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ORIGINAL PAPER

Simultaneous isolation of pure and intact chloroplasts and mitochondria from moss as the basis for sub-cellular proteomics

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Abstract The moss *Physcomitrella patens* is increasingly being used as a model for plant systems biology studies. While genomic and transcriptomic resources are in place, tools and experimental conditions for proteomic studies need to be developed. In the present study we describe a rapid and efficient protocol for the simultaneous isolation of chloroplasts and mitochondria from moss protonema. Routinely, 60–100 μg mitochondrial and 3–5 mg chloroplast proteins, respectively, were obtained from 20 g fresh weight of green moss tissue. Using 14 plant compartment marker antibodies derived from seed plant and algal protein sequences, respectively, the evolutionary conservation of the compartment marker proteins in the moss was demonstrated and purity and intactness of the extracted organelles confirmed. This isolation protocol and these validated compartment markers may serve as basis for sub-cellular proteomics in *P. patens* and other mosses.

Keywords Organelles · Compartment marker · Chloroplast proteins · Mitochondrial proteins · *Physcomitrella* · Bryophyte

Abbreviations

AOX	Alternative oxidase
Arf	ADP-ribosylation factor
Csp41b	Ribosome associated endonuclease
COX	Cytochrome *c* oxidase
CPX1	Coproporphyrinogen III oxidase
CRD1	Cyanobacterial homolog of plant CHL27 cyclase
Cyt *f*	Cytochrome *f* protein of thylakoid cytochrome b6/f-complex
GLN	Glutamine synthetase
Hsp70b	Stromal alfa-heat shock protein 70
Lhcb2	Light harvesting complex II chlorophyll a/b-binding protein
PsaD	Photosystem I reaction centre subunit II
PsbP	23 kDa protein of the oxygen evolving complex of photosystem II
VDAC	Voltage dependent anion channel
V-type ATPase	Vacuolar-type ATPase

Introduction

As the first non-seed plant with a completely sequenced genome (Rensing et al. 2008) the moss *Physcomitrella*

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patens has been established as a plant system to investigate the evolution of stress adaptation (Frank et al. 2007; Khandelwal et al. 2010) and of signalling events (Heintz et al. 2004, 2006) in early land plants. Along with these studies a wide range of high-throughput molecular biology tools has been developed and implemented in recent years (Richardt et al. 2007, 2010) paving the way for the use of this model organism for systems biology studies (Decker et al. 2006).

Focussing on plant organelles in a moss such as *P. patens* can be of special interest to obtain information on the evolution of metabolic compartmentalisation (Kopriva et al. 2007; Wiedemann et al. 2010), biosynthetic pathways (Stumpe et al. 2006) and protein sorting mechanisms (Kiessling et al. 2004, Mitschke et al. 2009, Richter et al. 2002). Of special interest are chloroplasts and mitochondria as they are semi-autonomous organelles of endosymbiotic origin with own DNA that encodes only for a small subset of proteins localised to these organelles. Hence, most of the proteins are nuclear-encoded and have to be imported into chloroplasts and mitochondria, respectively (Gray et al. 1999; Reski 2009; Strittmatter et al. 2010). The prediction of sub-cellular protein localisation, however, is error prone because the transit peptides are not well conserved (Bruce 2001) and prediction algorithms are usually trained on the basis of proteins from seed plants. Experimental data sets have shown that the tools currently available for the prediction of sub-cellular localisation can only identify about 50% of the proteins targeted to organelles (Heazlewood et al. 2004; Kleffmann et al. 2004). These limitations can only be overcome by the generation of species-specific training data sets for the respective organelles, the data sets being very much dependent on the specificity, i.e. correct prediction of the protein localisation (Baginski and Gruissem 2004; Salvi et al. 2008b). The generation of reliable data sets is, however, difficult as contaminations with proteins from other organelles and from the cytosol can never be ruled out during the isolation of single organelles.

Many protocols for the isolation of plant organelles in seed plants are established and have been used for subsequent high-throughput shotgun proteomic studies of *Arabidopsis* chloroplasts (Kleffmann et al. 2004; Baginski et al. 2005) and mitochondria (Heazlewood et al. 2004; Millar et al. 2001a, b; Sweetlove et al. 2007) or for example, the analysis of mitochondria in rice (Heazlewood et al. 2003; Huang et al. 2009). All these studies employ density gradients for the purification of organelles, sometimes combining it with free flow electrophoresis (FFE) to separate chloroplasts from mitochondria (Eubel et al. 2007; Huang et al. 2009; Lee et al. 2008). However, losses of about 50% of the organelle material can occur (Eubel et al. 2007), creating a need for the adaptation of existing protocols for each model species (Sweetlove et al. 2007).

For the moss *P. patens* protocols for the isolation of organelles via density gradients have been reported (Kabeya and Sato 2005; Kasten et al. 1997; Marienfeld et al. 1989). However, the moss material used in these experiments was always subjected to protoplastation, which besides from being a laborious and costly pretreatment of the material might also have an effect on the physiological status of the cell and, hence, its proteome.

The aim of this study was to set up a protocol for the simultaneous isolation of highly enriched fractions of pure and intact chloroplasts and mitochondria from protonema tissue of *P. patens*. Integrity and purity of these fractions as well as potential contaminations were assessed using a set of plant compartment marker antibodies. The protocol presented here enables for the isolation of intact chloroplasts and mitochondria and delivers protein yields that are sufficient for sub-cellular proteomic studies in *Physcomitrella*. Such studies can provide the basis for a large-scale analysis of protein sorting mechanisms in moss and, in addition, unravel the evolution of metabolic and biosynthetic processes occurring in plant organelles.

Materials and methods

Plant material and growth conditions

Protonema of *Physcomitrella patens* (Hedw.) Bruch & Schimp. was cultured in modified liquid Knop medium according to Reski and Abel (1985) containing 250 mg/l KH_2PO_4, 250 mg/l KCl, 250 mg/l $MgSO_4 \times 7\ H_2O$, 1,000 mg/l $Ca(NO_3)_2 \times 4H_2O$ and 12.5 mg/l $FeSO_4 \times 7H_2O$ (pH 5.8).

Protonema filaments were disrupted with an Ultra-Turrax (IKA, Staufen, Germany) at 18,000 rpm for 90 s before inoculation. Round-bottom flasks containing 5 l of medium were inoculated with 0.3 g dry weight and aerated with 0.3 vvm at 25°C under long day conditions [16 h light, 8 h dark, Osram TLD 36 W/25, 70 µmol/(m^2 s)]. After 7 days, moss was harvested and filter-dried using a vacuum pump and a Büchner funnel.

Organelle isolation

All following steps were performed at 4°C and, where applicable, on wet ice. Typically, two 5 l flasks were used per experiment, which corresponds to 20 g fresh weight of moss. The vacuum filtrated protonema was chopped in organelle isolation buffer [1% (w/v) polyvinylpolypyrrolidone (PVPP), 300 mM D-sorbitol, 50 mM HEPES, 2 mM Na-EDTA, 1 mM $MgCl_2$ and 0.1% BSA] in the presence of a protease inhibitor [0.1% (v/v) Sigma Plant Protease Inhibitor Cocktail P 9599] using a household vegetable

chopping device. After 100–150 strokes with this device the chopped moss was filtered through 3 layers of Miracloth (Calbiochem). The filtrate was transferred to a 50 ml centrifuge tube (Oak Ridge, Nalgene) and the chloroplasts were pelleted at 1,500×*g* for 10 min (Beckman Coulter Avanti Centrifuge J-25, fixed angle rotor Ja 25.50). The supernatant was decanted into new centrifuge tubes and used for the isolation of mitochondria (see below).

A workflow diagram outlining the isolation protocol is given in Fig. 1.

Chloroplast isolation

The crude chloroplast pellet was resuspended in 3 ml resuspension buffer (300 mM D-sorbitol, 50 mM HEPES, 2 mM Na-EDTA, 1 mM $MgCl_2$ and 0.1% BSA) using a fine paint brush. The resuspended sample was carefully loaded onto several Percoll density gradients (top to bottom: 5 ml 10% Percoll, 5 ml 40% Percoll, 5 ml 80% Percoll) prepared in resuspension buffer. The Percoll gradients were centrifuged for 20 min at 16,000×*g*. Broken chloroplasts accumulated at the 10–40% interface, while intact chloroplasts formed a band at the 40–80% interface. The fractions containing intact chloroplast were collected and combined using a thin glass pipette. The chloroplasts were washed twice with three volumes of washing buffer (300 mM D-sorbitol, 50 mM HEPES, 2 mM Na-EDTA, 1 mM $MgCl_2$) and centrifuged for 10 min at 1,500×*g*. Organelle purity was monitored via fluorescence microscopy. Pellets were frozen at −80°C.

Mitochondria isolation

Mitochondria were purified from the 1,500×*g* supernatant adapting a protocol of Kabeya and Sato (2005) using differential centrifugation and a Percoll density gradient. The supernatant was centrifuged at 3,000×*g* for 5 min followed by 6,000×*g* for 5 min in the same tube in order to pellet nuclei and cellular debris. Afterwards the resulting supernatant was decanted to a new tube and centrifuged at 18,000×*g* for 20 min yielding a greenish pellet enriched in mitochondria.

The pellet was carefully resuspended in 1 ml of washing buffer using a fine paint brush. Several pellets were combined in a potter (Braun, Melsungen, Germany) to a final volume of 4.8 ml and homogenised with 10 strokes. Subsequently 100% Percoll was added to the sample to a final concentration of 20%.

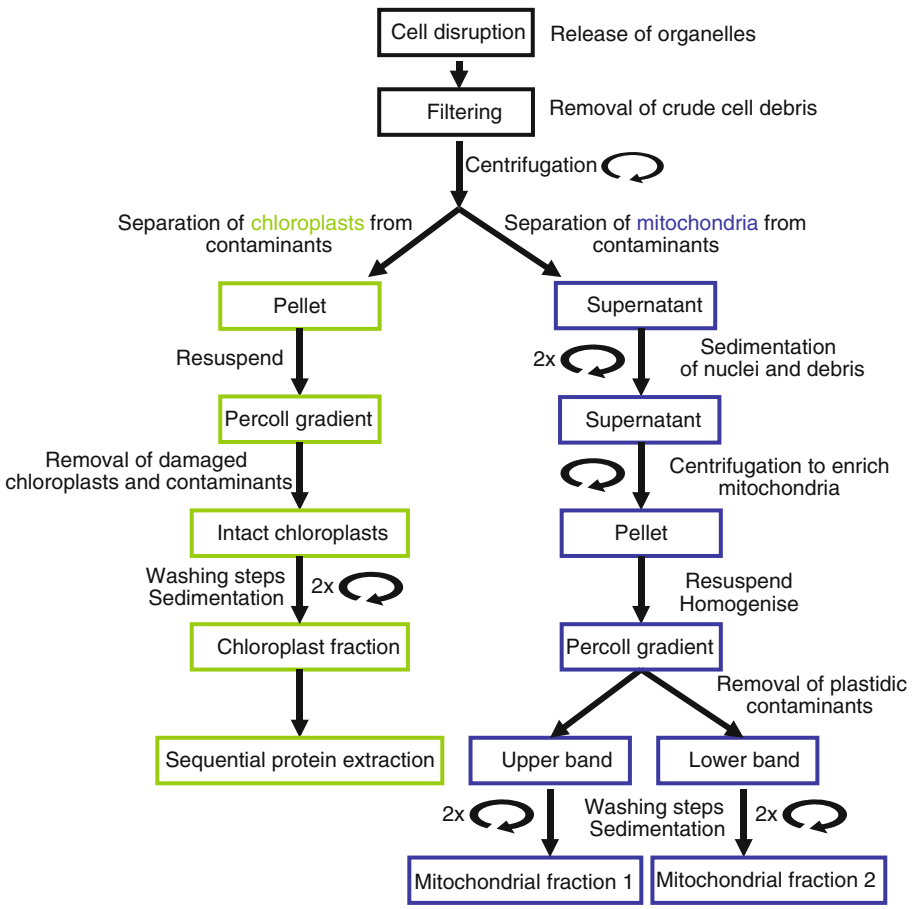

Fig. 1 Workflow of the isolation of chloroplasts and mitochondria. Schematic illustration of the different steps during the simultaneous isolation of chloroplasts and mitochondria from moss protonema. After cell disruption, filtration and the first centrifugation step, intact and highly enriched chloroplasts and mitochondria can be isolated from the pellet and the supernatant, respectively, using further differential centrifugation and Percoll density gradients

A stepwise Percoll density gradient was assembled by overlaying 5 ml of 80% Percoll in washing buffer with 5 ml of 33% Percoll in washing buffer. The homogenised sample in 20% Percoll was transferred on top of this gradient and centrifuged at 18,000×*g* for 1 h.

Two pale whitish mitochondrial bands were extracted at the 20–33% interface and the 33–80% interface, respectively. They are subsequently referred to as bands M1 and M2. Mitochondria were carefully collected using thin glass pipettes and freed of Percoll by adding 15 ml of washing buffer, gentle mixing and centrifugation at 18,000×*g* for 20 min (slow deceleration), repeated twice. The upper band yielded a still greenish, loose mitochondria pellet whereas the lower band formed a stable whitish pellet also containing residual PVPP. Pellets were frozen at −80°C.

Protein extraction

Proteins were extracted from frozen organelle pellets and from frozen protonema using lysis buffer (7.5 M urea, 2.5 M thiourea, 12.5% glycerol, 62.5 mM tris–HCl, 2.5% *n*-octylglycosid, 1.25 mM protease inhibitor) and subsequently precipitated with methanol/chloroform (modified after Wessel and Flügge 1984). The protein pellet was resuspended in urea buffer (6 M urea, 25 mM bis–tris, 0.2% *n*-octylglucopyranosid, pH 8.5), measured with a nanodrop (Thermo Scientific) and mixed with SDS sample buffer (Bio-Rad) for SDS-PAGE. The measured protein concentration was confirmed by Coomassie (Fermentas PageBlue) staining of SDS gels. Supplementary Fig. 1 shows an example of a silver-stained SDS-PAGE equally loaded with 4 µg organellar protein extracts prepared using lysis buffer.

Sequential chloroplast protein extraction was performed after a protocol adapted from Molloy et al. (1998) to obtain a soluble fraction (Cs) and a chloroplast membrane fraction (Cm). Frozen chloroplast pellets were resuspended in up to 500 µl chloroplast protein extraction buffer I [CPE I; 2.5 mM EDTA, 5 mM $MgCl_2$, 10 mM KCl, 20 mM HEPES, 300 mM D-sorbitol, 1% (w/v) PVPP, 0.1% (v/v) plant protease inhibitor] followed by subsequent incubation in an ultrasonic ice bath for 20 min. The supernatant was collected after centrifugation (20 min, 4°C, 20,000×*g*) representing fraction Cs. The residual green pellet was resuspended in up to 500 µl CPE II [CPE I with 1% (v/v) Triton-X 100], followed by a subsequent incubation in an ultrasonic ice bath for 20 min. The supernatant was collected, representing fraction Cm.

The soluble total protein fraction (Ts) was prepared using Tris-buffer [40 mM tris-(hydroxymethyl)-aminomethane, 0.5% (w/v) PVPP, 1% (v/v) plant protease inhibitor]. Frozen protonema (500 mg) was homogenised with mortar and pestle and thawed in the presence of Tris-buffer. The homogenate was incubated for 20 min in an ultrasonic ice bath. Subsequently the homogenate was centrifuged for 90 min at 121,000×*g* (Beckman Coulter Optima L-60, rotor type Ti 70.1) at 2°C. The clear supernatant was transferred into Teflon-centrifuge tubes (Oak-Ridge, Nalgene) and precipitated overnight with five volumes of acetone with 0.2% dithiothreitol (DTT) at −20°C. Afterwards the proteins were pelleted at 12,000×*g* for 10 min and 0°C. The protein pellet was resuspended in 20 ml acetone with 0.2% DTT and incubated for 1 h at −20°C. After centrifugation as described above, the air-dried protein was resuspended in lysis buffer [8 M urea, 4% 3((3-cholamidopropyl)-dimethylammonio)-propansulfat, 100 mM DTT, 40 mM Tris-(hydroxylmethyl)-aminomethane]. Protein concentration of the fractions was determined via Bradford assay (Bradford 1976) and mixed with SDS sample buffer (Bio-Rad) for SDS-PAGE.

Western blotting

After gel electrophoresis using ready-made SDS-gels (Ready gels, 12% Tris–HCl, Bio-Rad), the stacking gel was cut off and discarded. The remaining gel was immersed in 100 ml cathode-buffer [25 mM tris, 40 mM glycine, 10% (v/v) methanol] for 15 min. Transfer membranes (Immobilon-P, Millipore) were incubated in 100% methanol for 15 s, washed for 2 min in water and subsequently equilibrated for at least 5 min in 100 ml anode-buffer II [25 mM Tris, 10% (v/v) methanol]. Six pieces of filter paper (Grade GB003, Whatman) in the size of the membranes were incubated as follows: three in 100 ml cathode-buffer, two in 100 ml anode-buffer I [0.3 M Tris, 10% (v/v) methanol] and one together with the membrane in 100 ml anode-buffer II (filter paper on top and membrane below the paper).

The transfer sandwich was placed and assembled directly on the anode-plate of the semi-dry blotting device (Trans-Blot SD Semi-dry transfer cell, Bio-Rad). Assembly of the transfer sandwich from anode to cathode: two filter papers with anode-buffer I, one filter paper with anode-buffer II, membrane, separating gel and three filter papers with cathode-buffer. Transfer was performed at 1.2 mA/cm^2 for an hour.

Membranes were blocked with 4% blocking reagent (ECL Advance, GE Healthcare) in TBST [20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween 20] for an hour. Membranes were washed five times: two short immersions, then a 15 min wash followed by two 5 min washing steps. Incubation with primary antibodies was performed at room temperature for an hour followed by the previously described washing procedure. The final concentration for the primary antibody was determined

empirically for each antibody (see Table 1 for details). Membranes were incubated with the secondary antibody (anti-rabbit HRP-conjugate, Amersham) at a final concentration of 1:500,000 followed by washing steps. Reactive bands were detected using ECL detection (ECL Advance detection kit, GE Healthcare).

Antibody production

Polyclonal antibodies were raised in rabbits against KLH-conjugated peptides derived from conserved regions of the target protein across a range of species including mono- and dicotyledonous plants and *P. patens* or *Chlamydomonas reinhardtii*. The antibodies were validated using available positive and negative controls. A summary of antibody names, full names of their target proteins, predicted protein localisations, UniProt or TAIR accession numbers, organism of origin and expected/apparent molecular weights is given in Table 1.

Fluorescence microscopy

To stain mitochondria and verify mitochondrial integrity 200 µl of mitochondrial fractions (M1 and M2) were taken from the density gradient and stained with 200 nM MitoTracker Green FM and MitoTracker Red CMXRos (Molecular Probes), respectively, in washing buffer. After 15 min of gentle shaking at room temperature, samples were observed under a fluorescence microscope (Axioplan, Zeiss, Jena, Germany) using a bandpass 450–490 nm excitation filter with a 520 nm long-pass emission filter for MitoTracker Green FM stained samples and a bandpass 546/12 excitation filter with a 590 long-pass emission filter for MitoTracker Red CMXRos stained samples.

Results

Protein yield

Simple chopping of moss protonema yielded a cell homogenate that can be easily and rapidly fractionated for the simultaneous isolation of chloroplasts and mitochondria via differential centrifugation and Percoll density gradients (Fig. 2a, b). Twenty gram fresh weight of moss material (corresponding to 2 g dry weight) yielded protein amounts of 3–5 mg chloroplast protein and 60–100 µg mitochondrial protein, i.e. 150–250 µg of chloroplast protein and 3–5 µg mitochondrial protein per gram of fresh weight of moss material. After isolation, organelle pellets were frozen and the proteins from the respective pellets extracted for subsequent experiments.

Purity and integrity of mitochondria

Two distinct whitish bands were observed in the Percoll density gradient during purification of mitochondria, one at the 20–33% interface (M1) just below the green band of chloroplast debris and another one at the 33–80% interface (M2) (Fig. 2b). Samples from both bands were analysed by fluorescence microscopy and the presence of mitochondria was confirmed by MitoTracker Green staining (Supplementary Fig. 2a, b). Additional staining with MitoTracker Red CMXRos confirmed the existence of a membrane potential (Supplementary Fig. 2c, d).

Impurities in the mitochondrial fraction were assessed using antibodies against plastidic contaminants, namely, the membrane localised light-harvesting complex II type II chlorophyll *a*/*b*-binding protein (Lhcb2) and the plastidic stroma localised glutamine synthetase (GLN1/GLN2), and antibodies against Golgi apparatus and tonoplast membrane contaminants, namely, the ADP-ribosylation factor 1 (Arf1) and the Epsilon subunit of the tonoplast H+ ATPase (V-type ATPase) localised in Golgi and tonoplast, respectively (Fig. 3).

Immunoblot signals for the mitochondrial fraction from the 20–33% interface (M1) were weak for plastidic markers and absent for Golgi vesicle and tonoplast markers (Fig. 3a, b, f, g), while the mitochondrial fraction from the 33–80% interface (M2) showed only a weak positive signal for contamination with Golgi vesicles (Fig. 3f).

The signal strength for the three mitochondrial marker antibodies used, the plant alternative oxidase 1 and 2 (AOX1/2) and the cytochrome *c* oxidase subunit 2 (COX II) which are both localised in the inner mitochondrial membrane, and the antibody against the voltage-dependent anion-selective channel protein 1 (VDAC1), a protein of the outer mitochondrial membrane, was higher for the mitochondrial protein extracts from the M1 fraction (Fig. 3c, d). Integrity of the mitochondria of both fractions was confirmed by the positive immunoblot signal for VDAC1 (Fig. 3d).

Purity of chloroplasts

During the purification in the Percoll density gradient, the intact chloroplasts accumulated at the 40–80% interface, while the broken chloroplasts were found at the 10–40% interface (Fig. 2a).

To detect impurities in the chloroplast fraction (C) the same set of antibodies as for the mitochondrial fraction was used (Fig. 3). Immunoblots showed that the chloroplasts were free of contamination by Golgi (Fig. 3f) but were contaminated to some extent with tonoplast membranes (Fig. 3g). The signal strength of the mitochondrial marker antibodies against chloroplast proteins varied strongly. The

Table 1 Antibody and protein data

Antibody name/ product number[a]	Protein	Expected subcellular localisation	Protein used to raise antibody[b]	Organism	Expected/apparent MW (kDa)	Apparent MW in *P. patens* (kDa)	Dilution of primary antibody used for ECL detection
CPX1/AS06 123	Coproporphyrinogen III oxidase, isoform 1	Chloroplast	Q9S7V1	*C. reinhardtii*	41.4/38	38	1:2,000
CRD1/AS06 122	Cyanobacterial homolog of plant CHL27 cyclase	Chloroplast thylakoid and envelope membranes	Q9M591	*A. thaliana*	47/40	48	1:5,000
CSP41b/AS08 298	Ribosome associated endonuclease (CRB)	Chloroplast ribosome	Q9SA52	*A. thaliana*	42/39	42	1:5,000
Cyt *f*/AS06 119	Cytochrome *f* protein (PetA) of thylakoid Cyt b6/f-complex	Chloroplast thylakoid membrane	P23577	*C. reinhardtii*	34/31–32	34	1:100,000
HSP70B/AS06 175	Stromal α-HSP70	Chloroplast		*C. reinhardtii*	71.9	72	1:10,000
Lhcb2/AS01 003	LHCII type II chlorophyll *a*/*b*-binding protein	Chloroplast; thylakoid membrane	At2g05100 At2g05070 At3g27690 AF134124	*A. thaliana*	25	26	1:500,000
PsaD/AS09 461	PSI reaction centre subunit II	Chloroplast; thylakoid membrane	At1g03130 At4g02770	*A. thaliana*	17.9	18/20	1:5,000
PsbP/AS06 167	23 kDa protein of the oxygen evolving complex (OEC) of PSII	Chloroplast; thylakoid membrane	Q42029	*A. thaliana*	28/23	20/23	1:10,000
GLN1/GLN2/AS08 295	Glutamine synthetase	GLN1: cytoplasm	GLN1-1 GLN1-2	*A. thaliana*	39–40 (GLN1)	42–43	1:12,500
		GLN2: chloroplast	GLN1-3 GLN1-4		44–45 (GLN2)		
Arf1/AS08 325	ADP-ribosylation factor 1	Golgi apparatus	At2g47170; P36397	*A. thaliana*	21	19	1:4,000
AOX1/2/AS04 054	Plant alternative oxidase 1 and 2	Mitochondria inner membrane	At3g22370 At5g64210	*A. thaliana*	36–40	35	1:1,000
COX II/AS04 053A	Cytochrome *c* oxidase subunit 2	Mitochondria inner membrane	AtmG00160	*A. thaliana*	29.4	31	1:1,000
			P04373	*O. sativa*			
			Q1XGA9	*P. patens*			
VDAC1/AS07 212	Voltage-dependent anion-selective channel protein 1	Mitochondria outer membrane	At3g01280	*A. thaliana*	29	31	1:5,000
V-type ATPase/ AS07 213	Epsilon subunit of tonoplast H+ ATPase	Tonoplast membrane	At4g11150	*A. thaliana*	26	25	1:2,000

Summarised information on antibody names, full name of their target protein and predicted protein localisations. For the proteins used to raise the antibodies, UniProt or TAIR accession numbers, organism of origin and expected/apparent molecular weights are given. For the Western blots shown in this study, the apparent molecular weights in *P. patens* and the antibody dilutions used are depicted

[a] Agrisera product number (http://www.agrisera.com)

[b] Protein accession number, source: http://www.ncbi.nlm.nih.gov/genbank/

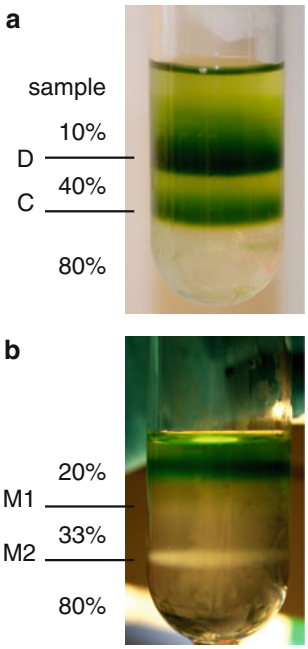

Fig. 2 Percoll gradients after centrifugation. Percentages of Percoll in buffer are given for the different layers. **a** Purification of intact chloroplasts, *D* debris, *C* intact chloroplasts **b** Purification of mitochondria, *M1* mitochondrial fraction 1, *M2* mitochondrial fraction 2; the greenish band in the upper part of the gradient consists of chloroplast debris

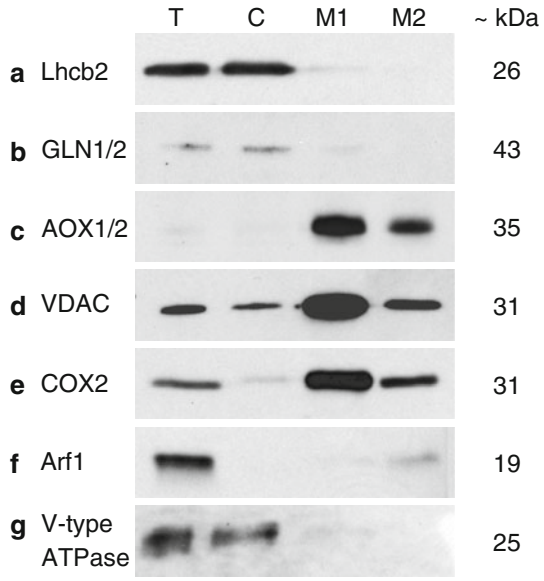

Fig. 3 Immunoblots assessing the organellar purity of urea-extracted proteins. *T* total protein, *C* chloroplast protein, *M1* mitochondrial fraction 1 protein, *M2* mitochondrial fraction 2 protein, loading amounts: 10 µg/lane (**a**, **e**), 2.5 µg/lane (**b**–**d**, **f**, **g**); for dilutions of primary antibodies, see Table 1

AOX1/2 antibody gave no signal whereas the signal for COX II was weak and the one for VDAC1 was clear (Fig. 3c–e). The immunoblot signals were all at the expected size as indicated by the manufacturer. The total protein extract (T) used as a control showed clear signals for each of the antibodies tested besides from the AOX1/2, which was very weak (Fig. 3a–g).

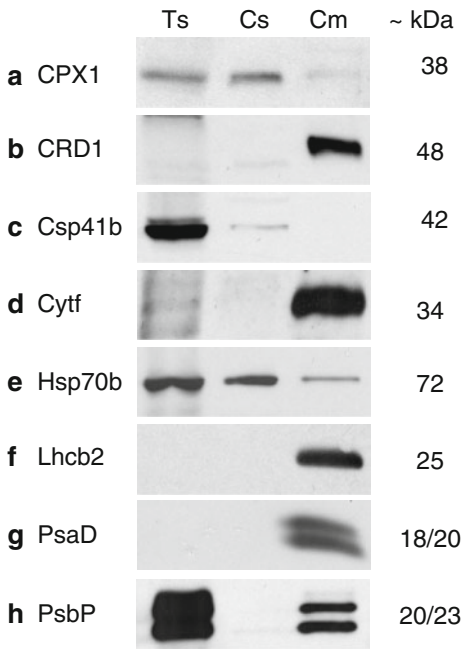

Fig. 4 Immunoblots from sequentially extracted proteins of chloroplasts. *Ts* total soluble protein, *Cs* chloroplast soluble protein, *Cm* chloroplast membrane protein; loading amounts: 5 µg/lane; for dilutions of primary antibodies, see Table 1

The success of the organelle isolation procedure was further assessed for chloroplast proteins via the application of a series of chloroplast specific antibodies against the sequentially extracted soluble (Cs) and membrane proteins (Cm) from intact chloroplasts (Fig. 4).

All the antibodies against chloroplast proteins not localised to the membranes, i.e. the coproporphyrinogen III oxidase, isoform 1 (CPX1), the ribosome associated endonuclease (CSP41b) and the stromal heat-shock protein (Hsp70b), showed a clear signal in the soluble chloroplast protein fraction whereas for the proteins extracted from the membrane fraction only the CPX1 and the Hsp70b resulted in a very weak and a weak signal, respectively (Fig. 4a, c, e).

Immunoblot signals for the antibodies against proteins localised to the chloroplast membranes, namely, the cyanobacterial homolog of the plant CHL27 cyclase (CRD1), the cytochrome *f* protein of the thylakoid Cyt b6/f-complex (Cyt *f*), the light-harvesting complex II type II chlorophyll *a*/*b*-binding protein (Lhcb2), the photosystem I reaction centre subunit II (PsaD) and the 23 kDa protein of the oxygen evolving complex of the photosystem II (PsbP) were all very strong in the protein extract from the chloroplast membrane fraction (Fig. 4b, d, f–h). For both

antibodies, PsaD and PsbP, two signals were obtained, namely, at 18 and 20 kDa for PsaD and at 20 and 23 kDa for PsbP. None of these five antibodies against membrane proteins of the chloroplast delivered a signal in the protein extracts of the chloroplast soluble fraction. For the protein extracts from the soluble total protein sample (Ts) immunoblot signals were obtained for all the proteins not localised to the membranes (CPX1, CSP41b, Hsp70b) (Fig. 4a, c, e). No signal was obtained for the membrane localised chloroplast proteins, besides for PsbP with which two highly saturated signals were recorded (Fig. 4h).

Discussion

Protein yield

Previous publications using mitochondria for proteomic studies (Heazlewood et al. 2004; Kruft et al. 2001; Millar et al. 2001a, b) did not indicate the amount of mitochondrial protein recovered per gram of fresh weight of starting material. It is certain that in moss a reduced amount of starting material is available as compared to seed plants. However, the optimised cultivation of moss protonema in aerated liquid culture allows for a yield of 60–100 µg mitochondrial protein per isolation. The protein yield from isolated chloroplasts is well in the range of previously reported studies. While Kley et al. (2010) reported a range of 149–499 µg *Arabidopsis* chloroplast proteins per gram of rosette leaves, Salvi et al. (2008a) recovered about 100–150 µg protein per gram using 400–500 g of leaf material. Previous protocols for organelle extraction in moss (Kabeya and Sato, 2005; Marienfeld et al. 1989) did not report protein yield as reference value. Taking into account that the moss material used in this study is derived from a liquid protonema culture, the amount of 20 g fresh weight of moss and the protein yield extracted from this material are very acceptable to routinely isolate both organellar fractions and perform subsequent proteomics analyses.

Purity and integrity of mitochondria

Surprisingly, the isolation protocol for mitochondria reported here revealed two bands of different densities, indicating two populations of mitochondria in moss protonema. In contrast, Kabeya and Sato (2005) reported only one single band at the 33–80% Percoll interface. It is possible that the homogenisation step using a potter before the density gradient purification causes disruption of some mitochondria. However, the clear signal in the immunoblot obtained for the mitochondrial outer membrane protein VDAC1 as well as the staining with Mitotracker Red CMXRos, which stains only intact mitochondria with membrane potential (Gilmore and Wilson 1999; Metivier et al. 1998; Poot et al. 1996) indicate that both mitochondrial fractions contain intact mitochondria. So far, very few reports of a second mitochondrial band in a density gradient exist (Logan et al. 2001; Segui-Simarro et al. 2008). While Logan et al. (2001) found two subpopulations of mitochondria differing in the degree of cristae organisation using electron microscopy techniques on germinating maize embryos, Segui-Simarro et al. (2008) reported for *A. thaliana* that in meristematic cells two subpopulations of mitochondria coexist. The mitochondria extracted from maize were obtained using a sucrose gradient where the organelles accumulated at a density of 22–28% sucrose and at a density of 37–42%, the latter "heavy" band being the described and expected one. These authors concluded that the mitochondria accumulating in the "light" fraction were remains of mitochondria that were active in the developing embryo during the maturation phase of seed development (Logan et al. 2001). A comparison of these results with those from *P. patens* would be far-fetched as there are no such developmental stages in moss. Observations by Segui-Simarro et al. (2008) in *Arabidopsis* were based on reconstructed thin-sections of electron micrographs and not on isolated mitochondria and, hence, have to be considered as an *in planta* finding. The two different mitochondrial fractions in moss as presented here are isolated from protonema without previous protoplastation, in contrast to the protocol of Kabeya and Sato (2005). Protoplastation may lead to a unification of the cells which in turn could reduce or abolish potential functional or morphological differences between organelles in distinct cell types. To date it is unclear if these two populations of mitochondria originate from mitochondria present in different cell types (as e.g. from caulonema and chloronema cells within the protonema tissue) or from two subpopulations of mitochondria from the same set of cells.

The results from the immunoblot analysis demonstrate that the plastidic contamination of both mitochondrial fractions is minimal or not existent, since the signal from the very abundant light-harvesting complex protein (Lhcb2) is absent in the lower mitochondrial fraction M2 and very weak in the upper mitochondrial band M1. In addition, this difference might be caused by residual contamination due to the green layer of broken cell debris accumulating above the M1 band in the density gradient.

The pronounced increase in signal intensity for the three mitochondrial markers in both mitochondrial protein fractions as compared to the total protein and chloroplast protein extracts, which is best observed for the AOX1/2 immunoblot results, confirms that the protocol besides yielding intact and pure mitochondria delivers a highly enriched fraction of organelles. Interestingly, the signal for

all mitochondrial markers used is stronger in the newly described M1 fraction which hints to differences in protein content between the mitochondrial fractions.

Potential contaminations of the M2 fraction by Golgi and a closer characterisation of both mitochondrial bands at the level of proteins will have to be analysed in subsequent proteomics studies.

Purity of chloroplasts

Comparable to Mason et al. (1991), the intact chloroplast fraction isolated from moss protonema accumulated at the 40–80% Percoll interface. Immunoblot results for the proteins extracted from this fraction suggest that the contamination of the chloroplast fraction with outer mitochondrial membranes represented by the VDAC protein is higher than with inner mitochondrial membrane proteins such as COX II and AOX1/2. The contamination with outer mitochondrial membranes might be caused by an attachment of membrane pieces deriving from broken mitochondria to intact chloroplasts during the isolation. A slight contamination due to the handling procedure cannot be ruled out as the removal of the intact chloroplasts from the density gradient with a Pasteur pipette requires penetrating the broken cell debris layer, which might automatically lead to a residual contamination of the glass pipette surface.

Immunoblotting results with the urea-extracted proteins using the plant compartment marker antibodies indicate that there is a contamination with mitochondria in the chloroplast fraction and vice versa. However, contaminations by the Golgi apparatus are absent while there is a clear signal from contaminants of the tonoplast.

The use of several different chloroplast protein markers clearly demonstrates that the method for extraction of a soluble and a membrane chloroplast protein fraction worked very well. All the proteins reported to be part of the chloroplast stroma resulted in clear immunoblot signals for the soluble protein fraction, whereas none of the membrane-localised chloroplast proteins gave a signal. On the other hand, the proteins extracted from the chloroplast membrane fraction resulted in a clear and strong immunoblot signal. The immunoreactive bands observed for CPX1 and Hsp70b in the chloroplast membrane protein fraction might be the result of contaminations due to handling of samples since during sequential chloroplast protein extraction it is not improbable that residues of the soluble fraction are still present in samples of membrane protein. Therefore, even though a residual contamination of soluble proteins in the membrane fraction is likely, there is no contamination of the soluble fraction with membrane proteins. Our protocol might therefore be used in subsequent studies to specifically analyse the proteins present in the chloroplast stroma and to investigate the proteins of a highly enriched thylakoid membrane protein fraction.

Specific enrichment using the sequential extraction method could be confirmed via the immunoblot results with total soluble proteins. None of the chloroplast membrane protein markers resulted in a signal for the total soluble proteins besides for the membrane localised PsbP.

Interestingly, the anti-PsbP as well as the anti-PsaD antibodies gave two signals in the immunoblot. The expected unique 23 kDa signal for *A. thaliana* matched with the apparent size of one of the bands for the *P. patens* chloroplast membrane protein extract, while the second minor band at 20 kDa had not been expected. Yi et al. (2007) also reported two signals for the PsbP protein from *A. thaliana*, which is a highly conserved protein in seed plants (Bricker and Burnap 2005; Roose et al. 2007). Although recent findings in *A. thaliana* have demonstrated that there are two putative genes that encode for PsbP-1 and PsbP-2, respectively, the minor band does not correspond to the PsbP-2 protein because it migrates at significantly lower apparent molecular mass (Goulas et al. 2006).

Similarly, although it has been found in other species such as *A. thaliana* that there can be a second immunoreactive band at 20 kDa, i.e. a slightly higher band than the expected unique 18 kDa band using the PsaD antibody, the origin of this second signal is unclear. The question whether the two signals found here with moss represent processed or modified forms of the proteins remains elusive and might be answered using sequencing techniques.

General remarks and conclusions

Our protocol for a simultaneous isolation of chloroplasts and mitochondria from the same biological sample offers several advantages as it will facilitate a direct comparison of organelles under identical physiological conditions. Protonema tissue has only few different cell types (Reski 1998) and thus is a rather homogeneous material, well suited for systems biology approaches (Decker et al. 2006). In addition, the rapid protocol using intact tissue rather than protoplasts described here, will promote such studies in *P. patens*. Furthermore, employing this protocol we found two distinct mitochondrial bands, a finding that has not been reported before for moss and will be investigated further.

The use of plant compartment marker antibodies designed for algae or seed-plants in this study supports two main points. On the one hand, it demonstrates that plant antibodies can be used across species if their target sequences are conserved and, on the other hand, it has shown for this study that both organellar fractions are highly enriched with only minor contaminations by other organelles. Both aspects paired with the good protein yields

obtained from the extractions will allow the analysis of sub-cellular proteomes of this non-flowering plant, thus helping to increase our understanding of the evolution of metabolic compartmentalisation, of biosynthetic pathways and of protein sorting mechanisms in land plants.

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