves off the presequences of nuclear encoded mitochondrial proteins upon their transport into the organelle, form an integral part of the cyochrome c reductase complex (complex III) of the respiratory chain in plants (Braun et al. 1992, Eriksson et al. 1994, Braun and Schmitz 1995). In several groups of eukaryotes, mitochondrial fatty acid biosynthesis takes place at complex I of the respiratory chain, since the acyl carrier protein (ACP) is integrated into this protein complex (Runswick et al. 1991, Sackmann et al. 1991, Zensen et al. 1992, Rasmusson et al. 1998, Heazlewood et al. 2003). Complex I from plant mitochondria includes even further side activities, because some additionally plant-specific proteins form part of this complex, like L-galactono-1,4-lactone dehydrogenase, which catalyses the terminal step of mitochondrial ascorbate biosynthesis pathway (Millar et al. 2003). Also the complexes II and IV of plant mitochondria include several plant specific subunits, whose functions are not known so far (Eubel et al. 2003, Millar et al. 2004).

Recently, a novel group of structurally related complex I subunits was described for plant mitochondria, which initially were called "ferripyochilin binding proteins" based on sequence homology to a putative bacterial enzyme (Heazlewood et al. 2003). However, this bacterial protein meanwhile turned out to be falsely annotated in sequence databases (Parisi et al. 2004). Novel sequence comparisons and computer modelling approaches led the suggestion that these plant specific complex I subunits represent γ-type carbonic anhydrases (Parisi et al. 2004). Although not functionally proven, this hypothesis currently is the

best working model. A proteomic approach to systematically identify complex I subunits of Arabidopsis after purification of this enzyme complex by 2D Blue-native gel electrophoresis and analysis by mass spectrometry allowed to identify four different isoforms of this protein (At1g47260, At5g66510, At5g63510 and At3g48680) (Heazlewood et al. 2003). A gene encoding a fifth isoform is present in the Arabidopsis nuclear genome (At1g19580). All five members of this protein family have calculated molecular masses of about 30 kDa, but migrate slightly faster on polyacrylamide gels, most likely due to cleavage of mitochondrial targeting sequences. Structurally related subunits were also identified in chromatographically purified complex I fractions from potato and broad bean (Leterme et al. 1993, Herz et al. 1994, Heazlewood et al. 2003) and in electrophoretically purified complex I from Chlamydomonas (Cardol et al. 2004).

In an attempt to better understand the function of these extra subunits of respiratory complex I, homozygous Arabidopsis knock-out lines were generated and characterized on a physiological and biochemical level. Here we report that deletion of the gene encoding At1g47260 has drastic effects on respiration in Arabidopsis cell suspension cultures. Oxygen consumption and growth rates are decreased by 30 – 50% and assembly of mitochondrial complex I by 90%. Amounts of singular complex I subunits are reduced, suggesting specific protein degradation or down-regulation of the corresponding nuclear and mitochondrial genes. At1g47260 is repressed under elevated CO_2 concentrations, supporting a role in mitochondrial carbon metabolism, and seems to be essential for assembly of complex I in plants.

Results

Generation of a homozygous knockout line for locus At1g47260 carrying a single T-DNA insertion

The gene encoding At1g47260 is split into five exons (Fig. 1A). *Knockout* line Salk_010194 (Kan^r) carries a T-DNA insertion within exon 4. The insertion was confirmed by PCR using primers derived from the left border sequence of the T-DNA and an internal region of AT1g47260 (data not shown). To functionally confirm the gene *knockout*, total mRNA from mutant and wild-type plants was extracted and used as template for RT-PCR to amplify transcripts of At1547260. A 700 bp amplification product was visible in wild-type but totally absent in some of the mutant plants. Latter plants were considered to be homozygous with respect to the T-DNA insertion in the gene encoding At1g47260. To exclude plants with secondary T-DNA insertions from future investigations, heterozygous *knockout* plants were

generated by crossing a homozygous ΔAt1g47260 plant with a wild-type plant. Subsequently, 20 seeds were selected, grown in soil and afterwards plants were self-pollinated for seed amplification. Finally, 150 seeds of each of the 20 parental lines were plated onto solid MS medium containing kanamycin. Lines including a single T-DNA insertion were recognized by a germination rate of about 75% (plants homo- and heterozygous with respect to the knock out for At1g47260), whereas lines with higher germination rates were considered to possibly have additional insertions. A *knockout* line carrying a single gene deletion was self-pollinated and homozygous plants with respect to the insertion in At1g47260 were again selected by RT-PCR as described above (Fig. 1B).

Characterization of the phenotype of ΔAt1g47260 plants

Knockout of the gene encoding At1g47260 results in absence of the corresponding transcript (Fig. 1B). However, comparison of the phenotypic properties and developmental stages between ΔAt1g47260 and wild-type plants did not reveal any differences. Under normal growth conditions, mutant plants were not distinguishable from wild-type plants concerning morphology of roots, leafs, stems and flowers (Fig. 2A, data not shown). Also, statistical evaluations of the duration of developmental stages according to Boyes et al. (2001) did not reveal any significant differences (Fig. 2B). Furthermore, the volume of produced seeds was very similar for mutant and wild-type plants. Both lines exhibited a very comparable fertility. Finally, mutant and wild-type plants were compared upon cultivation on solid agarose for two weeks in the total absence of light or under normal light conditions but in the presence of 10 or 100 μM salicylic acid. Also under these conditions, the two lines were phenotypically not distinguishable (data not shown). In summary, conditions to allow visualization of the molecular differences between wild-type and ΔAt1g47260 plants on a phenotytic or developmental level could not be defined so far.

Characterization of a cell suspension culture for ΔAt1g47260

Cell suspension cultures were established to compare mitochondria of mutant and wild-type plants on a molecular level. Cell suspension cultures proved to be a very helpful tool for mitochondrial research in Arabidopsis, because they allow the preparation of highly pure organelles (May and Leaver 1993, Davy de Virville 1994, Davy de Virville 1998, Werhahn et al. 2001). Proteome projects were initiated to

systematically characterize the protein complement of Arabidopsis mitochondria isolated from this source, which represent a very fruitful data background for the exploration of mitochondria in plants (Kruft et al. 2001, Millar et al. 2001, Werhahn and Braun 2002, Millar and Heazlewood 2003, Giege et al. 2003, Heazlewood et al. 2004, Brugière et al. 2004, Eubel et al. 2004). Cell cultures from mutant and wild-type lines have a similar particle size and colour (Fig. 3A). However, the growth rate of the ΔAt1g47260 cell line was reduced by 30 – 45% (Fig. 3B). Also, respiration of mutant cells (KCN sensitive oxygen consumption) was decreased by about 50% (Fig. 3C).

Deletion of At1g47260 causes drastic reduction of mitochondrial complex I

One-dimensional Blue-native PAGE was carried out to monitor and quantify respiratory protein complexes in mutant and wild-type Arabidopsis cell lines. Solubilization of protein complexes prior to gel electrophoresis was based on incubation of isolated mitochondria with digitonin, because this detergent proved to be most suitable for the stabilization of mitochondrial protein complexes and supercomplexes (Schägger and Pfeiffer 2000, Eubel et al. 2003). Deletion of the gene encoding At1g47260 leads to a dramatic reduction of complex I on Blue-native gels, which is estimated to lie in the range of 90% (Fig. 4A). Furthermore, the $I+III_2$ supercomplex (Eubel et al. 2003) is absent in the protein fractions of the mutant cell line. Remaining complex I has a molecular mass similar to the one from wild-type cells, possibly indicating that some of the other structurally related putative carbonic anhydrase subunits might replace the At1g47260 protein. Abundances of all other protein complexes is largely unchanged on the Blue-native gels between mutant and wild-type cells, except for the formate dehydrogenase complex, which is induced in protein fractions of mutant cells (Fig. 4A). Complex I activity is dramatically reduced in the protein fractions of the mutant cell line, as monitored by an *in-gel* activity assay for this protein complex (Fig. 4B).

Complex I from ΔAt1g47260 cells lacks only the subunit encoded by the deleted gene

Two-dimensional Blue-native / SDS PAGE was performed to compare the subunit composition of complex I in ΔAt1g47260 and wild-type Arabidopsis cells. Based on Coomassie-stained gels, the subunits of complex I and the I+III_2 supercomplex are hardly visible on the 2D gel for the mutant cell line (Fig. 5A). Therefore, 2D gels were blotted and immune-stained with an antibody generated against the over-expressed At1g47260 protein (Fig. 5C). The antibody strongly recognizes two closely co-migrating proteins in the 28 kDa range of wild-type cells which most likely represent At1g47260 and one of the structurally related other putative carbonic anhydrase subunits of complex I. The immune reaction mainly is visible in the region of complex I and the I+III_2 supercomplex, but exhibits some horizontal streaking on the 2D gels in Fig. 5C, possibly indicating that further carbonic anhydrase complexes of minor abundances occur in plant mitochondria independently of complex I. In ΔAt1g47260 cells, no immune-signal is visible (Fig. 5D). In conclusion, Coomassie and immune-stains of 2D separations of mitochondrial proteins on 2D Blue-native / SDS gels confirm the dramatic effect of the deletion of the gene encoding At1g47260 on complex I and the I-III_2 supercomplex. However, some low amount of complex I is still present in protein fractions of the mutant cell line, which can be visualised on 2D Blue-native / SDS gels upon silver staining (Fig. 6). Complex I of the mutant cell line has an identical subunit composition than the same complex in the wild-type line, but lacks a subunit in the 28 kDa range which most likely represents the At1g47260 protein.

Purification of mitochondrial complex I from mutant and wild-type cells by sucrose gradient centrifugation

Since a minor fraction of the At1g47260 protein was not associated with complex I or the I+III_2 supercomplex on Blue-native / SDS gels upon immune-staining, digitonin-solubilized mitochondrial fractions were separated by sucrose gradient ultracentrifugation to test the mitochondrial localization of the At1g47260 protein on the basis of an independent biochemical separation procedure. Conditions were optimized to resolve protein complexes in the 100 to 1500 kDa range. After ultracentrifugation, gradients were subdivided into 10 fractions and aliquots of all of them were analysed by 1D BN-PAGE to monitor the resolution of protein complexes. For wild-type cells, the I+III_2 supercomplex and singular complex I were mainly present in fractions 1 and 2, whereas complexes III and V had peaks in fraction 3 (Fig. 7A).

As expected, $I+III_2$ supercomplex and singular complex I were largely absent in the corresponding fractions of the sucrose gradient of ΔAt1g47260 cells (Fig. 7B). Direct analysis of the fractions of the sucrose gradients by 1D SDS-PAGE (Fig. 7 C, D) and subsequent immunoblotting (Fig. 7 E, F) revealed highest amounts of the At1g47260 protein in the fractions containing $I+III_2$ supercomplex and singular complex I. However, the immune-positive bands at 28 kDa also were visible in fractions containing smaller protein complexes. As expected, the corresponding immune signal was largely absent in the protein fractions of the ΔAt1g47260 cells (the faint signal at 28 kDa protein in fraction 1 most likely represents a crossreaction with one of the structurally related carbonic anhydrase subunits present in complex I). In conclusion, most of the At1g47260 protein is associated with complex I upon fractionation of mitochondrial protein complexes by Blue-native gel electrophoresis or sucrose gradient ultracentrifugation. However, a smaller proportion of the protein migrates slower upon sucrose gradient ultracentrifugation and faster on Blue-native gels. It currently cannot be decided whether the At47260 protein also occurs separately from complex I under *in vivo* conditions, or whether it artificially aggregates upon solubilization of mitochondrial proteins by digitonin.

Monitoring differences between the mitochondrial proteomes of ΔAt1g47260 and wild-type cells

2D fluorescence difference gel electrophoresis (2D DIGE) was employed to systematically monitor changes in the mitochondrial proteome of ΔAt1g47260 cells. For this procedure, digitonin-treated mitochondrial fractions from mutant and wild-type cell lines were incubated with two different fluorescent dyes, which covalently bind to proteins. Subsequently, the two protein fractions were mixed and resolved on a single 2D Blue-native / SDS gel. Finally the gel was scanned at two different wavelengths and protein ratios in the two fractions were quantified using the DeCyder software tool. On overlapping images, proteins of equal amounts are yellow, proteins reduced in the mutant are green and proteins increased in the mutant are red (Fig. 8A). As expected, $I+III_2$ supercomplex and singular complex I are reduced in the mutant cell line. Reduction was quantitatively determined and found to lie at about 90%, which is in accordance with previous estimations on the basis of Coomassie-stains (Fig. 4 and 5). The ATP synthase complex (complex V), dimeric complex III and complex IV as well as the HSP60 complex were unchanged in the mutant cell line.

Reduction of $I+III_2$ supercomplex and complex I in the mutant cell line could either be the consequence of decreased stability of these protein complexes during Blue-native PAGE, or of true reduction of their

subunits (by specific protein degradation or down-regulation of the corresponding nuclear and mitochondrial genes). To distinguish between these two possibilities, 2D DIGE was performed in combination with 2D IEF / SDS PAGE (Fig. 8B). About 50 proteins specifically were reduced in the mutant cell line (green spots) and a few others are present in increased amounts (red spots). Some of the reduced proteins were previously identified in the course of the Arabidopsis mitochondrial proteome project (Kruft et al. 2001, www.gartenbau.uni-hannover.de/genetik/AMPP) and represent known subunits of complex I (At5g37510, At5g52840, At3g48680 and At3g63510). The degree of reduction of these subunits was in a similar range like previously determined for the DIGE experiment on the 2D Blue-native / SDS gel. In conclusion, reduction of complex I in mutant cell lines is due to true down-regulation of complex I subunits and not to decreased stability of complex I during Blue-native PAGE. Some of the proteins reduced in the mutant cell line do not form part of complex I, e.g. adrenodoxin (mitochondrial ferredoxin, At5g52840).

Expression of alternative oxidoreductases is similar in ΔAt47260 and wild-type cells

Immunoblotting experiments were carried out to monitor changes in expression of alternative oxidase (AOX) and Rotenone-insensitive NAD(P)H dehydrogenases in the ΔAt47260 cell line. Total mitochondrial protein of mutant and wild-type cells was separated by 2D Blue-native / SDS PAGE and blotted onto nitrocellulose. Blots were immune-stained using antibodies directed against AOX from *Sauromatum guttatum* (Elthon et al. 1989) and the alternative NAD(P)H dehydrogenases NDA and NDB from potato (Rasmusson and Agius 2001). All three antibodies recognized proteins of expected molecular masses: dimeric AOX at about 65 kDa, NDB at about 60 kDa and NDA at about 53 kDa (Fig. 9C-9H). Stained proteins are represented by horizontal streaks, indicating their presence in higher order structures during first dimension Blue-native PAGE. Expression of all three enzymes was similar in wild-type and ΔAt47260 cells (Fig. 9C-9H). Also, oxygen consumption measurements using isolated mitochondria revealed similar rates of alternative (SHAM-sensitive) respiration for wild-type and mutant cell lines (data not shown).

Discussion

Reverse genetic approach to investigate the function of plant-specific subunits of complex I

Mitochondrial complex I was isolated and characterized previously for broad bean, potato, red beet, sugarbeet and wheat (Leterme and Boutry 1993, Herz et al. 1994, Rasmusson et al. 1994, Trost et al. 1995, Jänsch et al. 1996, Combettes and Grienenberger 1999, reviewed in Rasmusson et al. 1998). Very recently, subunits of this complex were systematically identified for Arabidospis, rice and Chlamydomonas by mass spectrometry (Heazlewood et al. 2003, Cardol et al. 2004). In the course of these studies, about 9 plant specific subunits of complex I were discovered. Several of them have low molecular masses (6 – 12 kDa) and it currently cannot be excluded that they correspond to known complex I subunits of animals or fungi but were not recognized due to low primary sequence conservation. However, another group of plant-specific complex I subunits includes three to five structurally related proteins in the 30 kDa range, which clearly are absent in complex I preparations from any other group of organisms. Based on sequence comparisons and computer modelling, these proteins were suggested to represent carbonic anhydrases of the γ-type (Parisi et al. 2004). Homozygous *knockout* plants were now generated to functionally characterize these subunits. Here we report the characterization of the first such mutant, which includes a T-DNA insertion in the gene encoding At1g47260. Initially, a peripheral localization of the protein was assumed due to its plant-specific occurrence. However, surprisingly the gene deletion causes a drastic reduction of complex I in cell suspension cultures by 90%, possibly reflecting an integral position of the protein (Fig. 4, 5, 8). Complex I subunits are shown to be reduced within the total mitochondrial proteome from Arabidopsis as monitored by 2D IEF / SDS PAGE, indicating their efficient degradation or the down-regulation of the corresponding nuclear and mitochondrial genes. Deletion of the gene encoding At1g47260 has clear effects on the composition of the mitochondrial proteome (Fig. 8).

Submitochondrial localization of At1g47260

While localization of At1g47260 and other structurally related proteins within complex I in plants has now been established by various biochemical and genetic investigations (Leterme et al. 1993, Herz et al. 1994, Heazlewood et al. 2003, Cardol et al. 2004, this study), an additional localization within

mitochondria detached from complex I can currently not be excluded. Immunological localization of At1g47260 on blots of 2D Blue-native / SDS gels revealed its presence within the $I+III_2$ supercomplex and complex I but also in other gel regions representing protein complexes of lower molecular mass (Fig. 5). A very similar result was obtained upon resolution of mitochondrial protein complexes by sucrose density centrifugation (Fig. 7). However, both experiments were based on solubilization of mitochondrial membrane proteins by digitonin, which might lead to some artificial aggregations of membrane proteins. Alternatively, presence of At1g47260 in gel regions representing smaller protein complexes might be caused by partial degradation of complex I into subcomplexes. Indeed the DIGE experiment revealed some complex I subunits in lower molecular mass regions on the Blue-native / SDS gel in Fig. 8. We conclude that possibly all of At1g47260 is localized within singular complex I or the supercomplex-bound form of this respiratory protein complex.

The function of At1g47260

At1g47260 and related subunits of complex I exhibit significant sequence identity to a prokaryotic type carbonic anhydrase, which first was identified in the archeabacterium *Methanosarcina thermophila* (Alber and Ferry 1994) and which was designated γ-type to distinguish it from previously defined other types of carbonic anhydrases (Hewett-Emmett and Tashian 1996). Sequence identity between the γ-carbonic anhydrase and the plant-specific complex I subunits lies in the range of 30% (Parisi et al. 2004). Furthermore, functionally important residues are completely conserved in At1g47260, e.g. three histidine residues involved in coordinating a Zn cofactor necessary of enzymatic activity. Structure of the γ-carbonic anhydrase from *Methanosarcina thermophila* was recently resolved by x-ray crystallography at atomic resolution (Iverson et al. 2000). Computer modelling allows to predict a very similar structure for At1g47260 (Parisi et al. 2004).

Involvement of At1g47260 in CO_2 metabolism is supported by transcriptome analyses for Arabidopsis. Currently, 294 DNA Arabidopsis microarray experiments are publically available at Stanford-Microarray Database (http://genome-www5.stanford.edu/cgi-bin/scriptIndex.pl). Concerning the gene encoding At1g47260, expression is very constant under all physiological conditions tested. However, the gene was clearly repressed (> 80%) if Arabidopsis was cultivated in the presence of an elevated CO_2 concentration

(700 ppm) (Fig. 10). The same result was obtained for At1g19580, which is one of the other plant-specific subunits of complex I related to At1g47260 (data not shown). …

Direct physiological evidence of carbonic anhydrase activity of At1g47260 is still lacking. Enzyme assays were carried out using At1g47260 protein overexpressed in *E. coli*, mitochondrial extracts from Arabidopsis, or complex I isolated by sucrose density gradient ultracentrifugation (data not shown). Also, an *in-gel* enzyme assay using Blue-native gels was employed to investigate carbonic anhydrase activity of complex I. This assay is based on monitoring a local pH shift within a gel at the location of a carbonic anhydrase band in the presence of excess of CO_2 due to its conversion to HCO_3^- (Galvez et al. 2000). So far, carbonic anhydrase activity of complex I from plants could not be demonstrated. Possibly, Coomasie-blue within gels interferes with this enzymatic acitivity. Furthermore, treatment of mitochondrial fractions with digitonin might not be compatible with carbonic anhydrase activity and the *E. coli* overexpressed form of At1g47260 might not have the correct three-dimensional conformation. It therefore currently can not be excluded that At1g47260 has a different physiological role within complex I of plants. However, carbonic anhydrase activity of At1g47260 is strongly supported by sequence comparison, computer modelling and the altered expression of its gene in the presence of varying CO_2 concentrations during cultivation of Arabidopsis. Indirect evidence for involvement of At1g47260 in mitochondrial one-carbon metabolism also comes from the observation that formate dehydrogense is upregulated in the ΔAt1g47260 line (Fig. 4).

What could be the physiological role of γ-carbonic anhydrases in plant mitochondria? Carbon metabolism of plant mitochondria has special features. Large amounts of CO_2 / HCO_3^- are liberated in plant mitochondria not only due to the decarboxylation of citric acid intermediates, but also by the conversion of two molecules glycine into serine during photorespiration and by the direct conversion of malate into pyruvate by the malic enzyme. Possibly carbonic anhydrases in plant mitochondria are important to avoid acidification of the mitochondrial matrix by HCO_3^-, which would reduce the electrochemical gradient across the inner mitochondrial membrane

For sure At1g47260 is very important for assembly of complex I. Deletion of the gene encoding this protein causes reduction of complex I levels by about 90%. The remaining complex I seems to have an identical molecular mass on Blue-native gels, indicating that the At1g47260 protein possibly is replaced to some degree by one or several of the structurally related plant-specific subunits of complex I.

The role of complex I in plant cells

Mutants concerning mitochondrial complex I in plants were characterized previously and gave interesting insights into the physiological role of this complex. A well investigated mutant is the "CMSII" line of tobacco, which was generated by the regeneration of plants through two successive cycles of protoplast culture (Li et al. 1988). The mutant carries a deletion on the mitochondrial genome in the region of the NAD7 gene encoding a 40 kDa subunit of complex I (Pla et al. 1995). As a result, NAD7 and other complex I subunits are missing and activity of complex I (Rotenone-sensitive oxygen uptake of isolated mitochondria) is reduced under certain conditions, especially if glycine is used as a substrate (Gutierres et al. 1997, Sabar et al. 2000). At the same time, oxidation rates for exogenous NADH and activity of alternative oxidase (AOX) are induced. Mutant plants develop slowly and exhibit partial male sterility, which becomes complete under low light. Furthermore, photosynthesis is reduced by 20 to 30% under atmospheric CO_2 levels (Dutilleul et al. 2003a). Absence of complex I was also reported to affect the redox balance within plant cells, induce enzymes of the cellular antioxidant response and cause a higher tolerance to abiotic and biotic stresses (Dutilleul et al. 2003b, Noctor et al. 2004).

Another interesting complex I mutant is the "NCS2" line of maize, which carries a deletion in the mitochondrial NAD4 gene and has reduced complex I activity (Marienfeld and Newton 1994). Mutant plants can be maintained at a heteroplasmic state and show an impaired development resulting in striped leaves. Mitochondria of mutant plants include a smaller version of complex I, which is enzymatically active (Karpova and Newton 1999). Compensatory increase in the activities or amounts of exogenous NAD(P)H dehydrogenase was not observed. Recently, a mutant affecting a nuclear encoded 18 kDa FeS subunit of complex I from Arabidopsis was found to cause reduced capacity for cold acclimation and accumulation of reactive oxygen species (Lee et al. 2002).

Surprisingly, ΔAt1g47260 plants did not show a phenotype under all conditions tested, although the complex I level in cell suspension cultures is reduced by about 90%. Furthermore, levels of alternative NAD(P)H dehydrogenases and alternative oxidase are not enhanced in the cultures (Fig. 9). Most likely complex I activity is not rate limiting for respiratory electron transport in Arabidopsis under the conditions applied. Consequently, plants also had normal fertility. In contrast, oxygen consumption and growth rate of a mutant cell suspension culture was clearly reduced, indicating a limiting role of complex

I during optimal growth conditions. Interestingly, mitochondrial ferredoxin is downregulated in the mutant, pointing to an altered redox state of the mitochondrial matrix.

The ΔAt1g47260 line certainly is a very interesting tool to further address the function of complex I in plant cells. Also, it might offer to investigate cross-talk between the mitochondrial and the nuclear genome in plants, because deletion of the gene encoding At1g47260 seems to result in specific down-regulation of other nuclear and mitochondrial genes.

Materials and Methods

Selection of an Arabidopsis knockout line for At1g47260

Seeds of an Arabidopsis *knockout* line for locus At1g47260 were obtained from ABRC (T-DNA insertion line Salk_010194, Kan^r). Mutant and wild-type plants (Arabidopsis ecotype "Columbia") were grown in soil in a growth chamber at 16-h light (22°) and 8-h dark (20°) periods and 50% relative humidity. Plants carrying the T-DNA insertion at At1g47260 were selected by PCR using genomic DNA extracted from leaves by the CTAB method and primers 5`-GCGTGGACCGCTTGCTGCAACT-3` (specific for the T-DNA left border) and 5`-CACTCGAGTGGGAACCCTAGGA-3` (specific for At1g47260). PCR settings were 1 cycle 94 °C for 1 min, 35 cycles 94 °C for 1 min, 55 °C for 2 min, 72 °C for X min, and a final extension at 72 °C for 10 min. An amplification fragment of 925 bp was obtained for mutant plants.

RNA Extraction and RT-PCR

RNA of mutant and wild-type plants was isolated from leaves using the Trizol method. CopyDNA fragments were amplified by the OneStep RT-PCR kit (Qiagen®) using 1 µg RNA and primers APF1minus40 (5'-ATACATATGAGGCATCGGACGTTG-3') and PAF5th (5'-GACTCGAGTTAGAAGTACTGAG-3') for At1g47260 or primers ATHACT2M (5´-CTT GGAGATCCACATCTGC-3´) and ATHACT2P (5´-TGGCATCACACTTTCTACA-3´) for actin2. RT-PCR settings were 1 cycle 43°C for 30 min, 1 step 95°C for 3 min and 40 cycles for 45s 95°C, 45s 55°C,

45s 72 °C, and a final step at 72 °C for 5 min. Amplification products were separated by gel electrophoresis on 1% agarose gels

Selection and characterization of homzygeous mutant lines carrying a single T-DNA insertion

Plants lacking the At1g47260 transcript were considered to be homozygous with respect to the T-DNA insertion at the corresponding locus. One plant was selected and backcrossed with a wild-type plant to generate heterozygous plants. Twenty individual seeds from this crossing were selected, grown in soil (pots of 15 cm Ø) and resulting plants were self-pollinated for seed amplification. Subsequently, 150 surface-sterilized seeds of each of the 20 lines were germinated on petri dishes containing solid MS medium including 50µg l^{-1} kanamycin. After one week, the ratio of resistant / non-resistant seedlings from each petri dish was determined. Arabidopsis lines showing a ratio of 3:1 were considered to include a single T-DNA insertion. One line was selected, grown in the green house and seeds were collected after self-pollination. Homozygous plants with respect to the insertion at At1g47260 were again selected by RT-PCR as described above.

For phenotype characterizations, wild-type and mutant plants were sowed in soil (pots of 15 cm Ø), placed for 3 days at 4°C to synchronize germination and finally grown until senescence at a 16 h / 8 h light-dark period, 22 and 20°C day/night temperatures and 60% humidity. All measurements were taken day by day for 20 individuals per genotype. Growth stages were classified according to Boyes et al. 2001..

Cell suspension cultures

Arabidopsis suspension cultures were established from wild-type and ΔAt1g47260 Arabidopsis lines as outlined by May and Leaver (1993): plants were grown under sterile conditions on solid MS medium. About one week after germination, short stem pieces were cut and transferred onto solid B5 medium (Gamborg et al, 1968) supplemented with glucose (20 g/l), kinetin (50 mg/l), 2,4D MES (0.5 g/l) and grown at 24 °C in the dark. After another three to four weeks, callus was transferred into liquid suspension cell medium (MS medium supplemented with 3 % sucrose, nicotinic acid [0.5 mg/l],

pyridoxol-HCl [0.5 mg/l], thiamine-HCl [100 µg/l], myo-inositol [100 mg/l], 2,4-dichlorphenoxyacetic acid [1 mg/l] and ampicillin [100 mg/l], pH 5.7) and cultivated in the dark at 24°C, 30% humidity and gentle shaking (90 rpm). Cells were transferred once a week into fresh suspension cell medium. Growth rates of the suspension cell cultures were determined by weight determinations at days 3, 5 and 7. Oxygen uptake of suspension cells were analysed using a Clark-type oxygen electrode with an reaction chamber volume of 2 ml (Oxygraph, Hansatech, Norfolk, England) in a reaction buffer containing 0.3 M mannitol, 10 mM K_2HPO_4 (pH 7.2), 10 mM KCl, 5 mM $MgCl_2$, +/- 5mM KCN.

Preparation of mitochondria

Starting material for mitochondrial preparations were about 100 g of Arabidopsis cells from cell suspensions. Cell suspension cultures were filtered trough two layers of gaze and dissolved in grinding buffer (450 mM sucrose, 15 mM MOPS [3-(*N*-morpholino)-propanesulfonic acid], 1.5 mM EGTA, 0.2 % [w/v] bovine serum albumin [BSA], 0.6 % [w/v] polyvinylpyrrolidone 40, 0.2 mM phenylmethylsulfonyl fluoride [PMSF] and 14.3 mM ß-mercaptoethanol/KOH, pH 7.4). Cells were homogenized three times for 15 s in a Waring blender (once at high speed; twice at low speed) and subsequently mitochondria were enriched by a three-step centrifugation procedure at 3000xg for 5 minutes (organelles in supernatant), 8200xg for 5 minutes (organelles in supernatant) and 17000xg for 10 minutes (organelles in pellet). Afterwards, mitochondria were resuspended in washing buffer (300 mM sucrose, 10 mM MOPS, 1 mM EGTA and 0.2 mM PMSF/KOH, pH 7.2), dispersed using a Dounce homogenizer (two strokes) and placed on top of 30 ml Percoll gradients (10 ml of 18 %, 23 % and 40 % Percoll [v/v] in 0.3 M sucrose and 10 mM MOPS/KOH, pH 7.2). After ultracentrifugation for 45 min at 70000xg the mitochondria can be isolated from the 23 % / 40 % interphase of the gradients. Percoll was subsequently removed by sedimentation of the organelles three times at 14500xg for 10 minutes and resuspension in storage buffer (400 mM mannitol, 1 mM EGTA, 10 mM Tricine and 0.2 mM PMSF/KOH pH 7.2). Purified organelles were either directly used for biochemical analyses or shock frozen and stored at –80°C.

Gel electrophoresis procedures

1D SDS-PAGE was carried out according to Schägger and von Jagow (1987) and 1D Blue-native PAGE as outlined by Schägger (2001). Protein solubilization for Blue-native PAGE was performed using digitonin at a concentration of 5 µg per µg mitochondrial protein as given in Eubel et al. 2003. Blue-

native separation of protein complexes was carried out in gradient gels of 4.5 – 16 % polyacrylamide. 2D Blue-native / SDS PAGE followed a protocol given by Schägger (2001). Conditions for 2D IEF / SDS PAGE were adopted from Kruft et al. 2001. All 1D and 2D gels were either stained using the Coomassie-colloidal procedure (Neuhoff et al. 1985, 1990) or by silver (Heukeshoven and Dernick 1986). Activity staining for complex I was carried out according to Zerbetto et al. (1997).

2D fluorescence difference gel electrophoresis (2D DIGE)

2D DIGE was performed using CyDye Fluors from Amersham Biosciences, Freiburg, Germany. Freshly prepared mitochondria from mutant and wild-type Arabidopsis lines (about 500 µg mitochondrial protein / line) were centrifuged for 10 minutes at 14000xg.

For 2D Blue-native / SDS PAGE, the pellet was resuspended in 50 µl of a buffer containing 30 mM HEPES / pH 7.4, 150 mM potassium acetate, 10 % glycerole and 5 % digitonin. After incubation for 20 minutes on ice, the samples were centrifuged for 10 minutes at 18300xg for removal of unsolubilized material. The pH value required for the labelling reaction was adjusted by adding two volumes of the same solubilization buffer set to a pH of 9.0. Checking of pH value was performed by using pH strips. 10µl of the individual protein samples were incubated for 30 minutes in the dark with 1 µl of a 400 µM Cy2Dye or Cy5Dye solution (Amersham Biosciences) prepared according to the manufacturer's instructions for minimal labelling conditions. Labelling reactions were stopped by addition of 1 µl lysine solution (10 mM) and incubation for 10 minutes in the dark. Samples from mutant and wild-type plants were now mixed, supplemented with 1 µl of a 5 % Coomassie-blue solution (5 % Serva blue G, 750 mM aminocaproic acid) and directly loaded onto the gel. Conditions for 2D Blue-native / SDS PAGE were adopted from Schägger (2001)

For 2D IEF / SDS PAGE, samples were resuspended in 50µl "lysis solution" (8 M urea, 4 % CHAPS, 40 mM Tris base, 0.1 mM PMSF). After checking the pH value, minimal staining and termination of the reaction of 10 µl of each sample were performed as described for BN-PAGE. Subsequently 10 µl "lysis solution" plus 100 mM DTT were added to each sample. Samples were pooled, supplemented with 300µl rehydration buffer (8 M urea, 2 % CHAPS, 40 mM Tris base, 40 mM DTT, 10 µl of IPG buffer, a trace of bromophenol blue) and finally loaded onto an IPG strip pH 3-10 NL (Amersham Biosciences). Conditions for 2D IEF / SDS PAGE were taken from Kruft et al. (2001).

After completion of electrophoresis runs, 2D gels were scanned using the Typhoon laser scanner at two different wavelength (Amersham Biosciences). Protein quantification was carried out using the DeCyder software tool (Amersham Biosciences).

Antibody production and immunoblotting experiments

An antibody directed to the C-terminal region of At1g47260 (amino acid 208 to 278) was generated by over-expressing a corresponding DNA fragment in *E. coli* using the pET24a expression vector (Novagen, Madison, WI). The DNA fragment was amplified by PCR using 5'-TTGGATCCATCTCGCACAGATTC-3' and 5'-GAGGATCCGTAGAAGTACTG-3' primers. Isolation of overexpressed protein and rabbit immunization was carried out following standard procedures. Production of a second antibody directed against the full-length sequence of At1g47260 was described previously (Perales et al. 2004). For immunoblotting experiments, gels were blotted onto nitrocellulose membranes in transfer buffer (20 mM Tris base, 20% [v/v] methanol, and 150 mM glycine). Blots were incubated over night with antibodies directed against At1g47260, alternative oxidase (AOX) or the Rotenone insensitive NAD(P)H dehydrogenases NDA and NDB in incubation buffer (100 mM TrisHCl/pH 7.4, 0.1 % Tween20, 150 mM NaCl). Staining of immune-positive bands was carried out using the Vectastain ABC-Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Separation of mitochondrial protein complexes by sucrose gradient ultracentrifugation

About 0.1 g freshly prepared mitochondria from Arabidopsis (protein concentration: 10 mg/ml) were centrifuged for 10 minutes at 14000xg and resuspended in a buffer containing 30 mM HEPES/pH 7.4, 150 mM potassium acetate, 10 % glycerine and 5 % digitonin. After incubation for 1 hour the sample was centrifuged for 10 minutes at 25000xg for removal of unsolubilized material. The supernatant (about 1 ml) was transferred onto a single sucrose gradient (volume: 11.5 ml) composed of 15 mM TrisBase/pH 7.0, 20 mM KCl, 0.2 % digitonin and 0.3 to 1.5 M sucrose and centrifuged at 150000xg for 20 h. Afterwards fractions of 800 µl were removed from the gradient from bottom to top using a needle connected via a hose to a peristaltic pump and collected automatically. 50 µl of each fraction was

supplemented with Coomassie blue solution and loaded onto a 1D Blue-native gel to separate mitochondrial protein complexes.

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Figure legends:

Figure 1: Characterization of Arabidopsis knock-out line SALK_010194. A: Location of the T-DNA within gene At1g47260. B: Confirmation of gene *knockout* by RT-PCR. Total mRNA was isolated from mutant and wild-type plants and the open reading frame of locus At1g47260 was amplified by RT-PCR. As a control, actin2 mRNA was amplified. Sizes of a DNA length standard are given to the left.

Figure 2: Comparison of phenotype and development between wild-type and ΔAt1g47260 plants. A: Phenotyes of wild-type and ΔAt1g47260 plants after growing for 22 days on soil. B: Duration of developmental stages of wild-type and ΔAt1g47260 plants (in days).

Figure 3: Comparison of phenotype, growth rate and O_2 consumption rate of suspension cell cultures for wild-type and ΔAt1g47260 plants. A: Suspension cell cultures 5 days after inoculation. B: Fresh weight of mutant and wild-type cell lines 3, 5 and 7 days after inoculation. C: KCN sensitive O_2 consumption of mutant and wild-type cell lines 3, 5 and 7 days after inoculation.

Figure 4: Complex I is strongly reduced in ΔAt1g47260 cell lines. Total mitochondrial protein from mutant and wild-type Arabidopsis lines was separated by one-dimensional Blue-native PAGE and either Coomassie stained (A) or activity stained for complex I (B). Proteins were solubilized with digitonin (5 g / g protein). The molecular masses of standard proteins are given to the left (in kDa) and the identities of protein complexes to the right of the gel strips. I+III_2: supercomplex formed of complexes I + dimeric complex III, I: complex I, H: HSP60 complex, V: ATP synthase complex, III_2: dimeric complex III, FDH: formate dehydrogenase complex.

Figure 5: Two-dimensional resolution of mitochondrial protein complexes of wild-type and ΔAt1g47260 cell lines by Blue-native / SDS PAGE. A,B: Coomassie-stained gels. C, D: Immunoblot of the boxed regions in A and B developed with an antibody directed against At1g47260. Molecular masses of standard proteins are given to the right (in kDa) and identities of protein complexes above the gels (for designations see Figure 4).

Figure 6: Subunit composition of complex I from wild-type and ΔAt1g47260 cell lines. The protein complex was separated by 2D Blue-native polyacrylamide gel electrophoresis and visualized by silver staining. Schemes indicating complex I subunits are given to the right of the gel strips. Molecular masses of standard proteins are given on the right (in kDa).

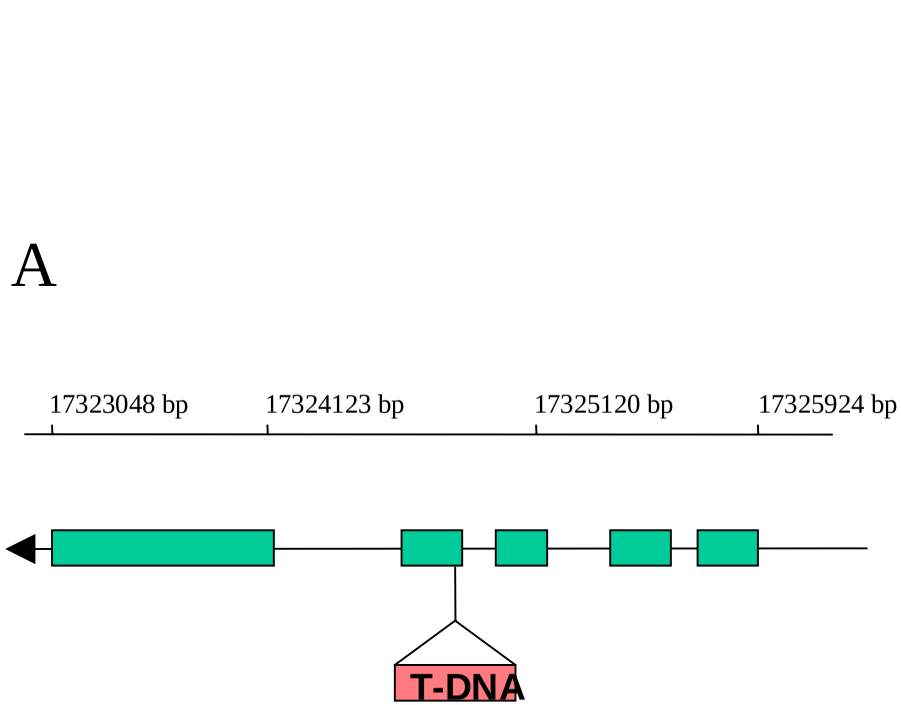
Figure 7: Analysis of complex I from wild-type and ΔAt1g47260 cell lines after purification by sucrose density gradient ultracentrifugation. Sucrose gradients were fractionated into 10 fractions (fraction 1: bottom of the gradient, fraction 10: top of the gradient). All fractions were analysed by 1D Blue-native PAGE and Coomassie-staining (A, B), 1 D SDS PAGE and Coomassie-staining (C, D) and 1 D SDS PAGE, western blotting and immune-staining (E, F). The immune-stained region in E, F corresponds to the boxed region in C, D. Identities of protein complexes are given in the middle between A and B (for designations see Figure 4), the molecular masses of standard proteins between C and D (in kDa).

Figure 8: Analysis of the mitochondrial proteomes of wild-type and ΔAt1g47260 cell lines by 2D fluorescence difference gel electrophoresis (DIGE). A: 2D Blue-native-SDS PAGE, B: 2D IEF / SDS PAGE. Proteins were detected by fluorescence scanning (proteins from the wild-type cell line are given in green, proteins from ΔAt1g47260 cell lines in red). Yellow spots are equally expressed in both cell lines, green spots are of lower abundance in ΔAt1g47260 and red spots of lower abundance in wild-type cell lines. Molecular masses of standard proteins are given to the right. Designations beneath the gel in A refer to identities of protein complexes (see Figure 4) and numbers beneath the gel in B to pI values. Proteins identified in the course of the Arabidopsis mitochondrial proteome project (http://www.gartenbau.uni-hannover.de/genetik/AMPP) are indicated in B.

Figure 9: Immunological quantification of the amounts of alternative oxidase (AOX) and two different "Rotenone" insensitive NAD(P)H dehydrogenases (NDA, NDB) in wild-type and ΔAt1g47260 cell lines. Total mitochondrial protein was separated by 2D Blue-native SDS PAGE (A, B), electroblotted and subsequently immune-stained using antibodies directed against NDA (C, D), NDB (E, F) and AOX (G, H). The immune-stained regions in C, D, E, F, G and H correspond to the boxed regions in A, B. Numbers on the right correspond to molecular masses of standard proteins (in kDa), designation on top of the gels to identities of protein complexes (see Figure 4).

Figure 10: Summary of results of micro array gene expression experiments available at the Stanford Microarray Database for locus At1g47260 (http://genome-www5.stanford.edu/cgi-bin/scriptIndex.pl). X-axis: normalized red/green intensity, y-axis: frequency.

Fig. 1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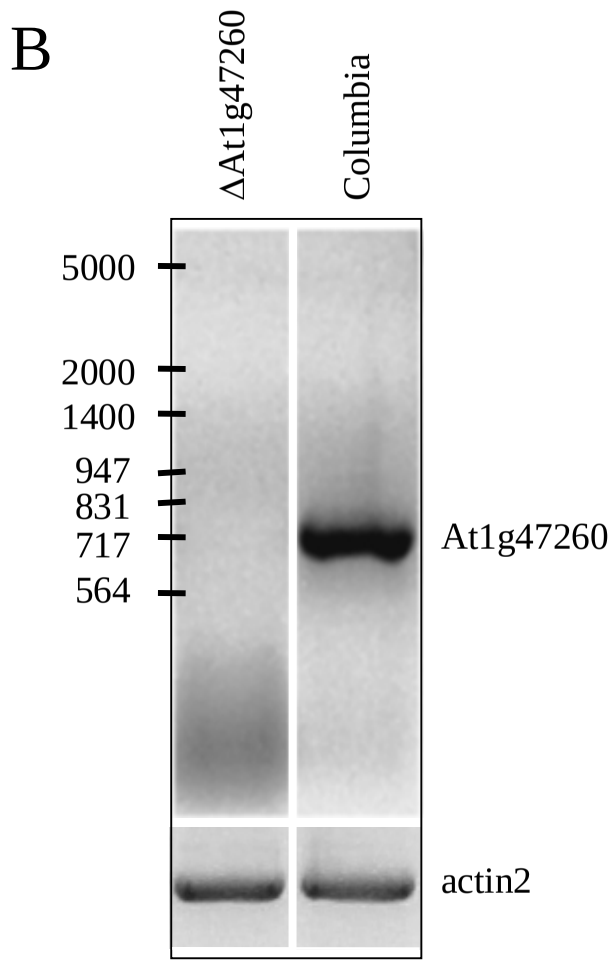
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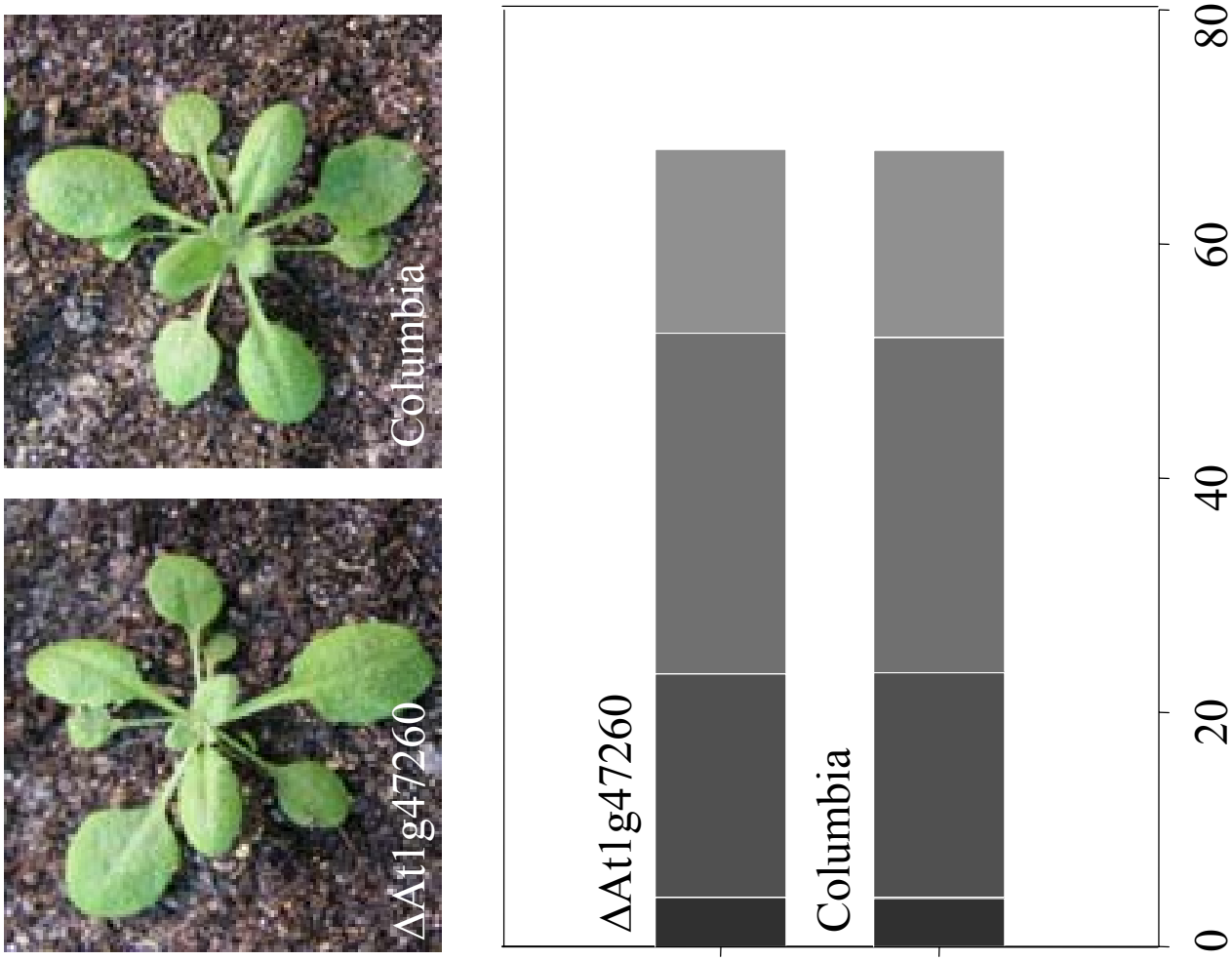
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ΔAt1g47260
Columbia
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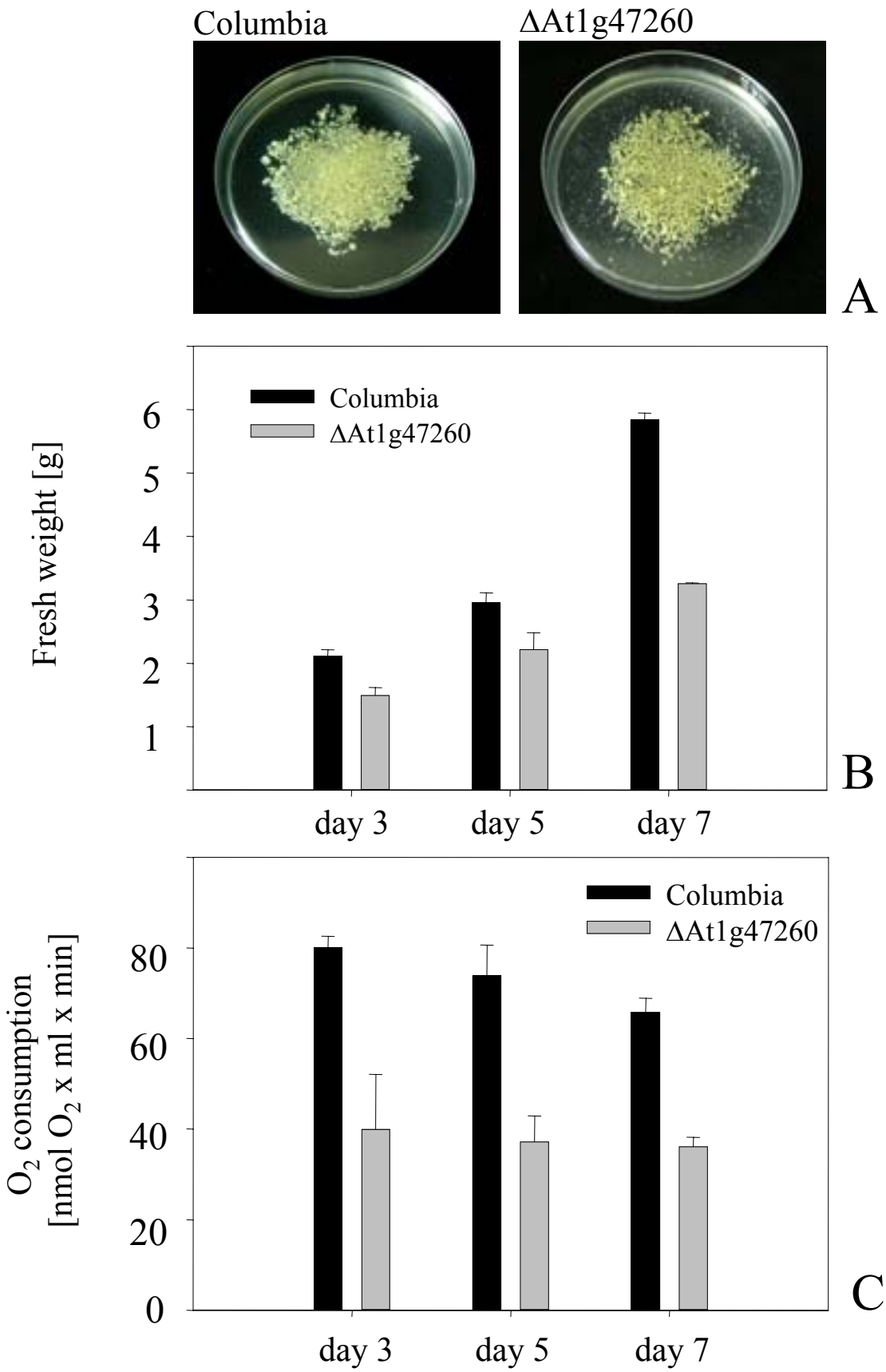
60
80

Fig. 2

Fig 3


Columbia
ΔAt1g47260
A
Fresh weight [g]
Columbia
ΔAt1g47260
1
2
3
4
5
6
day 3
day 5
day 7
B
O2 consumption
[nmol O2 x ml x min]
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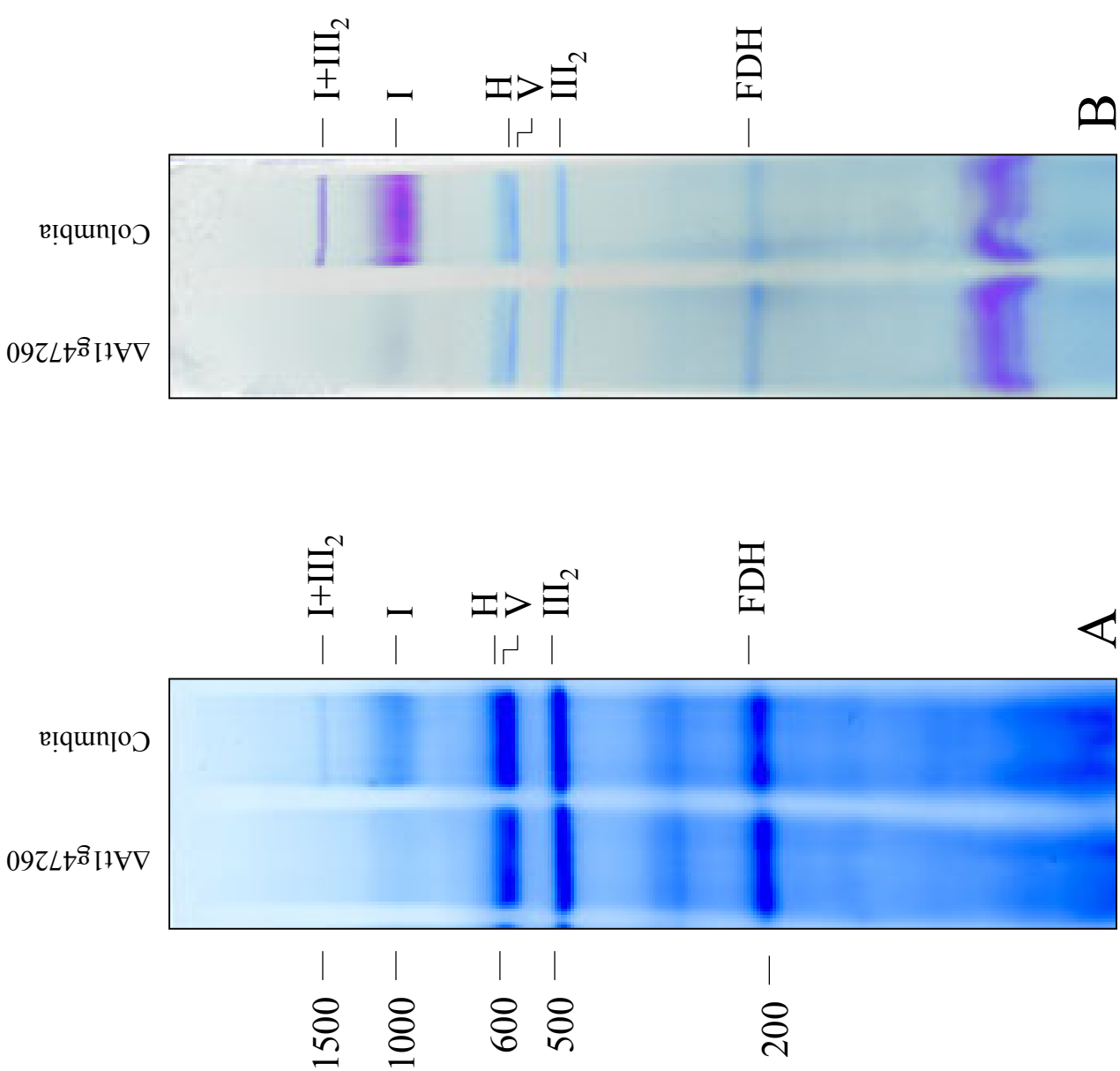
20
40
60
80
day 3
day 5
day 7
C


A
B
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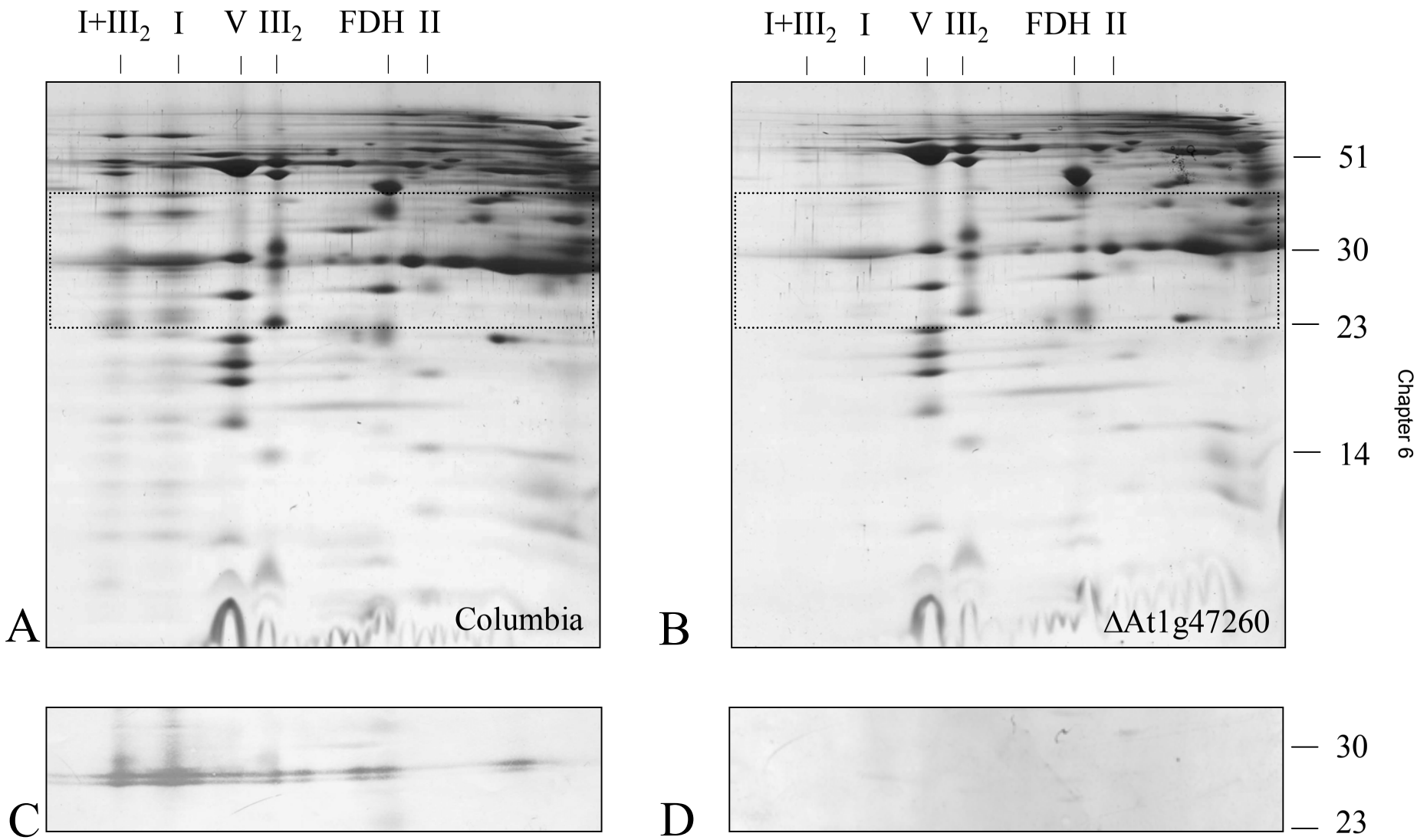
1000
600
500
200
I+III2
I
H
V
III2
FDH
ΔAtlg47260
Columbia

Fig. 4

Fig. 5


I+III2
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V
III2
FDH
II
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14
C
D
30
23

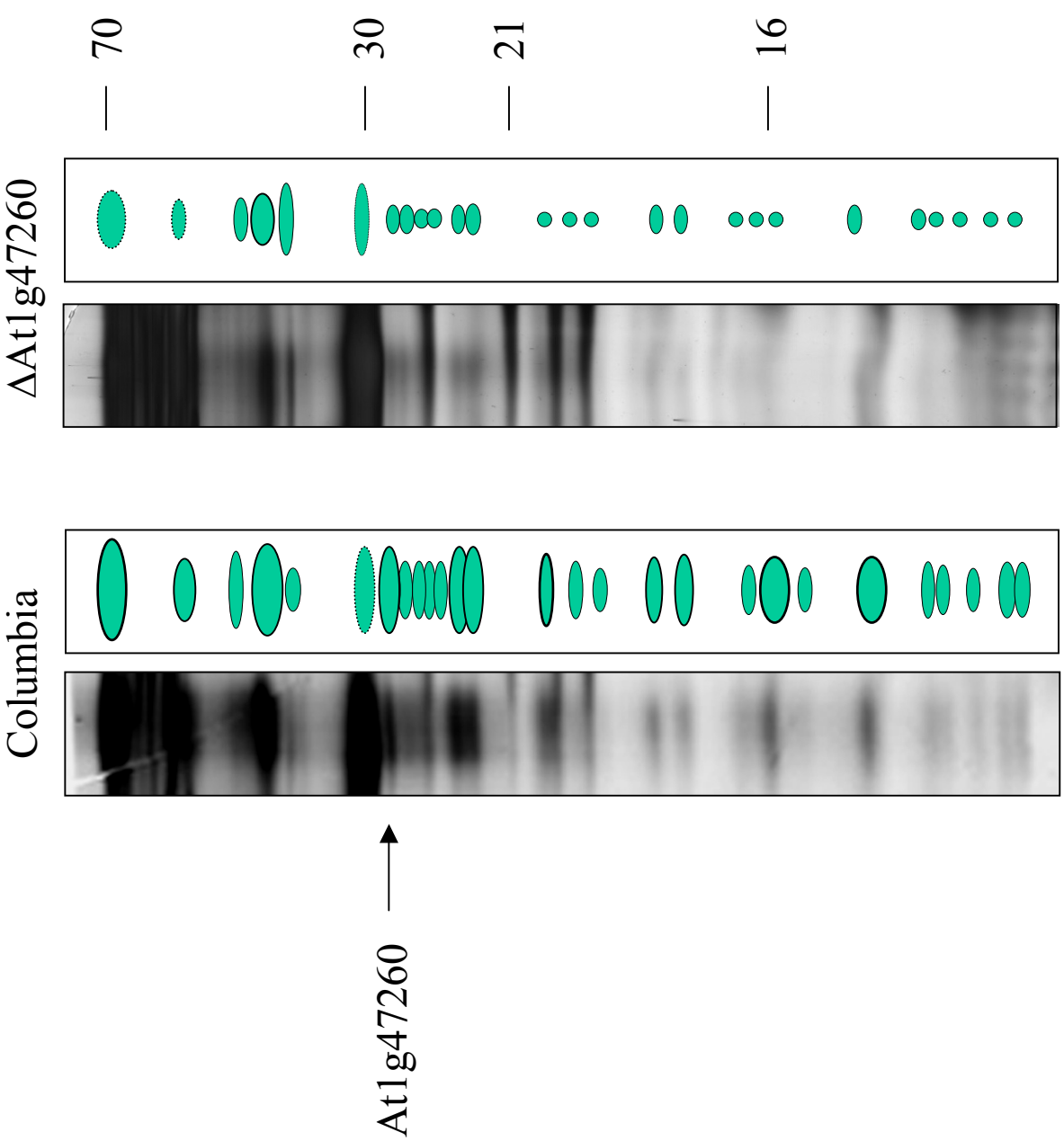
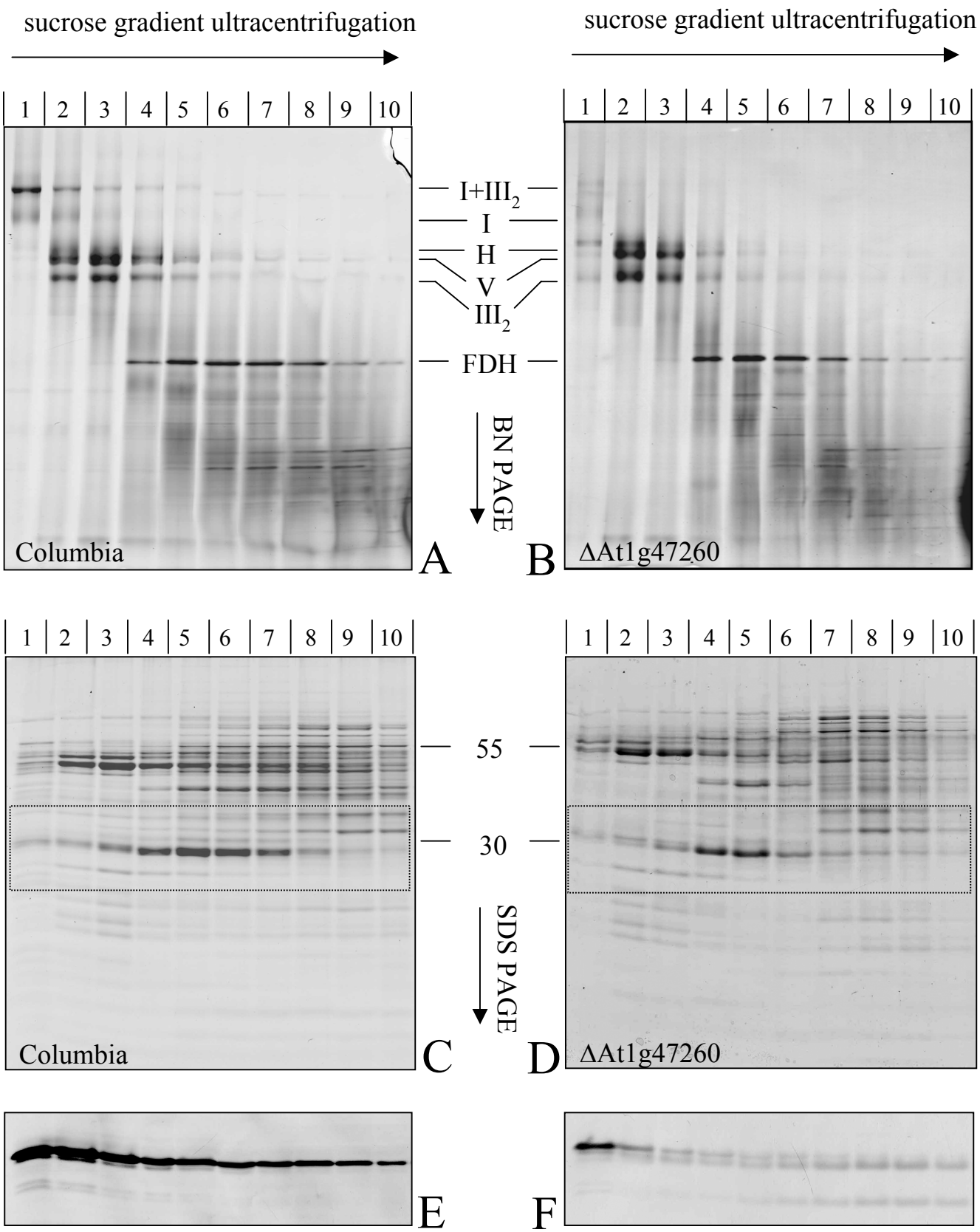


Fig. 6

Fig. 7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sucrose gradient ultracentrifugation
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I+III2
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H
V
III2
FDH
BN PAGE
Columbia
A
B
ΔAt1g47260
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
55
30
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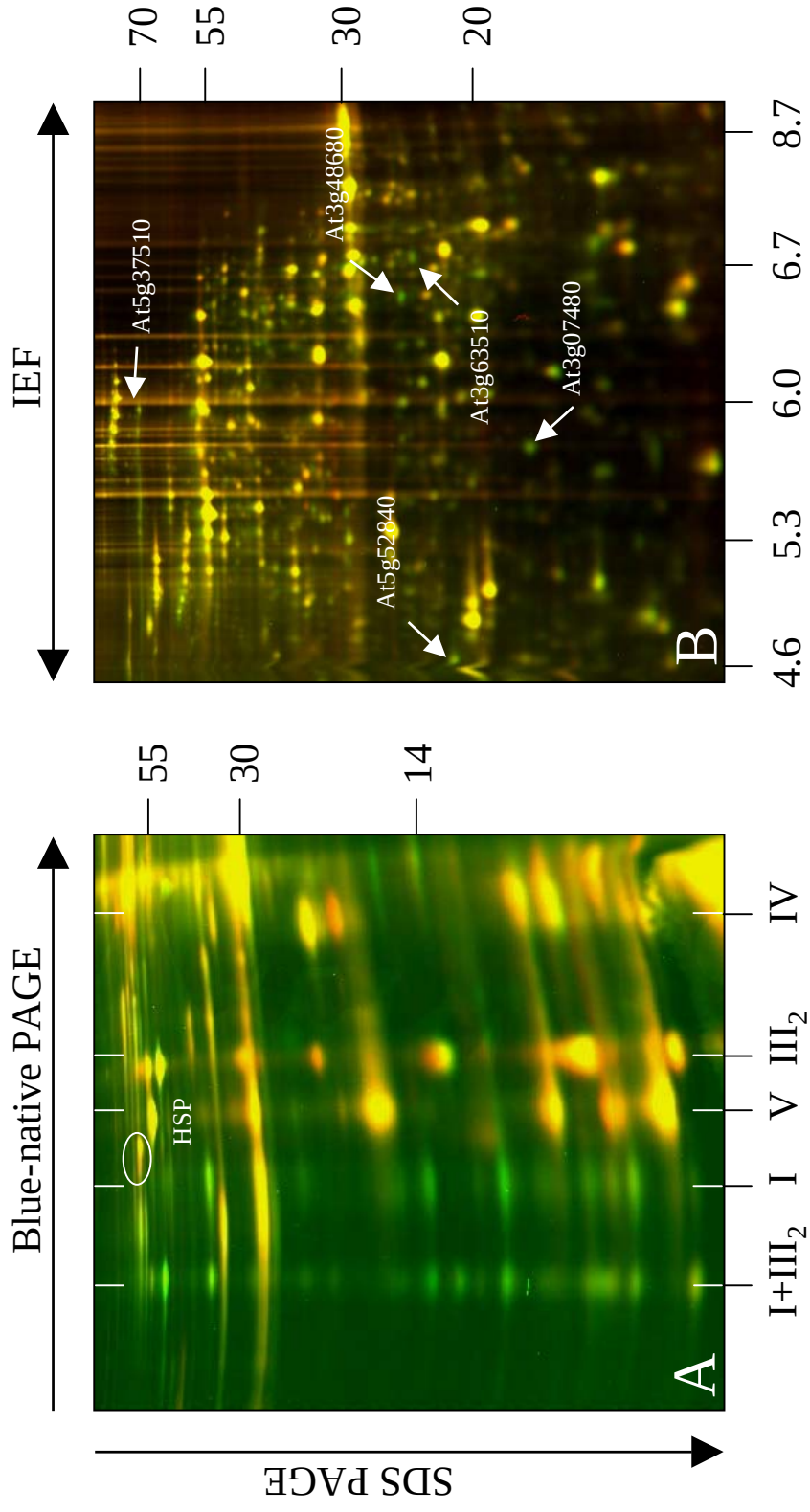
Columbia
C
D
ΔAt1g47260
E
F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14
HSP
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V
III2
IV
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20
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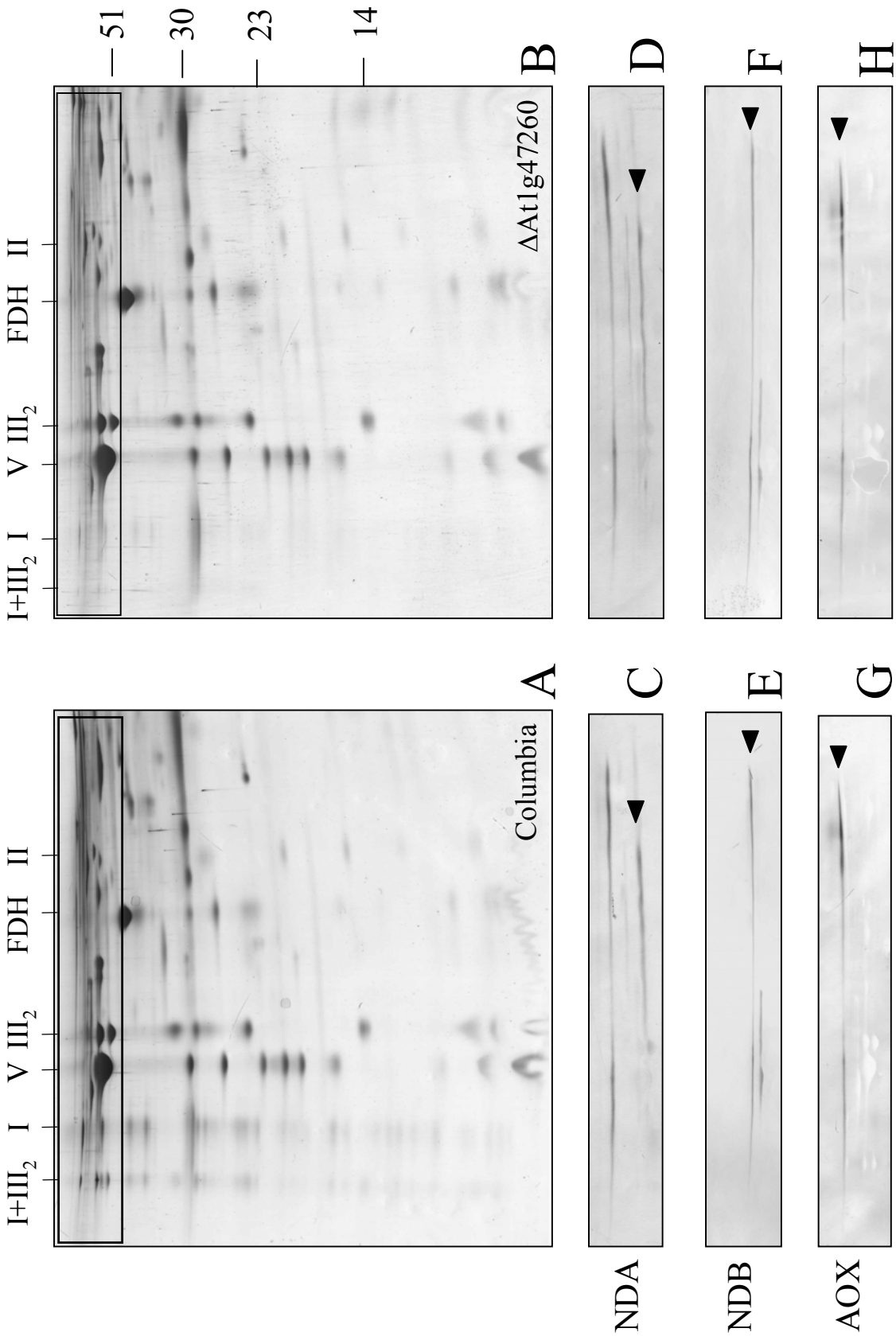
4.6
5.3
6.0
6.7
8.7
B

Fig. 8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II
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NDA
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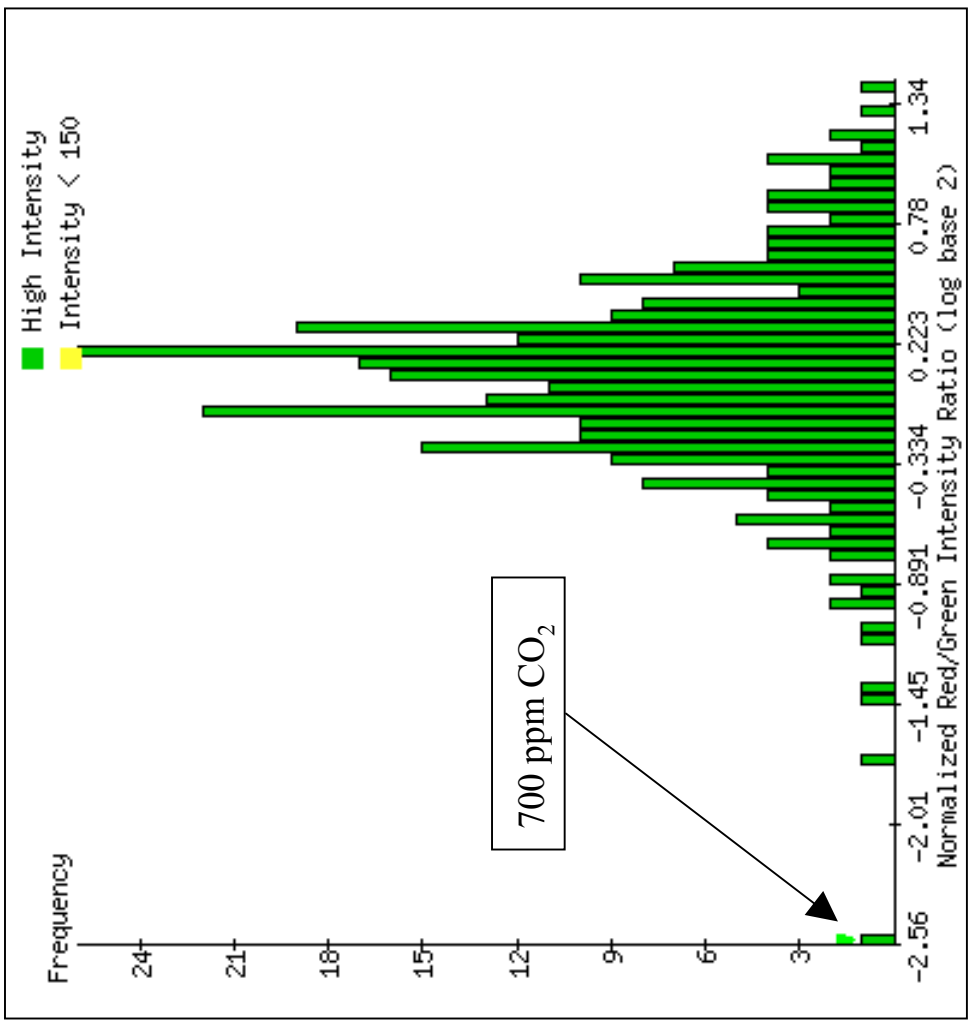
D
NDB
E
F
AOX
G
H

Fig. 9


High Intensity
Intensity < 150
Frequency
24
21
18
15
12
9
6
3
700 ppm CO_2
-2.56
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-0.891
-0.334
0.223
0.78
1.34
Normalized Red/Green Intensity Ratio (log base 2)

Fig. 10

Supplementary Discussion and Outlook

Careful solubilization of plant mitochondria with digitonin and subsequent separation of the protein complexes by BN-PAGE led to the discovery of new supramolecular structures formed by components of the respiratory chain.

For Arabidopsis, bean and barley, only supercomplexes consisting of NADH dehydrogenase and cytochrome c reductase were found (Chapter 2). One supercomplex has a I_1III_2 composition and a molecular mass of approximately 1500 kD, another supercomplex of about 3000 kD probably has a I_2III_4 composition.

In freshly harvested potato tubers and etiolated potato stems, additonal supercomplexes containg also cytochrome c oxidase were found and are supposed to have III_2IV_1, III_2IV_2, $I_1III_2IV_1$, $I_1III_2IV_2$ and $I_1III_2IV_4$ compositions (Chapter 3).

Digitonin solubilization of mitochondria also allows separation of the respiratory complexes II and IV on 2D BN/SDS-PAGE. Surprisingly, complex II of Arabidopsis consists of 8 subunits, twice as many as known for bacteria, yeast and mammals. For complex IV, six putative additional subunits were identified (Chapter 5). So far, nothing is known about the functions of these additional polypeptides. The respiratory complexes I and III are known to contain plant specific subunits performing side activities and one can speculate that the supplementary complex II and IV subunits also are not involved in electron transfer or proton translocation.

One of the plant specific subunits of complex I, encoded by the gene with accession number at1g47260, seems to be highly important for the stability of the complex. Knockout of the gene results in an almost complete loss of singular complex I and the I_1III_2 supercomplex and leads to impaired respiration and growth rates in cell suspension cultures (Chapter 6).

Since the existence, composition and possible functions of respiratory supercomplexes have already been discussed in chapters 2 to 6, this discussion will focus on special properties of the plant respiratory chain and putative implementations for the regulation of alternative respiration. Additionally the applicability of the solubilization technique used will be discussed and new results on the structure of the I_1III_2 supercomplex, which are not included in chapters two to six, will be presented.

Special Properties of Plant Respiratory Supercomplexes and Regulation of Alternative Respiration in Plant Mitochondria

Apart from differences in the ratio of singular to supercomplex-bound complexes, the organization of the potato respiratory chain resembles its counterparts found in bacteria and mammals (Chapter 3) but is different to the one found in *Saccharomyces cerevisae* due to lack of complex I in this organism. Arabidopsis, bean and barley either seem to lack significant amounts of complex IV containing respiratory supercomplexes or these structures are more sensitive to dissociation (Chapter 2). Therefore, on BN gels only supercomplexes that comprise the complexes I and III were found in significant amounts.
Although some fungi also possess alternative oxidoreductases, the combination of several rotenone insensitive NAD(P)H dehydrogenases and cyanide insensitive terminal oxidases is a general feature of plant mitochondria and unique to this kingdom. For this reason, a discussion on the structure of the plant respiratory chain has to consider the presence of these enzymes, especially because they do not form part of supercomplexes (Chapter 2).

The alternative oxidase and the cytochrome c pathway, which includes cytochrome c reductase and oxidase as well as cytochrome c, compete for ubiquinol produced by complex I, the alternative NAD(P)H dehydrogenases and complex II (Day et al. 1996). The alternative oxidase is believed to perform several functions: i) it prevents the formation of reactive oxygen species (ROS) by balancing the Q_r/Q_t ratio, ii) it keeps the citric acid cycle running during state 4 respiration, and iii) it might be useful for the plant during inhibition of the cytochrome pathway by naturally occuring cyanide, nitric oxide, sulfide, high concentrations of CO_2, low temperatures or limited phosphorus concentrations (for review, see Millenaar and Lambers 2002).
But how is the energy wasting action of this dimeric enzyme regulated? Apart from gene expression and protein turnover, two major parameters that influence the activity of the alternative oxidase *in vitro* are known to be the concentration of α-keto acids like pyruvate (Rhoads et al. 1998, Vanlerberghe et al. 1998) and the reduction state of a conserved Cystein residue at the N-terminus of each monomer (Umbach and Siedow 1993). However, *in vivo* these regulatory mechanisms probably are not relevant because the pyruvate concentration within the mitochondrium usually is two to ten times higher than the concentration for half maximum stimulation (Millenaar et al. 1998). Additionally, the oxidized, less active version of the dimer is found only rarely in mitochondria. Very little is known about the regulation of

AOX by the Q_r/Q_t ratio, although it seems to have a certain effect (Millenaar and Lambers 2002).

The discovery of respiratory supercomplexes in plant mitochondria could provide a new mechanism for AOX regulation. The presence of the highly abundant I_1III_2 supercomplex in plant mitochondria might reduce access of AOX to its substrate ubiquinone by substrate channeling of ubiquinone inside the particle. For substrate channeling between the complexes I and III, no ubiquinone pool is necessary. However, AOX activity most likely depends on the presence of such a pool. How do substrate channeling and the existence of an ubiquinone pool fit together? In mammalian mitochondria, which also possess supercomplexes (Schägger 2002), the presence of an ubiquinone pool has been shown (Kröger and Klingenberg 1973, Bianchi et al. 2004), leading to a conflict between physiological and structural data. A model of temporary interactions of the complexes I and III has been proposed for mammalian mitochondria, allowing the existence of both, ubiquinone pool and I_1III_2 supercomplex (Bianchi et al. 2004). In mitochondria of plants, apart from complex II, various rotenone insensitive NADH dehydrogenases (and other enzymes) can additionally reduce ubiquinone and do not participate in the I_1III_2 supercomplex (Chapter 2), making the existence of an ubiquinone pool even more likely. On the other hand, the interaction of the complexes I and III seems to be very stable, as indicated by the high rate of incorporation of the singular enzymes into the supercomplex. Depending on the organism, on BN-gels, 50 to 90 % of complex I is integrated into the supercomplex. Excluding artificial protein aggregation as the reason for supercomplex formation, the amount of singular complex I might be even lower *in vivo* due to potential dissociation of the supercomplex during sample preparation. This makes a dynamic association between the complexes I and III unlikely.

Two of the seven alternative NAD(P)H oxidases of Arabidopsis recently were found to be induced by light, independantly of the state of carbon metabolism. (Escobar et al. 2004). They most likely form the basis of an overflow protection mechanism for the standard respiratory chain in a situation of excess NADH supply due to photorespiration. The decarboxylation of glycine, which is coupled to the generation of NADH, seems to be not or only weakly affected by the complex I inhibitor rotenone. Additionally, the cyanide resistant electron pathway increases in the presence of ATP. The ATP/ADP ratio in the cytosol of barley protoplasts increases in the presence of CO_2 but not in CO_2-free medium, indicating an electron flow via the alternative non-phosphorylating pathways under conditions in which photorespiration is induced (Igamberdiev et al. 1997). However, studies on the electron flow

between classical and alternative pathways using inhibitors have to be considered with caution because they most likely do not reflect electron partitioning *in vivo* (Day et al. 1996).
In case of substrate channeling within the I_1III_2 supercomplex, these results suggest a regulation of AOX activity by the supply of reduced ubiquinone via the alternative NADH dehydrogenases. Only fine trimming of AOX activity might then be performed by association or dissociation of the I_1III_2 supercomplex. Dissociation possibly leads to a reduced electron transport efficiency via this pathway and to an increased level of reduced ubiquinone. This would shift the equilibrium of electron transport between the classical and alternative way towards the AOX.

However, there is also the possibility of a physiological aggregation of the complexes I and III only for structural reasons without featuring substrate channeling. Stabilization of the individual respiratory complexes and a more efficient packing of the proteins in the inner mitochondrial membrane could be the advantages of the supercomplex over singular complexes. Of course, these considerations are also valid in the case of substrate channeling.

A striking difference between (most) plant and mammalian respiratory supercomplexes is the participation of complex IV. Only in freshly harvested potato mitochondria, small amounts of complex IV containing supercomplexes were found. Two reasons might explain this difference: i) supercomplexes containing complex IV really are absent in most plants, ii) the interactions between complex IV and the other complexes are more labile in most plants compared to mammalian (and yeast) mitochondria or have a more temporary nature and are therefore hardly detectable on BN gels. The latter theory is supported by the discovery of faint bands representing a III/IV supercomplex in Arabidopsis (data not shown). A more dynamic association of complex IV with the I_1III_2 supercomplex might represent another mechanism to influence AOX activity by contributing to an improved electron flow through the classical pathway in an associated condition, or by partially inhibiting electron flow in the dissociated form. Again, the equilibrium between the two pathways will be shifted towards AOX when complex IV is not associated with the I_1III_2 supercomplex. In potato respirasomes, electron flow through this supercomplex might be regulated by the conversion from small to large form of complex IV, or *vice versa*. Activity of the larger, Cox VIb containing complex, is higher than the activity of the smaller form.
In conclusion, regulation of alternative respiration remains an unanswered question. The existence of an ubiquinone pool and the finding that electrons of NADH generated by

photorespiration are transferred to oxygen via the alternative, non-phosphorylating pathway do not indicate such a mechanism, at least not for the photorespiratory pathway. Investigation of the structure of the I_1III_2 supercomplex will generate further information about the feasability or unlikeliness of substrate channeling in plant respiratory supercomplexes and therefore will shed new light on regulation of alternative oxidase.

Solubilization of Respiratory Supercomplexes by Digitonin - Artificial Aggregates or Defined Physiological Associations?

The glycoside digitonin is a non-ionic detergent which is supposed to interact specifically with sterols like Cholesterol. Because the inner mitochondrial membrane is assumed to lack this kind of lipids completely, it is normally used to solubilize proteins or protein complexes of the outer membrane, i.e. the TOM complex. However, obviously there is an interaction of digitonin with components of the inner mitochondrial membrane because solubilizations employing this detergent lead to the presence of several protein complexes of the inner membrane on BN gels. As indicated by the high amounts of digitonin needed compared to DDM or Triton X100, this interaction seems to be rather weak.
A putative role of digitonin in formation of articial aggregations of protein complexes can not be entirely excluded at the moment but the results obtained so far are pointing into the opposite direction:

i) Only specific stoichometric interactions were observed, mainly between the respiratory complexes I and III. In Arabidopsis, the only supercomplexes present on BN/SDS or BN/BN gels displayed I_1III_2 and I_2III_4 compositions. In barley and bean, just the I_1III_2 supercomplex was visible. Only in potato, small amounts of supercomplexes containing cytochrome c oxidase were found. Again, these supercomplexes showed a defined stoichometric organisation. The demonstrated tissue specifity for supercomplexes further favors the idea of supercomplexes over artificial aggregates.

ii) In no case participations of complex II, alternative oxidoreductases or other mitochondrial protein complexes have been observed, although several of them are also known to be at least partly hydrophobic.

iii) Supercomplexes contain active complex I and, in the case of potato, additionally complex IV in active form as demonstrated by *in gel* activity stains. This might not be the case in unspecific aggregation.

iv) Coomassie-coated proteins tend to repel each other due to the high amount of negative charges added to their surface.

v) The incubation time of the mitochondria in digitonin-containing solubilization solution has no effect on the abundance of the protein complex or supercomplex bands on the gel (Heinemeyer et al., unpublished data). When Coomassie is added before the digitonin, the result also remains unchanged (Eubel et al., unpublished data). This again indicates no unspecific aggregation caused by digitonin.

vi) Flux control measurments clearly indicate an interaction of the complexes I and III (Genova et al. 2003).

vii) Electron microscopy and average image analysis of the digitonin solubilized I_1III_2 supercomplex of Arabidopsis reveals a highly ordered association of the two single respiratory complexes, which is not expected for unspecific aggregation of proteins (Eubel et al., unpublished data. See also below).

ix) In mammals, several mutants of the *cyt b* gene not only show defects in complex III assembly but also in the formation of complex I and the I_1III_2 supercomplex. This indicates a role of complex III in the formation and/or stabilization of complex I (Acin-Perez et al. 2004).

A Short Look through the Microscope

Digitonin solubilization of plant mitochondrial supercomplexes and subsequent BN-PAGE revealed the presence of a highly abundant supercomplex comprising a single copy of NADH dehydrogenase and dimeric cytochrome c reductase. To confirm the presence of this supercomplex and to get a detailed impression about of its topology, digitonin was again employed to solubilize Arabidopsis mitochondria. Separation of the different protein complexes and supercomplexes was then carried out by sucrose density centrifugation. Fractions of the gradients were tested for their content by BN-PAGE and those fractions containing the I_1III_2 supercomplex were used for negative stain electron microscopy. For single particle analysis, several hundred images of the supercomplex were taken, classified and averaged to generate a detailed image of the structure. The combination of electron

microscopy and single particle analysis has already been succesfully applied to reveal the supramolecular structure of photosystem I (Kruip et al. 1993, Bibby et al. 2001) and photosystem II (Boekema et al. 1995, Boekema et al. 1999, Boekema et al. 1998, Yakushevska et al. 2001) of several species. Figure 1 shows a preliminary picture of the I_1III_2 supercomplex of *A. thaliana*. Being made of just a few hundred pictures, the image will become even more detailled when several thousand pictures are included in the analysis. This will allow to determine the orientation of the singular complexes to each other, the contact site of the two complexes, the distance between oxidation and reduction site of ubiquinone and further support or rebut the idea of substrate channeling in this particular supercomplex.

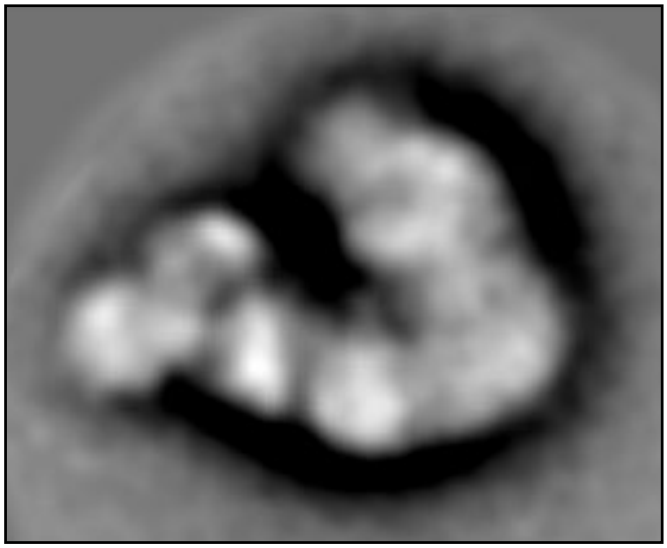

Figure 1: Average picture of the Arabidopsis I_1III_2 supercomplex. Isolated mitochondria were solubilized with 5 g/g digitonin and the protein complexes subsequently separated by sucrose density centrifugation. Uranyl acetate staining was performed prior to electron microscopy. Several hundred images were used to create an average picture of the supercomplex. (Image taken by N. Dudkina and E.J. Boekema, Groningen Univerity.)

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