Complex I–complex II ratio strongly differs in various organs of *Arabidopsis thaliana*

**Katrin Peters · Markus Nießen · Christoph Peterhänsel ·
Bettina Späth · Angela Hölzle · Stefan Binder ·
Anita Marchfelder · Hans-Peter Braun**

Abstract In most studies, amounts of protein complexes of the oxidative phosphorylation (OXPHOS) system in different organs or tissues are quantified on the basis of isolated mitochondrial fractions. However, yield of mitochondrial isolations might differ with respect to tissue type due to varying efficiencies of cell disruption during organelle isolation procedures or due to tissue-specific properties of organelles. Here we report an immunological investigation on the ratio of the OXPHOS complexes in different tissues of *Arabidopsis thaliana* which is based on total protein fractions isolated from five Arabidopsis organs (leaves, stems, flowers, roots and seeds) and from callus. Antibodies were generated against one surface exposed subunit of each of the five OXPHOS complexes and used for systematic immunoblotting experiments. Amounts of all complexes are highest in flowers (likewise with respect to organ fresh weight or total protein content of the flower fraction). Relative amounts of protein complexes in all other fractions were determined with respect to their amounts in flowers. Our investigation reveals high relative amounts of complex I in green organs (leaves and stems) but much lower amounts in non-green organs (roots, callus tissue). In contrast, complex II only is represented by low relative amounts in green organs but by significantly higher amounts in non-green organs, especially in seeds. In fact, the complex I–complex II ratio differs by factor 37 between callus and leaf, indicating drastic differences in electron entry into the respiratory chain in these two fractions. Variation in amounts concerning complexes III, IV and V was less pronounced in different Arabidopsis tissues (quantification of complex V in leaves was not meaningful due to a cross-reaction of the antibody with the chloroplast form of this enzyme). Analyses were complemented by *in gel* activity measurements for the protein complexes of the OXPHOS system and comparative 2D blue native/SDS PAGE analyses using isolated mitochondria. We suggest that complex I has an especially important role in the context of photosynthesis which might be due to its indirect involvement in photorespiration and its numerous enzymatic side activities in plants.

Keywords Oxidative phosphorylation ·
Respiratory chain · OXPHOS complexes ·
Mitochondria · Stoichiometry

K. Peters · H.-P. Braun (✉)
Faculty of Natural Sciences, Institute of Plant Genetics,
Leibniz Universität Hannover, 30419 Hannover, Germany
e-mail: braun@genetik.uni-hannover.de

M. Nießen · C. Peterhänsel
Faculty of Natural Sciences, Institute of Botany, Leibniz
Universität Hannover, 30419 Hannover, Germany

B. Späth · A. Hölzle · S. Binder · A. Marchfelder
Molekulare Botanik, Universität Ulm, 89081 Ulm, Germany

Introduction

Mitochondria represent an important site for ATP production in most eukaryotic cells. Generation of ATP is carried out by the oxidative phosphorylation (OXPHOS) system, which consists of five multi-protein complexes (complex I–V) in the inner mitochondrial membrane as well as cytochrome c and ubiquinone (Hatefi 1985). Complexes I to IV form part of the electron transport chain (ETC) also termed respiratory chain. This chain catalyzes

NADH oxidation by reduction of oxygen to water. The transport of electrons by complex I, III and IV is coupled to the translocation of protons from the matrix to the intermembrane space. The resulting proton gradient is used by the ATP-synthase (complex V) to produce ATP. The OXPHOS system in plants differs from the one in most mammals because some of its respiratory chain complexes have several extra protein subunits which introduce additional functions into these complexes (Millar et al. 2011). For example, complex I from plants includes more than 10 extra subunits (Klodmann and Braun 2011; Klodmann et al. 2010). Some of these proteins resemble known enzymes like gamma-type carbonic anhydrases (γ-CA) or ʟ-galactone-1,4-lactone dehydrogenase (GLDH).

Furthermore, the plant OXPHOS system is especially multifaceted because it involves additional 'alternative' oxidoreductases. As a consequence, respiratory electron transport is highly branched in plants. Enzymes catalyzing these alternative pathways are type II NAD(P)H dehydrogenases and the alternative oxidase (AOX). Former ones can bypass complex I, whereas AOX bypasses complex III and IV (Rasmusson et al. 2008). The transport of electrons via these alternative pathways is not coupled with proton translocation across the inner mitochondrial membrane and therefore not directly involved in respiratory ATP production. The activity of the alternative electron pathway enzymes therefore results in a lower ATP production of the respiratory system and thus leads to a decrease of respiratory energy conservation (Rasmusson and Wallström 2010). This especially is important under high light conditions due to excess formation of reduction equivalents within chloroplasts (by photosynthesis) and mitochondria (by glycine to serine conversion in the context of photorespiration) (Rasmusson et al. 2008). In fact, expression of the genes encoding AOX and alternative NAD(P)H dehydrogenases is light regulated (Rasmusson and Escobar 2007). Thus, the alternative respiratory pathways are considered to be the basis for an overflow protection mechanism for the OXPHOS system which prevents production of reactive oxygen species (ROS) due to over-reduction of the ETC in the light. Besides the alternative oxidoreductases also complex I is important with respect to ROS defence because mutations within complex I subunits affect the redox balance of the entire plant cell (Dutilleul et al. 2003a, b).

Despite all information on components of the OXPHOS system, little so far is known about the abundance and stoichiometry of the five OXPHOS complexes in plants. In contrast, several investigations on this subject were carried out for the respiratory chain complexes in mammals. It was shown that a number of genetic disorders result in defects in mitochondrial electron transfer (e.g. Alzheimer's and Parkinson's Diseases). Knowing the accurate stoichiometry

of the OXPHOS complexes is necessary to develop structural models and to more extensively understand the relation of structure and function with respect to the mitochondrial OXPHOS system. Using various methods, several insights were obtained into the stoichiometry of the OXPHOS complexes in *Bos taurus* (Hatefi 1985; Capaldi et al. 1988; Schägger and Pfeiffer 2001). Recently, different ratios for OXPHOS complexes were reported for various human tissues related to mutations within a mitochondrial elongation factor (Antonicka et al. 2006).

Here we report an investigation on the ratio of OXPHOS complexes in plants. To achieve reliable results, experiments were not carried out on the basis of isolated mitochondria but on the level of total protein fractions which were isolated from five different organs of Arabidopsis and from callus. A new set of antibodies was generated for immunological detection and quantification of the five OXPHOS complexes in plants. This study reports drastic differences in the ratio of OXPHOS complexes in different organs, especially with respect to complex I, which seems to be of special importance for photosynthesis.

Materials and Methods

Material

Organs were harvested from 7 weeks old *Arabidopsis thaliana* plants, sub-variety Columbia (Col-0). Plants were grown on soil under long day conditions (16 h light, 8 h dark) at 22 °C during the day and 20 °C at night. Flowers and seeds were collected from several plants cultivated under identical conditions. For the analyses of the leaves the whole rosette was harvested. Roots of the plants were cut off and leftovers of soil were washed out. Callus cultures of *Arabidopsis thaliana* (Col-0) were established as described by May and Leaver (1993). Callus was maintained as suspension culture as outlined previously (Sunderhaus et al. 2006).

Phenolic extraction of proteins

Extraction of proteins from different organs of *Arabidopsis thaliana* was performed following the protocol of Hurkman and Tanaka (1986) modified by Colditz et al. (2004). Dried protein pellets were resuspended in 1x 'Sample buffer tricine' (10 % (w/v) SDS, 30 % (v/v) glycerol, 100 mM Tris, 4 % (v/v) β-mercaptoethanol, 0.006 % (w/v) bromophenol blue, pH 6.8) for Tricine-SDS-PAGE (Schägger and von Jagow 1987). Samples were either directly loaded onto a SDS-gel or stored at −80 °C. Protein concentration of the fractions was determined by the use of the Bradford protein assay (Bradford 1976).

Native extraction of membrane proteins

Fresh plant material (5 g) was ground with a mortar on ice in 5 ml cold 'grinding buffer' (0.3 M mannitol, 50 mM Tris–HCl, 1 mM EDTA, 0.2 mM PMSF, pH 7.4) plus sea sand. Homogenate was filtered by the use of a gaze. Afterwards, samples were centrifuged for 1 min at $70 \times g$ at 4 °C. Supernatants were transferred into new 2 ml-Eppendorf tubes and centrifuged again for 20 min at $18,300 \times g$ at 4 °C. Pellets were resuspended in 500 µl 'BN-solubilization buffer' (30 mM HEPES, 150 mM potassium acetate, 10 % (v/v) glycerol, pH 7.4) plus 5 % (w/v) digitonin), followed by a 20 min incubation step on ice. Samples were centrifuged for 10 min at $18,300 \times g$ at 4 °C. Supernatants were transferred into new Eppendorf tubes and stored at −80 °C or directly loaded onto a blue native gel (see below).

Isolation of mitochondria

Mitochondria from callus cultures were isolated as described by Werhahn et al. (2001). Isolation of mitochondria from green leaves was performed according to the protocol of Keech et al. (2005).

Gel electrophoresis procedures

One-dimensional blue native PAGE (1D BN-PAGE) and two-dimensional blue native/SDS PAGE (2D BN/SDS-PAGE) were performed according to Wittig et al. (2006). One-dimensional Tricine-SDS-PAGE was carried out as described by Schägger and von Jagow (1987). Solubilization of mitochondrial protein was performed using digitonin at a concentration of 5 g/g mitochondrial protein (Eubel et al. 2003).

Western blotting

Proteins separated on polyacrylamide gels were blotted onto nitrocellulose membranes for antibody staining using the Trans Blot Cell from BioRad (Munich, Germany). Blotting was carried out as described by Kruft et al. (2001). Immuno-histochemical stains were performed by using the VectaStain ABC Kit (Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions.

For quantitative Western blotting experiments proteins were separated by SDS-PAGE using precast gels (Mini-Protean TGX^{TM} 10 % Tris–HCl, Bio-Rad, Munich, Germany). After separation, proteins were transferred on a nitrocellulose membrane using a liquid electroblotting apparatus (Mini-Protean Tetra Cell, Bio-Rad, Munich, Germany). The membrane was afterwards blocked in TTBS buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl,

0.05 % Tween 20) with 1 % BSA, and then incubated overnight in TTBS with an antibody directed against the 51-kDa subunit (complex I), the SDH 1-1 subunit (complex II), the alpha subunit of the mitochondrial processing peptidase (alpha MPP; complex III), the COX2 subunit (complex IV) or the beta subunit of complex V. After washing with TTBS, the membrane was incubated with a secondary monoclonal antibody directed against rabbit immunoglobulins. This secondary antibody is coupled to horseradish peroxidase (HRP) (Goat anti-Rabbit IgG HRP conjugate, Millipore, Billerica, MA, USA). HRP finally converts the 'Lumi-Light^{PLUS} Western Blotting Substrate' (Roche, Mannheim, Germany) into a fluorescence-emitting form. Light signals were recorded with the Lumi-Imager (Roche, Mannheim, Germany). Quantification of proteins was carried out using the AIDA Image Analyser Software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

Production of antibodies

Antibodies directed against the 51-kDa subunit (complex I), the SDH 1-1 subunit (complex II), the alpha subunit MPP subunit (complex III), the COX2 subunit (complex IV) and the beta subunit of complex V were produced as polyclonal peptide specific antibodies by Eurogentec (Seraing, Belgium) (for details see online resource 1). The antibody against the 51-kDa subunit (complex I) was additionally produced as polyclonal anti-protein antibody by Eurogentec (Seraing, Belgium). For this approach, the 51-kDa subunit was over-expressed in *E. coli*, purified from inclusion bodies and separated by 1D Tricine-SDS-PAGE.

Gel staining procedures

Polyacrylamide gels were stained with Coomassie Brilliant Blue G250 (Neuhoff et al. 1988, 1990). *In gel* enzyme activity stains for mitochondrial respiratory chain complexes I, II and IV were carried out as described in Zerbetto et al. (1997) and Jung et al. (2000).

Results

Specificity of the IgGs directed against the OXPHOS complexes from Arabidopsis

This work aims to determine the ratio of the five OXPHOS complexes in different organs of *Arabidopsis thaliana*. An immunological approach was chosen for this purpose. In a first step, a new set of antibodies was

generated. In order to produce antibodies with broad application spectra, surface exposed subunits were selected as targets for each complex (online resource 2). This ensures their use under native and non-native (denaturing) conditions. For the immunization of rabbits, two surface exposed peptides of one subunit of each OXPHOS complex were selected. In the case of complex I, the over-expressed 51-kDa subunit was additionally used for immunization because the anti-peptide IgGs proved to have low quality (data not shown).

Immunoblotting experiments were carried out to test the specificity of the generated antibodies. For this approach, total protein of an Arabidopsis callus culture was isolated by phenol extraction and resolved by 1D SDS-PAGE. Immunoblotting experiments revealed signals at the expected molecular mass (Fig. 1). Furthermore, all IgGs also specifically reacted with the target OXPHOS complexes resolved by native gel electrophoresis (online resource 3).
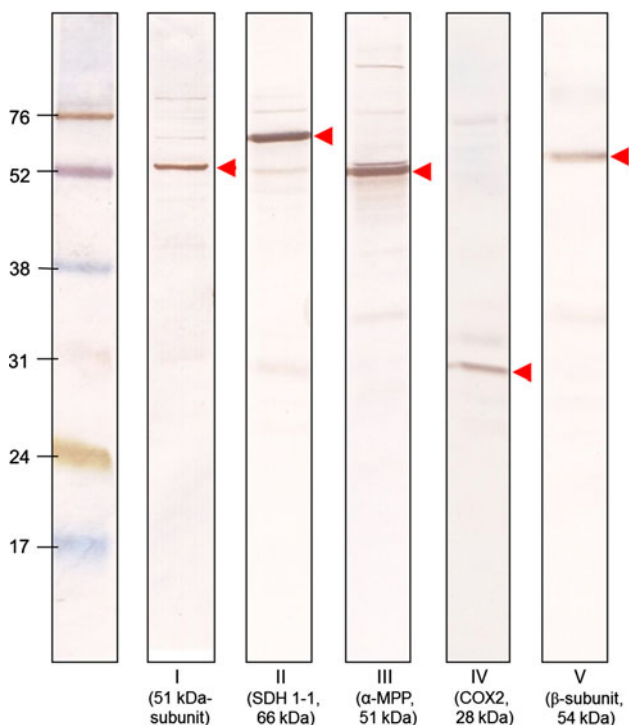
Quantification of OXPHOS complexes in different tissues of Arabidopsis

After specificity had been verified for all IgGs, quantification of OXPHOS complexes was carried out by immunoblotting analyses. For this investigation, total protein fractions were isolated by phenol extraction from five Arabidopsis organs (leaves, stems, roots, flowers and seeds) and from callus. Gels were loaded with protein equivalent corresponding to an identical amount of fresh weight (FW). In a pre-experiment, isolated protein fractions were evaluated by 1D SDS-PAGE and Coomassie-staining (Fig. 2). As expected, the protein concentration varies depending on the analysed fraction. Protein concentration is highest in seeds and flowers. The seed storage proteins are visible in the seed fraction (20–40 kDa range) and the large subunit of RubisCO in the above 50 kDa range in the photosynthetically active fractions (leaves, stems and flowers).

Next, protein fractions from all organs were resolved by 1D SDS-PAGE and evaluated by immunoblotting. Again,
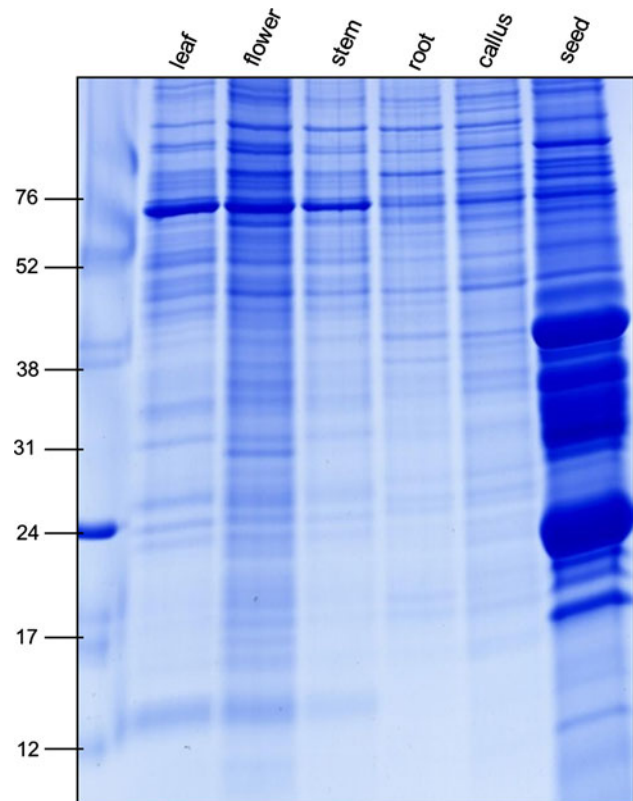

Fig. 1 Antibody specificity. Total protein (extracted from 5 mg FW) of an *Arabidopsis thaliana* Col-0 callus culture was separated by SDS-PAGE and blotted onto nitrocellulose. *Each Blot* was incubated with an IgG (dil. 1:1000) directed against one subunit of one of the five OXPHOS complexes. Detection of immune signals was carried out by immuno-histochemical staining. The molecular masses of standard proteins (High-range Molecular Weight Rainbow Marker, GE Healthcare, Munich, Germany) are given on the left (in kDa), the target proteins of the antibodies and their molecular masses (without presequences) are given at the *bottom* of the blots (I, complex I; II, complex II; III, complex III, IV, complex IV; V, complex V). *Red arrows* indicate the expected immune signals

Fig. 2 Separation of total protein of different *Arabidopsis* organs. Total protein (extracted from 5 mg FW) of different tissues of *Arabidopsis thaliana* Col-0 was separated by SDS-PAGE and stained with Coomassie colloidal. The molecular masses of standard proteins (High-range Molecular Weight Rainbow Marker, GE Healthcare, Munich, Germany) are given on the *left* (in kDa), the different tissues are indicated *above* the gel

defined protein amounts were loaded with respect to fresh weight of the plant material. Furthermore, defined dilutions were resolved for all fractions. Starting point of dilutions depended on the protein concentration of the tissue. Normally, dilution series started with a total protein extract equivalent to 0.6 mg fresh weight. At least three technical replicates were performed for each sample and each complex. One representative set of Western blots for each complex and each organ is shown in Fig. 3. The antibody directed against the COX2 subunit of complex IV cross reacts with storage proteins in the seed fraction. In this case, quantification was difficult but not impossible (online resource 5).

Results of all sets of Western blots were integrated to calculate relative quantities of the OXPHOS complexes in the different protein fractions. Quantification was based on chemiluminescence detection with subsequent evaluation by a software tool. Since signals for all OXPHOS complexes were highest in flowers, their amounts in this fraction were defined to be 100 %. *Note:* No statements on absolute quantities of OXPHOS complexes in Arabidopsis were obtained during this study because the intensity of the immune signals of the different antibodies most likely differs (the antibodies probably have differing specificities with respect to their target proteins).

A quantitative evaluation of the immune signals is shown in Fig. 4. Results either refer to fresh weight of the analysed tissue (Fig. 4a, b) or to total protein amount (online resource 6, part c and d). All complexes are present in highest amounts in flowers. However, decrease in amount with respect to flowers varies substantially for the individual complexes. Besides in flowers, abundance of complex I is especially high in leaves and very low in callus as well as root fractions. The opposite is true for complex II: It is relatively abundant in callus and seeds but of very low abundance in leaves. Complex V also is highly abundant in leaves. However, this result turned out not to be meaningful because the IgG directed against the beta subunit of the mitochondrial ATP-synthase complex cross-reacts with the beta subunit of the chloroplast ATP-synthase (online resource 4). Although plastidic ATP-synthase also is present in none green tissue (Green and Hollingsworth 1994), previous investigations indicate that expression of ATP synthase genes is regulated by light (Bolle et al. 1996). Our data for tissue other than leaves therefore are assumed to mainly reflect ATP-synthase of mitochondria. However, results for ATP synthase have to be taken with caution. Variation in abundance for complexes III and IV is less severe within the investigated fractions.

Activity of OXPHOS complexes from different Arabidopsis organs

In order to investigate the activity of the OXPHOS complexes in various organs of Arabidopsis, total protein fractions were isolated under native conditions and resolved by one-dimensional blue native PAGE (Fig. 5). Dimensions of the fractions were defined based on identical amounts of fresh weight for each tissue. Activity of OXPHOS complexes subsequently was visualized by *in gel* activity stains. As a control, isolated mitochondria from *Arabidopsis thaliana* callus were resolved on the same gels
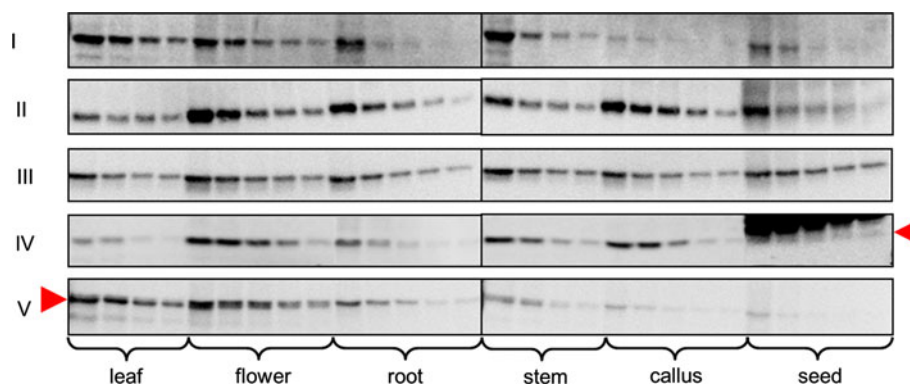

Fig. 3 Western blot analysis for quantification of OXPHOS complexes. Total protein from six different tissues of *Arabidopsis thaliana* Col-0 (extracted from 0.6 mg FW, respectively) was separated by 1D SDS-PAGE and subsequently transferred onto nitrocellulose membrane. *Blots* were incubated with specific IgGs directed against one subunit of each OXPHOS complex. The following dilutions of the extracted protein fractions were loaded onto the gel: leaf: 1/1, 1/2, 1/4, 1/8; flower: 1/2, 1/4, 1/8, 1/16, 1/32; root: 1/1, 1/2, 1/4, 1/8, 1/16; stem: 1/1, 1/2, 1/4, 1/8; callus: 1/2, 1/4, 1/8, 1/16, 1/32; seed: 1/2, 1/4, 1/8, 1/16, 1/32. Immune signals were detected by chemiluminescence with subsequent quantification. The identities of the OXPHOS complexes recognized by the five IgGs are given on the *left*, the identities of the analyzed protein fractions at the bottom of the blots. In case more than one immune signal is visible on the blots, the signal representing the targeted subunit is indicated by a *red arrowhead*. The very strong signal close to complex IV in seeds in some cases was visible on our blots and is due to a cross reaction of the COX2 antibody with one of the seed storage proteins (see also online resources 5)
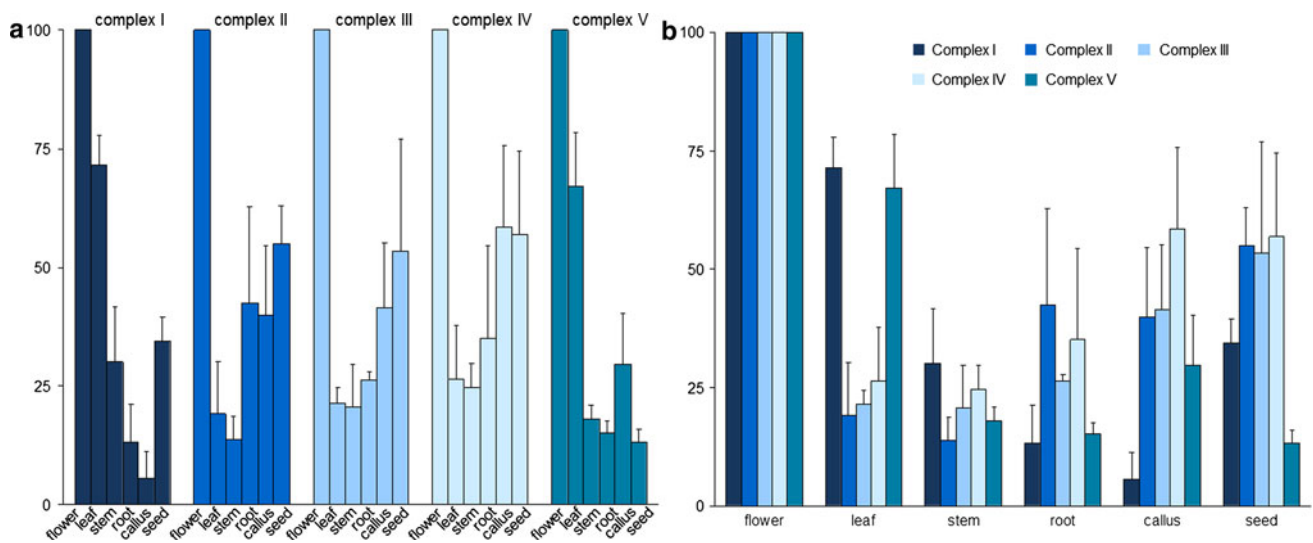
Fig. 4 Quantification of OXPHOS complexes in different tissues of *Arabidopsis thaliana*. Data are based on immune signals obtained by Western blotting (Fig. 3) with subsequent quantification of signals. Results refer to three replicates for each tissue and each complex. Since all five OXPHOS complexes were most abundant in flowers, this tissue was set as a standard (100 %). **a** Relative amounts of OXPHOS complex per FW (*y*-axis), analyzed tissue (*x*-axis).

Identities of the complexes are given above the *graph* and by *colors* (complex I: *dark-blue*, complex II: *middle-blue*, complex III: *light-blue*, complex IV: *very-light-blue*, complex V: *turquoise*). **b** Same as **a**, but data sorted according to tissues. The color code for the five complexes is the same as in part **a**. **c** and **d** (see online resource 6): Same as **a** and **b** but quantification of OXPHOS complexes is related to total protein amount of the fractions

(Fig. 5, left lane on each gel). As expected, signals in the mitochondrial fractions were extremely strong due to enrichment of the OXPHOS complexes. In contrast, signals for the OXPHOS complexes were much weaker in the total protein fractions of tissues due their relative low abundance. Furthermore, extraction of total proteins from seeds proved to be difficult. Besides these limitations the following results were obtained:

Three bands are detectable by the *activity stain for complex I* (Fig. 5b). The two bands in the upper part of the gel represent complex I and the I + III$_2$ supercomplex. Complex I activity is detectable in all Arabidopsis organs but highest in flowers. Activity in leaves is detectable but seems not to be higher than in the other fractions. This indicates that increased amount of complex I in leaves not necessarily causes an increase in complex I activity. Interestingly, the I + III$_2$ supercomplex is of low abundance in flowers. Recently, dissociation of complex I from the supercomplex was shown to occur under hypoxic conditions as well under low pH and was interpreted to represent a regulatory mechanism which leads to increased alternative respiration (Ramírez-Aguilar et al. 2011). Since flowers normally do not suffer from hypoxic conditions low amounts of I + III$_2$ supercomplex rather reflect a different regulatory mechanism which might be related to protecting the respiratory chain from over reduction during phases of high OXPHOS activity typically occurring during flower development.

The signal in the low molecular mass range of the gel reveals activity either of an alternative NADH-dehydrogenase or other enzymes with NADH acceptor activity, e.g. dihydrolipoamide dehydrogenase. It is also possible that this activity derives from extra-mitochondrial enzymes since activity assays are based on total protein fractions of the individual organs.

Complex II activity assay reveals two bands in the mitochondrial control fraction (Fig. 5c). The upper band represents the known 8-subunit version of complex II described before for higher plants (Eubel et al. 2003; Millar et al. 2004). The band in the lower part of the gel is a smaller form of complex II which only contains 4 subunits (Huang et al. 2010). Highest complex II activity was found in callus followed by the flower fraction. Activity in all other fractions was at the limit of detection.

Complex IV activity, apart from the control, is highest in the callus and flower fractions (Fig. 5d). Some high molecular mass complexes also are visible on the gels which represent complex IV containing respiratory super-complexes described before (Eubel et al. 2004; Welchen et al. 2011).

We conclude that *in gel* activity of the three investigated OXPHOS complexes correlates with their relative amounts determined by immunoblotting. However, there are exceptions which most likely indicate the presence of unknown post-translational regulation mechanisms.
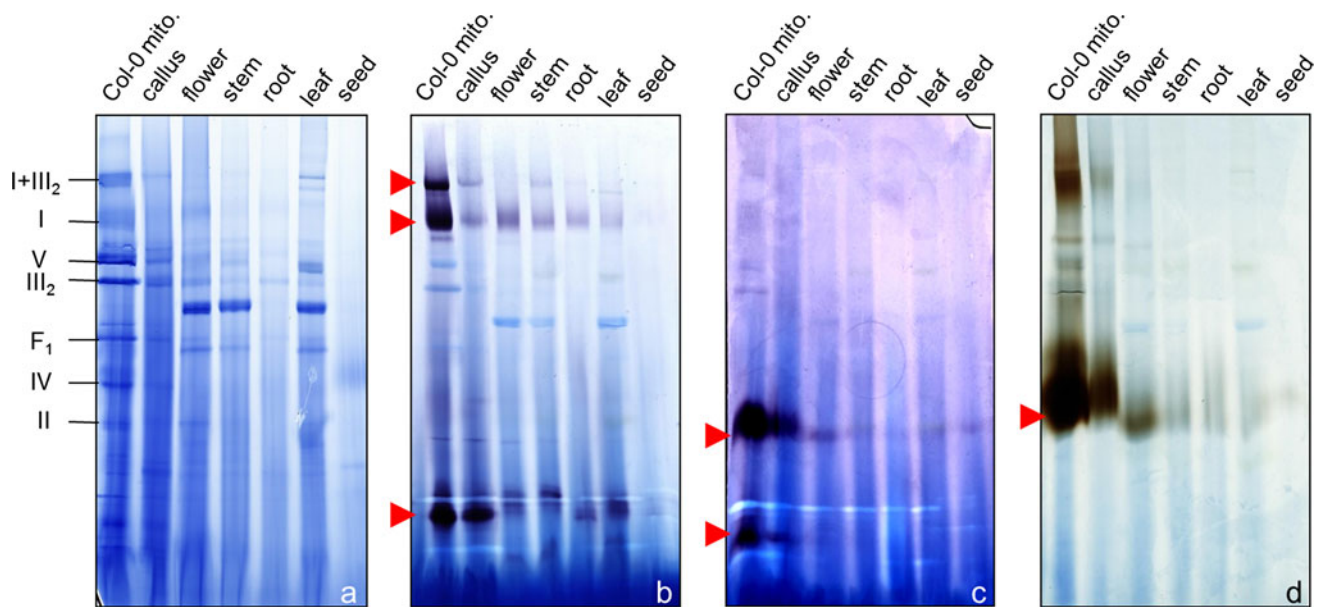
Fig. 5 In-gel activity of the OXPHOS complexes I, II and IV. Total membrane protein from different tissues of *Arabidopsis thaliana* Col-0 was extracted under native conditions (starting material: 285 mg FW, respectively) and separated by 1D BN-PAGE. Isolated mitochondria from an *Arabidopsis thaliana* Col-0 callus culture were taken as control (0.5 mg). Identities of resolved OXPHOS complexes are given on the left of the gels: I, complex I; V, complex V; III_2, dimeric complex III; F_1, F_1 part of complex V; IV, complex IV; II, complex II; $I + III_2$, supercomplex composed of complex I and dimeric complex III. Tissues are indicated above the gels. **a** Coomassie colloidal stain, **b** complex I activity stain, **c** complex II activity stain, **d** complex IV activity stain. *Red arrows* indicate activity signals

Abundance of OXPHOS complexes from mitochondria isolated from different Arabidopsis organs

Quantification of OXPHOS complexes was performed on the basis of total protein fractions and not on isolated mitochondria in order to avoid misinterpretations due to varying properties of mitochondria in the analysed tissues (e.g. variable densities which might result in differential isolation efficiencies). However, in a final experiment, quantity of OXPHOS complexes was compared by 2D BN/SDS-PAGE in two mitochondrial fractions isolated from (1) callus cultures and (2) green leaves of Arabidopsis plants (Fig. 6). Although quantity of the resolved protein complexes were not evaluated by software tools, higher complex I abundance in the mitochondria from leaves is very obvious. In contrast, complex II abundance in callus seems not to be increased with respect to leaves as found by analyses using total protein fractions. This result possibly indicates differential loss of mitochondrial sub-fractions, which may vary with respect to content of individual OXPHOS complexes, during the mitochondrial isolation procedures.

Correlation between protein abundance and gene expression

Does protein amount in different Arabidopsis tissues correlate with expression of the corresponding genes? To answer this question, the protein abundance data shown in Fig. 4 were compared to expression data downloaded from the Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). In case isogenes are present for a subunit, mean values for expression were calculated for the analysis. The COX2 subunit was not included into the investigation because its transcription is not precisely known (COX2 is encoded by the mitochondrial genome). Expression data for the beta-subunit of the mitochondrial ATP-synthase are also not shown because comparability of the two datasets is complicated due to the cross reaction of the IgG (online resource 4). Since protein abundance in flowers was set as a standard, no conclusions can be drawn for this organ. The protein-transcript comparison reveals a striking correlation for the complex II subunit SDH1 (Fig. 7). In contrast, correlation for the other subunits is low. We conclude that gene expression data not necessarily reflect abundance of proteins, which again indicates further levels of regulation during protein biosynthesis besides regulation of transcription. Similar results recently were found by a study comparing the proteomes of isolated mitochondria from different Arabidopsis organs with corresponding transcriptomic data (Lee et al. 2008, 2011).

Discussion

This study aims to investigate the ratio of OXPHOS complexes in various organs of *Arabidopsis thaliana*.
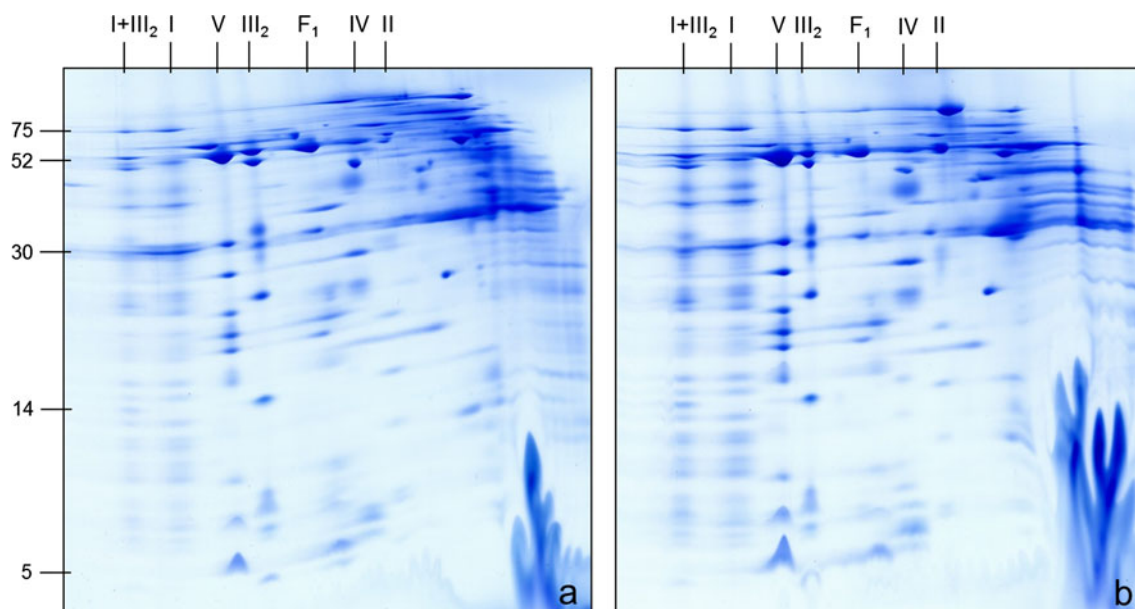
Fig. 6 Quantitative comparison of OXPHOS complexes in isolated mitochondria from callus culture and green leaves. Total mitochondrial membrane protein (0.5 mg) from an *Arabidopsis thaliana* Col-0 callus culture (**a**) and *Arabidopsis thaliana* Col-0 green leaves (**b**) was separated by 2D BN/SDS-PAGE and stained with Coomassie colloidal. Identities of resolved protein complexes and supercomplexes are given above the gel (for nomenclature see Fig. 5). The molecular masses of standard proteins (in kDa) are given on the left of the gel
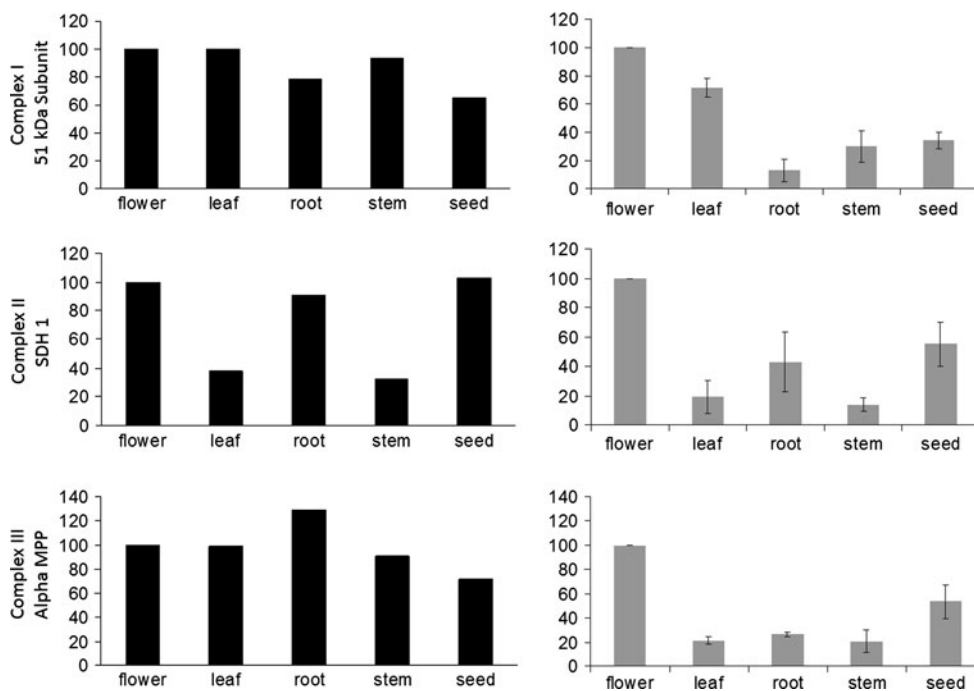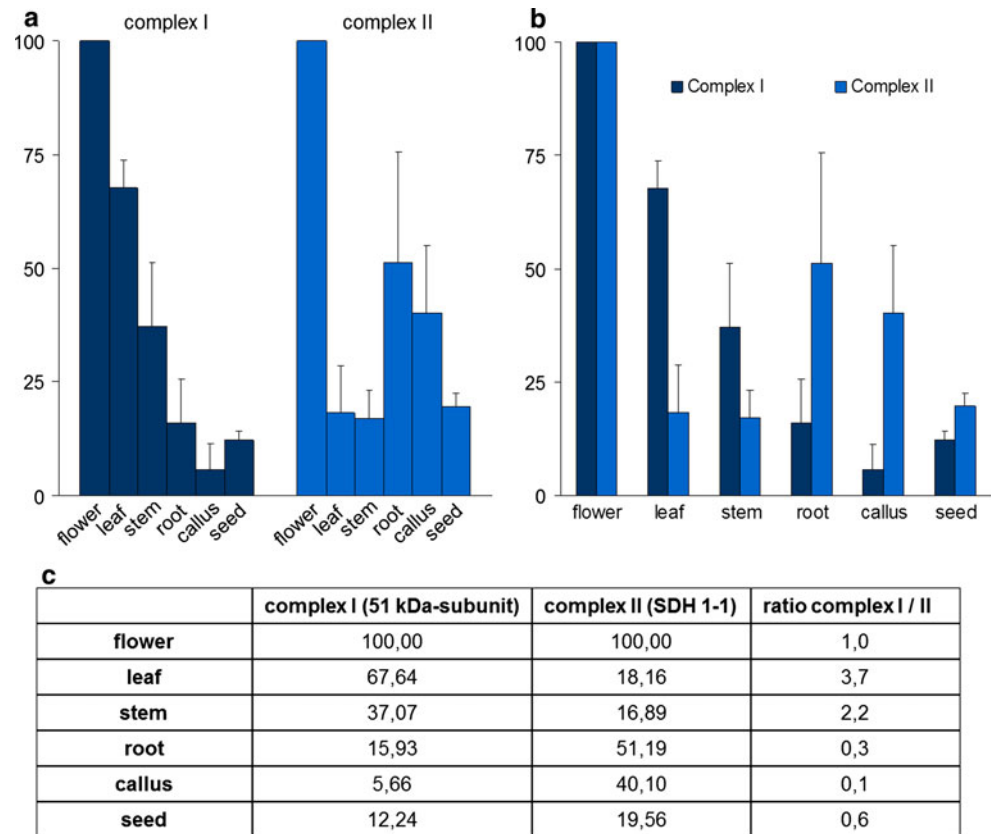

Fig. 7 Correlation of protein amount and expression of corresponding genes in different tissues of *Arabidopsis thaliana*. Relative amounts of four subunits of three different OXPHOS complexes per fresh weight were taken from Fig. 4a. Corresponding gene expression data were downloaded from the Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Expression data are given on the *left* (*black bars*), protein quantification data on the *right* (*gray bars*). For better comparability, expression data also are given as relative amounts of transcripts with respect to amounts in flowers. Identities of tissues are given below the *bars*, identities of the analyzed OXPHOS subunits on the *left*. Data for complex IV are not shown because expression of the gene encoding the COX2 subunit is not available from the database (COX2 is encoded on the mitochondrial genome)

Fig. 8 Complex I–complex II ratios in different tissues of *Arabidopsis thaliana* Col-0. Data represent a subset of results presented in Fig. 4. **a** Relative amounts of complex I and II (reference: amounts in flowers) per total protein of the fraction. The analyzed tissues are given below the graphs. **b** Same as a, but data sorted by tissues. **c** Complex I–complex II ratio in *numbers*

	complex I (51 kDa-subunit)	complex II (SDH 1-1)	ratio complex I / II
flower	100,00	100,00	1,0
leaf	67,64	18,16	3,7
stem	37,07	16,89	2,2
root	15,93	51,19	0,3
callus	5,66	40,10	0,1
seed	12,24	19,56	0,6

Three different lines of evidence indicate drastic changes in complex I–complex II ratio: (1) quantitative immunoblotting using total protein fractions (2) *in gel* activity stains using total protein fractions and (3) analyses by 2D BN/SDS-PAGE using isolated mitochondria. Since highest amounts for all OXPHOS complexes were detected in flowers, their quantity within this fraction was set to be 100 %. Complex I is especially abundant in green organs (leaves and stems) and shows a decrease in abundance in non-green fractions (roots and callus). In contrast, complex II is relatively abundant in callus and seeds but displays low amounts in leaves and stems. Figure 8 summarizes the differences in abundances of complexes I and II as obtained by quantitative immunoblotting. Also, abundances of complexes III and IV vary in different organs but variation is less extreme (Fig. 4). The complex I–complex II ratio is of special interest because these two complexes represent the main entrance points of electrons into the mitochondrial electron transport chain. However, electrons also enter the respiratory chain via the alternative oxidoreductases in plants. Dynamic changes in abundances of these enzymes were not part of the current investigation but are known to occur, e.g. due to light-regulated expression of their genes (Rasmusson et al. 2008).

The ratio of mitochondrial OXPHOS complexes extensively was analysed for other groups of organisms, especially in mammals (Hatefi 1985; Capaldi et al. 1988; Schägger and Pfeiffer 2001; Antonicka et al. 2006; Benard et al. 2006). Initial investigations based on spectrophotometric quantification of prosthetic groups of the OXPHOS complexes revealed ratios for the complexes I, II, III, IV and V in the range of 1:2:3:6–7:3–5 in beef (Hatefi 1985). In a careful investigation, which employed spectrophotometric and electrophoretic-densitometric methods, Schägger and Pfeiffer (2001) basically confirmed these data and presented even more precise values on the ratios of the OXPHOS complexes in beef, which are 1.1:2.1:3:6.7:3.5. Benard et al. (2006) investigated the ratio of OXPHOS complexes in different rat tissues using various biochemical methods like immunological techniques as well as spectrophotometry. Ratios for complexes II, III and IV were found to be 1–1.5:3:6.5–7.5, which are consistent with the previous values published for beef. Using quantitative immunoblotting, strong differences with respect to the ratio of OXPHOS complexes were reported for human mitochondria from various tissues (Antonicka et al. 2006). All complexes are highly abundant in muscle and heart, whereas complexes I, III, IV and V only show low abundance in liver and fibroblasts. The amount of complex II is almost the same for all tissues.

In contrast, little is known about the ratio of OXPHOS complexes in plants. Using analyses by 1D or 2D PAGE,

mitochondrial proteins of different organs were studied in spinach, sugar beet, maize, pea, potato, wheat and Arabidopsis (Sahlstrom and Ericson 1984, Newton and Walbot 1985, Remy et al. 1987, Lind et al. 1991, Rios et al. 1991, Colas des Francs-Small et al. 1992, Jänsch et al. 1996, Bardel et al. 2002, Lee et al. 2008, 2011). Results are based on investigations using isolated organelles. In potato, abundances of complexes I, III, IV and V were estimated to be in a similar range (Jänsch et al. 1996). However, in the latter study only mitochondria from potato tubers were analysed.

Our current investigation, which is based on total protein fractions, reveals strong differences in the ratio of the protein complexes of the OXPHOS system in different organs of the model plant Arabidopsis. Although no data on the absolute ratios of the complexes were obtained (the antibodies used for this study most likely differ in specificity), relative differences are very clear, especially with respect to complexes I and II. Most strikingly, complex I is of high relative abundance in photosynthetically active organs (leaves, stems, flowers). It so far only was known that the alternative NAD(P)H dehydrogenases are up-regulated in the light. In contrast to complex I, complex II amounts are especially high in callus, indicating an important function of this complex in this tissue type. Very recently, Gleason et al. (2011) reported that complex II contributes to localized ROS production in mitochondria and thereby regulates the plant stress and defence response (which should be of special relevance in callus).

Why complex I is especially important in the context of photosynthesis? There possibly are several reasons. The main function of complex I, re-oxidation of NADH in the mitochondrial matrix, is particularly essential in the light because additional NADH is formed by glycine to serine conversion during photorespiration. Indeed, NADH formed by photorespiration is the main substrate of the respiratory chain under high light, and not the NADH formed by the citric acid cycle like in mammalian systems (Tcherkez et al. 2008). Previous investigations on complex I mutants already revealed the importance of mitochondrial complex I for maintenance of the redox balance in leaves (Dutilleul et al. 2003a, b).

In addition, complex I of plant mitochondria is known to include side functions. Most notably, it includes five subunits very much resembling bacterial gamma-type carbonic anhydrases (reviewed in Braun and Zabaleta 2007; Klodmann and Braun 2011). It was suggested that these enzymes form part of a pathway to recycle photorespiratory CO_2 for photosynthesis (Braun and Zabaleta 2007, Zabaleta et al. 2012). This side function of complex I, although not experimentally proven, could well explain the special importance of complex I during photosynthesis. Finally, L-galactone-1,4-lactone dehydrogense (GLDH),

which catalyses the terminal step of ascorbate biosynthesis in plants, is associated with mitochondrial complex I. Ascorbate especially is needed in the chloroplast during photosynthesis for photoprotection (Apel and Hirt 2004). However, it so far was not shown that GLDH forms part of the fully assembled complex I in plants, but rather of its assembly intermediates (Millar et al. 2003; Pineau et al. 2008; Schertl et al. 2012). Therefore, the importance of complex I for ascorbate biosynthesis so far remains elusive. Further investigations will be necessary to fully understand the special importance of complex I in the context of photosynthesis.

References

Antonicka H, Sasarman F, Kennaway NG, Shoubridge EA (2006) The molecular basis for tissue specificity of the oxidative phosphorylation deficiencies in patients with mutations in the mitochondrial translation factor EFG1. Hum Mol Genet 15:1835–1846

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399

Bardel J, Louwagie M, Jaquinod M, Jourdain A, Luche S, Rabilloud T, Macherel D, Garin J, Bourguignon J (2002) A survey of the plant mitochondrial proteome in relation to development. Proteomics 2:880–898

Benard G, Faustin B, Passerieux E, Galinier A, Rocher C, Bellance N, Delage JP, Casteilla L, Letellier T, Rossignol R (2006) Physiological diversity of mitochondrial oxidative phosphorylation. Am J Physiol Cell Physiol 291:1172–1182

Bolle C, Kusnetsov VV, Herrmann RG, Oelmüller R (1996) The spinach AtpC and AtpD genes contain elements for light-regulated, plastid-dependent and organ-specific expression in the vicinity of the transcription start sites. Plant J 9:21–30

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Braun HP, Zabaleta E (2007) Carbonic anhydrase subunits of the mitochondrial NADH dehydrogenase complex (complex I) in plants. Physiol Plant 129:114–122

Capaldi RA, Halphen DG, Zhang YZ, Yanamura W (1988) Complexity and tissue specificity of the mitochondrial respiratory chain. J Bioenerg Biomembr 20:291–311

Colas des Francs-Small C, Ambard-Bretteville F, Darpas A, Sallantin M, Huet JC, Pernollet JC, Remy R (1992) Variation of the polypeptide composition of mitochondria isolated from different potato tissues. Plant Physiol 98:273–278

Colditz F, Nyamsuren O, Niehaus H, Eubel H, Braun HP, Krajinski F (2004) Proteomic approach: identification of *Medicago truncatula* proteins induced in roots after infection with the pathogenic oomycete *Aphanomyces euteiches*. Plant Mol Biol 55:109–120

Dutilleul C, Driscoll S, Cornic G, de Paepe R, Foyer CH, Noctor G (2003a) Functional mitochondrial complex I is required by tobacco leaves for optimal photosynthetic performance in photorespiratory conditions and during transients. Plant Physiol 131:264–275

Dutilleul C, Garmier M, Noctor G, Mathieu C, Chétrit P, Foyer CH, de Paepe R (2003b) Leaf mitochondria modulate whole cell

redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. Plant Cell 15:1212–1226

Eubel H, Jänsch L, Braun HP (2003) New insights into the respiratory chain of plant mitochondria. Supercomplexes and unique composition of complex II. Plant Physiol 133:274–286

Eubel H, Heinemeyer J, Braun HP (2004) Identification and characterization of respirasomes in potato mitochondria. Plant Physiol 134:1450–1459

Gleason C, Huang S, Thatcher LF, Foley RC, Anderson CR, Carroll AJ, Millar AH, Singh KB (2011) Mitochondrial complex II has a key role in mitochondrial-derived reactive oxygen species influence on plant stress gene regulation and defense. Proc Natl Acad Sci USA 108:10768–10773

Green CD, Hollingsworth MJ (1994) Tissue-specific expression of the large ATP synthase gene cluster in spinach plastids. Plant Mol Biol 25:369–376

Hatefi Y (1985) The mitochondrial electron transport and oxidative phosphorylation system. Ann Rev Biochem 54:1015–1069

Huang S, Taylor NL, Narsai R, Eubel H, Whelan J, Millar AH (2010) Functional and composition differences between mitochondrial complex II in Arabidopsis and rice are correlated with the complex genetic history of the enzyme. Plant Mol Biol 72:331–342

Hurkman WJ, Tanaka CK (1986) Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol 81:802–806

Jänsch L, Kruft V, Schmitz UK, Braun HP (1996) New insights into the composition, molecular mass and stoichiometry of the protein complexes of plant mitochondria. Plant J 9:357–368

Jung C, Higgins CM, Xu Z (2000) Measuring the quantity and activity of mitochondrial electron transport chain complexes in tissues of central nervous system using blue native polyacrylamide gel electrophoresis. Anal Biochem 15:214–223

Keech O, Dizengremel P, Gardeström P (2005) Preparation of leaf mitochondria from Arabidopsis thaliana. Physiol Plant 124:403–409

Klodmann J, Braun HP (2011) Proteomic approach to characterize mitochondrial complex I from plants. Phytochemistry 72:1071–1080

Klodmann J, Sunderhaus S, Nimtz M, Jänsch L, Braun HP (2010) Internal architecture on mitochondrial complex I from Arabidopsis thaliana. Plant Cell 22:797–810

Kruft V, Eubel H, Jänsch L, Werhahn W, Braun HP (2001) Proteomic approach to identify novel mitochondrial proteins in Arabidopsis. Plant Physiol 127:1694–1710

Lee CP, Eubel H, O'Toole N, Millar AH (2008) Heterogeneity of the mitochondrial proteome for photosynthetic and non-photosynthetic Arabidopsis metabolism. Mol Cell Proteomics 7:1297–1316

Lee CP, Eubel H, O'Toole N, Millar AH (2011) Combining proteomics of root and shoot mitochondria and transcript analysis to define constitutive and variable components in plant mitochondria. Phytochemistry 72:1092–1108

Lind C, Hallden C, Moller IM (1991) Protein synthesis in mitochondria purified from roots, leaves and flowers of sugar beet. Physiol Plant 83:7–16

May MJ, Leaver CJ (1993) Oxidative stimulation of glutathione synthesis in Arabidopsis thaliana suspension cultures. Plant Physiol 103:621–627

Millar AH, Mittova V, Kiddle G, Heazlewood J, Bartoli C, Theodoulou F, Foyer C (2003) Control of ascorbate synthesis by respiration and its implications for stress responses. Plant Physiol 133:443–447

Millar AH, Eubel H, Jänsch L, Kruft V, Heazlewood JL, Braun HP (2004) Mitochondrial cytochrome c oxidase and succinate

dehydrogenase complexes contain plant specific subunits. Plant Mol Biol 56:77–90

Millar AH, Whelan J, Soole KL, Day DA (2011) Organization and regulation of mitochondrial respiration in plants. Annuv Rev Plant Biol 62:79–104

Neuhoff V, Stamm R, Eibl H (1988) Clear background and highly sensitive protein staining with coomassie blue dyes in polyacrylamide gels: a systematic analysis. Electrophoresis 6:427–448

Neuhoff V, Stamm R, Pardowitz I, Arold N, Ehrhardt W, Taube D (1990) Essential problems in quantification of proteins following colloidal staining with coomassie brilliant blue dyes in polyacrylamide gels, and their solution. Electrophoresis 11:101–117

Newton KJ, Walbot V (1985) Maize mitochondria synthesize organ-specific polypeptides. Proc Acad Natl Sci USA 82:6879–6883

Pineau B, Layoune O, Danon A, de Paepe R (2008) L-galactono-1,4-lactone dehydrogenase is required for the accumulation of plant respiratory complex I. J Biol Chem 283:32500–32505

Ramírez-Aguilar SJ, Keuthe M, Rocha M, Fedyaev VV, Kramp K, Gupta KJ, Rasmusson AG, Schulze WX, van Dongen JT (2011) The composition of plant mitochondrial supercomplexes changes with oxygen availability. J Biol Chem 286:43045–43053

Rasmusson AG, Escobar MA (2007) Light and diurnal regulation of plant respiratory gene expression. Physiol Plant 129:57–67

Rasmusson AG, Wallström SV (2010) Involvement of mitochondria in the control of plant cell NAD(P)H reduction levels. Biochem Soc Trans 38:661–666

Rasmusson AG, Geisler DA, Møller IM (2008) The multiplicity of dehydrogenases in the electron transport chain of plant mitochondria. Mitochondrion 8:47–60

Remy R, Ambard-Bretteville F, Colas des Francs C (1987) Analysis by two-dimensional gel electrophoresis of the polypeptide composition of pea mitochondria isolated from different tissues. Electrophoresis 8:528–532

Rios R, de Buyser J, Henry Y, Ambard-Bretteville F, Remy R (1991) Two-dimensional electrophoretic comparison of mitochondrial polypeptides from different wheat (Triticum aestivum L.) tissues. Plant Sci 76:159–166

Sahlstrom S, Ericson I (1984) Comparative electrophoretic studies of polypeptides in leaf, petiole and root mitochondria from spinach. Physiol Plant 61:45–50

Schägger H, Pfeiffer K (2001) The ratio of oxidative phosphorylation complexes I–V in bovine heart mitochondria and the composition of respiratory chain supercomplexes. J Biol Chem 276:37861–37867

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379

Schertl P, Sunderhaus S, Klodmann J, Gergoff G, Bartoli CG, Braun HP (2012): L-galactono-1,4-lactone dehydrogenase (GLDH) forms part of three subcomplexes of mitochondrial complex I in Arabidopsis thaliana. J Biol Chem, in press

Sunderhaus S, Dudkina N, Klodmann J, Heinemeyer J, Perales M, Zabaleta E, Boekema E, Braun HP (2006) Carbonic anhydrases subunits form a matrix-exposed domain attached to the membrane arm of complex I in plants. J Biol Chem 281:6482–6488

Tcherkez G, Bligny R, Gout E, Mathé M, Cornic G (2008) Respiratory metabolism of illuminated leaves depends on CO_2 and O_2 conditions. Proc Natl Acad Sci 105:797–802

Welchen E, Klodmann J, Braun HP (2011) Biogenesis and supramolecular organization of the oxidative phosphorylation system in plants. In: Kempken F (ed) Plant mitochondria, advances in plant Biology, 1st edn. Springer, New York, pp 327–355

Werhahn WH, Niemeyer A, Jänsch L, Kruft V, Schmitz UK, Braun HP (2001) Purification and characterization of the preprotein

translocase of the outer mitochondrial membrane from Arabidopsis thaliana: identification of multiple forms of TOM20. Plant Physiol 125:943–954

Wittig I, Braun HP, Schägger H (2006) Blue native PAGE. Nat Protoc 1:418–428

Zabaleta E, Martin MV, Braun HP (2012) A basal carbon concentrating mechanism in plants? Plant Sci 187:97–104

Zerbetto E, Vergani L, Dabbeni-Sala F (1997) Quantification of muscle mitochondrial oxidative phosphorylation enzymes via histochemical staining of blue native polyacrylamide gels. Electrophoresis 18:2059–2064