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journal homepage: www.elsevier.com/locate/yabioIsolating intact chloroplasts from small *Arabidopsis* samples for proteomic studiesJeannette Kley^a, Martin Heil^{b,*}, Alexander Muck^a, Aleš Svatoš^a, Wilhelm Boland^a^aMax Planck Institute for Chemical Ecology, 07743 Jena, Germany^bDepartamento de Ingeniería Genética, Cinvestav-Irapuato, CP 36821, Irapuato, Guanajuato, Mexico

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ABSTRACT

We have established a method for the isolation of chloroplasts from *Arabidopsis thaliana* that allows proteomic studies in the context of biotic stress with small amounts of starting material. Employing a 50% Percoll layer to separate crude filtrates, the required leaf material was reduced to 2–3 g, yielding more than 300 µg of chloroplast proteins. The quality of this fraction was confirmed by immunological, enzymatic, and gel-based assays. This protocol provides intact chloroplasts from *Arabidopsis* plants with a high degree of integrity and purity as well as sufficient protein recovery, thereby enabling studies of plant–herbivore or plant–pathogen interactions.

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The chloroplast of higher plants harbors photosynthesis as well as the metabolism of carbohydrates, amino acids, and lipids [1]. Moreover, the biosynthetic pathways of phytohormones, such as jasmonic and salicylic acid, are partially located in this organelle [2,3]. Because both hormones serve as signals that orchestrate induced resistance of plants [4] to insects [5] and pathogens [6,7], the chloroplast also participates in the regulation of interactions among plants and their heterotrophic enemies. Thus, isolating chloroplasts and studying their proteome will provide information on the level of proteins of many central physiological processes of higher plants.

Arabidopsis thaliana is one of the most important model organisms in plant biology. Unfortunately, the isolation of intact chloroplasts from *Arabidopsis* is not trivial. The plant is small, and its chloroplasts are more fragile than those from other species [8]. Several attempts were made to improve the isolation of intact *Arabidopsis* chloroplasts. The use of protoplasts as introduced by Somerville and coworkers [9] appeared to be very promising. However, the treatment with cell wall-degrading enzymes modulates the response to biotic stress and is not applicable to investigate interactions of plants with insects or pathogens [10].

Various protocols use mechanical disruption of leaf material, causing not only the breakup of whole cells but often also the breakup of chloroplasts themselves. As a consequence, large amounts of starting material are required. Commonly, protocols require a minimum of 100 g [11–13]. Such amounts of leaf material are available when comparing abiotic stress conditions [14] but

make it virtually impossible to investigate the impact of biotic stresses on the chloroplast proteome or to study within-plant differences. A relatively low amount of leaf material from *Arabidopsis* seedlings (7.5–12.0 g) was used by Aronsson and Jarvis [15], and this protocol was also recommended for proteomic studies [16]. However, *Arabidopsis* seedlings are not feasible to study, for example, effects of herbivory that cause the destruction of parts of a leaf.

The goal of the current study was to develop a protocol for the isolation and purification of intact chloroplasts from *A. thaliana* from a minimal amount of leaf material. Besides an adequate protein yield, the intactness and purity of the chloroplast proteome were of particular importance. To check the quality of the method, we used characteristic marker enzymes to examine the chloroplast fraction for cytosolic and mitochondrial impurities, and we tested chloroplast integrity. Our protocol allows the isolation of physically intact chloroplasts from less than 3 g of *A. thaliana* leaves and yields up to 350 µg of pure chloroplast proteins per gram of leaf material. In addition, this method is suitable for the analysis of total soluble proteins of *A. thaliana* chloroplasts.

Materials and methods

Plant growing conditions

Arabidopsis thaliana accession Col-0 plants were cultivated as described previously [17] with the following modifications. Nitrogen concentration was not varied and was supplied as 1 g of Osmocote Exact Mini (Scotts, Heerlen, the Netherlands) per liter substrate. Plants were grown individually in 0.4-L pots and harvested at 38 days of age.

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Isolation of chloroplasts

1× Chloroplast (Clp)¹ buffer containing 0.3 M sorbitol, 50 mM Hepes/KOH (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM MgCl₂, 10 mM NaHCO₃, and freshly added 0.5 mM dithiothreitol (DTT) was prepared 5× concentrated in advance and was sterile filtrated (Steritop, 0.22 µm, Millipore, Billerica, MA, USA). Prior to chloroplast isolation, for each sample, two polypropylene tubes (15 ml, Roth, Karlsruhe, Germany) were supplied with a 50% Percoll layer by mixing 2.5 ml of Percoll (Sigma–Aldrich, Taufkirchen, Germany) and 2.5 ml of 2× Clp buffer (DTT included) in each tube. The solutions were cooled to 0–4 °C. For extraction, three independent samples (a, b, and c) were processed at 0–4 °C in parallel as follows. Rosette leaves (2.0–3.0 g fresh weight [FW]/sample) were harvested and crushed in 23 ml of 1× Clp buffer for 5 s at low speed using a small blending device (12–37 ml) of a Waring blender (VWR, Darmstadt, Germany). After filtration through a polyester mesh (Petex, pore size 38 µm, Sefar, Thal, Switzerland), the homogenate was carefully loaded onto the 50% Percoll layers with the help of a serological pipette and was centrifuged for 10 min at 2000g in a swing out rotor, acceleration set 5, brakes set off (Sigma, Osterode, Germany). Upper layers containing broken chloroplasts were removed using a serological pipette, and the chloroplast pellet was washed once by carefully adding 14 ml of 1× Clp buffer and inverting the tubes. After centrifugation for 5 min at 1000g (swing out rotor, acceleration and deceleration set 9), the supernatant was removed and the chloroplast pellet was frozen in liquid N₂.

Protein extraction and quantification

For comparison of the protein patterns that are revealed from intact and broken chloroplasts, broken chloroplasts were generated according to van Wijk and coworkers [18] as follows. Intact chloroplasts were lysed in 1 ml of lysis medium and centrifuged for 20 min at 10,000g. For enzyme assays and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), proteins were extracted following the protocol of Zrenner and coworkers [19] but using protease inhibitor cocktail (P9599, Sigma–Aldrich) instead of phenylmethylsulfonyl fluoride (PMSF). Then 1 ml of extraction buffer was added to 100 mg of N₂-homogenized leaf tissue to the intact or broken chloroplast pellet. After vortexing and centrifuging at 20,000g, the supernatant was divided into 150-µl aliquots and frozen immediately.

For two-dimensional (2D) electrophoresis, 3 ml of 16% trichloroacetic acid (TCA) in acetone containing 20 mM DTT was added to each chloroplast fraction and vortexed every 15 min while leaving on ice for 1 h. The fractions were incubated overnight at –20 °C and then centrifuged for 10 min at 10,000g. The pellet was subjected to three washing steps of at least 20 min on ice in 9 ml of 80% acetone, including 20 mM DTT, before it was dried and resolved in rehydration solution (7 M urea, 2 M thiourea, and 4% Chaps).

The dissolved proteins were quantified relative to bovine serum albumin (BSA) using the modified Bradford assay [20].

¹ Abbreviations used: Clp, chloroplast; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; DTT, dithiothreitol; FW, fresh weight; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; 2D, two-dimensional; TCA, trichloroacetic acid; BSA, bovine serum albumin; UGPase, UDP–glucose pyrophosphorylase; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence; SHMT, serine hydroxymethyltransferase; IPG, immobilized pH gradient; HPLC, high-performance liquid chromatography; UTP, uridine-5'-triphosphate; MTP, microtiter plate; FA, formic acid; MS/MS, tandem mass spectrometry; RbL, 1,5-bisphosphate carboxylase; LHCP, light harvesting complex protein.

Enzyme assay for UDP–glucose pyrophosphorylase

UDP–glucose pyrophosphorylase (UGPase) was used as a cytosolic marker enzyme and assayed following the protocol of Zrenner and coworkers [19]. In a 96-well microplate, 200 µl of assay buffer was added to 20 µg of protein in 30 µl of extraction buffer. After the addition of 5 µl of 50 mM Na₄P₂O₇·10H₂O, the NADPH+H⁺ generation was determined at OD₃₄₀ using a SpectraMax microplate reader (Molecular Devices, Ismaning, Germany). The rate of NADPH+H⁺ formation was a measure for the activity of UGPase in each sample.

SDS–PAGE and Western blotting

One-dimensional gel electrophoresis was carried out according to Laemmli [21]. Protein (10 µg) was separated by 12% SDS–PAGE. Either gels were stained with colloidal Coomassie (Roti-Blue, Roth) or proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Roth) using a Hoefer SemiPhor (Pharmacia, Freiburg, Germany) cell. Immunodetection was performed by enhanced chemiluminescence (ECL) [22] using a primary rabbit antibody raised against serine hydroxymethyltransferase (SHMT) from potato.

2D electrophoresis and protein profiling

After supplying proteins in rehydration solution with 50 mM DTT and 2% immobilized pH gradient (IPG) buffer (GE Healthcare, Munich, Germany), 150 µg of chloroplast proteins was applied to IPG strips (pH 3–11, 13 cm, GE Healthcare) by rehydration loading overnight. The first dimension (step 1: 300 V at 0.001 kV h; step 2: 3500 V at 2.9 kV h; step 3: 3500 V at 14.1 kV h) was performed in a Multiphor II unit (Pharmacia) followed by 12% SDS–PAGE in a Hoefer SE 600 cell as the second dimension. Gels were stained with colloidal Coomassie (Roti-Blue). After 2D electrophoresis, protein spots were manually picked by using disposable 200-µl pipette tips cut by a high-performance liquid chromatography (HPLC) tubing cutter (3 mm). The gel plugs were transferred to 96-well microtiter plates (MTPs) and processed as described previously [23].

The tryptic peptides were reconstituted in 6 µl of 0.1% aqueous formic acid (FA) and 3% acetonitrile and were analyzed on a nano-ACQUITY nano-UPLC SYNAPT HDMS System (Waters, Milford, MA, USA). The tandem mass spectrometry (MS/MS) spectra were collected at 1-s intervals (50–1700 *m/z*). Here 650 fmol/µl human Glu–fibrinopeptide B in 0.1% FA/acetonitrile (1:1, v/v) was infused at a flow rate of 0.5 µl/min through the reference NanoLockSpray source every 30th scan to compensate for mass shifts in the MS and MS/MS fragmentation mode.

The data were collected by MassLynx (version 4.1) software (Waters). ProteinLynx Global Server Browser (version 2.3) software (Waters) was used for baseline subtraction and smoothing, deisotoping, and database searching. The peptide fragment spectra were searched against the Uni-Prot subdatabase restricted to taxonomy of the term *A. thaliana* (downloaded on 2 April 2009 from <http://www.uniprot.org>). The following settings were used for protein identification from MS/MS fragment spectra: peptide mass tolerance (15 ppm), minimum peptides found (2), estimated calibration error (0.002 Da), possible missed cleavages (1), and peptide modifications (carbamidomethylation of cysteines, oxidation of methionines, and deamidation of asparagine and glutamine).

Results and discussion

Protein yield

We harvested 2–3 g of rosette leaves per sample. Following TCA extraction, on average 324 ± 175 µg of chloroplast proteins per

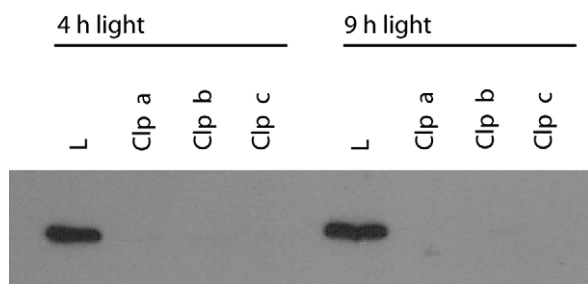


Fig. 1. Immunoblot of mitochondrial SHMT in total leaf extracts (L) and independent chloroplast fractions (Clp a–c) after 4 and 9 h of illumination. In each lane, 10 μ g of protein was loaded. An ECL detection system was applied at 1-min film exposure.

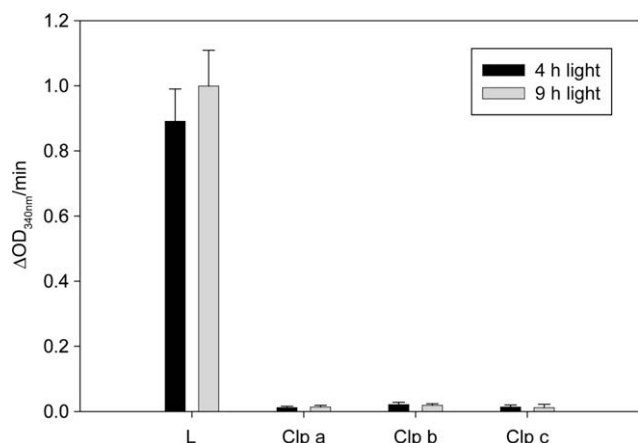


Fig. 2. Rate of NADPH+H⁺ formation as a measure of UGPase activity (cytosolic marker enzyme) in total leaf extracts (L) and independent chloroplast fractions (Clp a–c) after 4 and 9 h of illumination. OD_{340} + standard deviation ($n = 3$) was determined in 20 μ g of protein.

gram of leaf material was recovered after 4 h of illumination, whereas $352 \pm 65 \mu$ g was recovered after 9 h of illumination. The

varying yields most likely represented the original protein concentrations and reflect the effects of abiotic factors such as light exposition. Salvi and coworkers [13] reported 50–60 mg of chloroplast protein recovery from 400 to 500 g of leaf material, corresponding to 120 to 125 μ g/g leaf material. As compared with this method [13], our protocol used much lower amounts of starting material. The protein yield was, however, approximately three times higher proportionally and, thus, was sufficient for proteomic analyses.

Purity of the chloroplast fraction

Mitochondria are common contaminants of chloroplast preparations [24]. To detect impurities, a specific antibody against mitochondrial SHMT was used. SHMT is involved in serine formation during photorespiration [25]. SHMT was found in the total leaf extracts but was below the detection level in the isolated chloroplast fractions (Fig. 1), indicating that mitochondrial contaminants were not present in our chloroplast preparations.

The cytosol is another source of potential contaminants of chloroplast preparations [26]. To monitor cytosolic impurities, an enzyme assay for UGPase was applied. UGPase reversibly catalyzes the conversion of glucose-1-phosphate and uridine-5'-triphosphate (UTP) to UDP-glucose [27]. On average, $1.8 \pm 0.5\%$ of the original activity of the total leaf was detected in chloroplasts harvested after 4 h of light (Fig. 2, black bars), and $1.5 \pm 0.4\%$ was found in chloroplasts harvested at the end of the day (Fig. 2, gray bars), demonstrating that the degree of contamination by the cytosolic compartment was below 2%.

Integrity of the chloroplasts

Besides purity, the physical integrity of the chloroplasts is another important prerequisite for analyses that aim to monitor their entire proteome. A simple method to estimate the intactness of isolated chloroplasts is to quantify the ratio of major soluble stroma and thylakoid proteins as documented by SDS-PAGE [28]. A high ratio of the large subunit of ribulose 1,5-bisphosphate carboxylase (RbCL, localized in the stroma) to the light harvesting com-

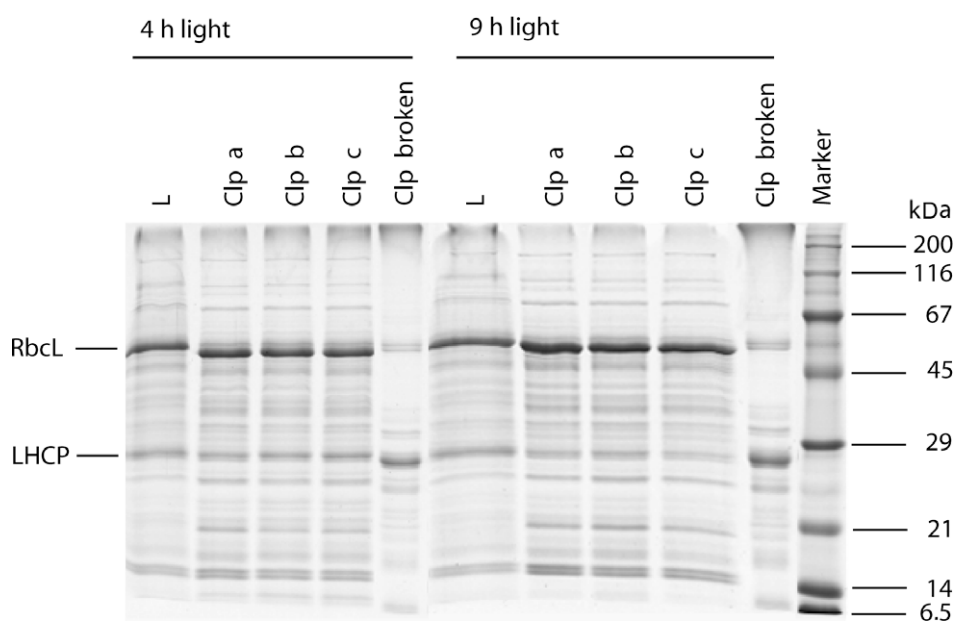


Fig. 3. Ratio of the major soluble stroma protein (RbCL) to the major thylakoid membrane proteins (LHCP) as an indicator of chloroplast integrity. Here 5 μ g of protein of total leaf extracts (L) and independent chloroplast fractions (Clp a–c) and 0.7 μ g of protein of a broken chloroplast fraction (Clp broken) after 4 and 9 h illumination were separated by 12% SDS-PAGE followed by colloidal Coomassie staining.

plex proteins (LHCs, bound to thylakoid membranes) was observed in all chloroplast fractions (Fig. 3). This ratio was similar to the proportions found in the total leaf extracts and opposite to the proportions observed in the fraction obtained from broken chloroplasts. Two further bands at 21 and 14 kDa, which were abundant in the samples obtained from intact chloroplasts, disappeared almost completely from the fraction of broken chloroplasts (Fig. 3). The latter band probably represents the small subunit of the RbcL (protein spots b, c, d, and e in Fig. 4 and Supplemental Table 1 in supplementary material), which is also located in the stroma. The band at 21 kDa most likely corresponds to spots 96 and 97, which were annotated as subunits E and D of photosystem I, that is, peripheral thylakoid membrane proteins that are localized at the side that is exposed to the stroma [29,30]. These proteins also appear to get lost during chloroplast lysis. By contrast, we observed no hints on soluble stroma proteins being lost from the fraction of intact chloroplasts during the isolation procedure, illustrating the physical integrity of the purified chloroplasts.

Other chloroplast isolation protocols suggested harvesting plant material during the early light period to reduce starch content because starch granules could affect chloroplast integrity [8,18]. In our case, however, chloroplasts isolated at the end of the light period exhibited similar RbcL/LHCP patterns as chloroplasts extracted

during the first part of the day (Fig. 3), demonstrating that starch granules had no detectable effect on our method.

A more detailed analysis of protein pattern was performed by 2D gel electrophoresis (Fig. 4). Of 122 spots, 102 different protein monomers could be identified by LC-MS/MS (Supplemental Table 1), and 99 of these could be assigned unambiguously to the chloroplast. Also, 16 were stromal proteins of the Calvin cycle (Fig. 4). Most prominent were the two subunits of glyceraldehyde-3-phosphate dehydrogenase, a typical stromal marker protein [31]. These findings strongly support the physical integrity of our chloroplast preparation.

Only 3 of the 102 proteins were of unknown or nonplastidial origin (Fig. 4). Catalase 2 was shown to be located in the peroxisome [32] but was also detected in other chloroplast preparations [14,33] and reported to possess a chloroplast target sequence [14]. In addition, myosinase TGG2, previously reported in chloroplast studies [30,33], has been considered as a contaminant because it was also found in the peroxisome [32]. Furthermore, both proteomic and immunocytological methods indicated a localization of this enzyme in vacuoles [34]. The eukaryotic elongation factor 1 α was identified and should be a cytosolic protein [35]. However, this protein also has been detected in other chloroplast preparations [30,33].

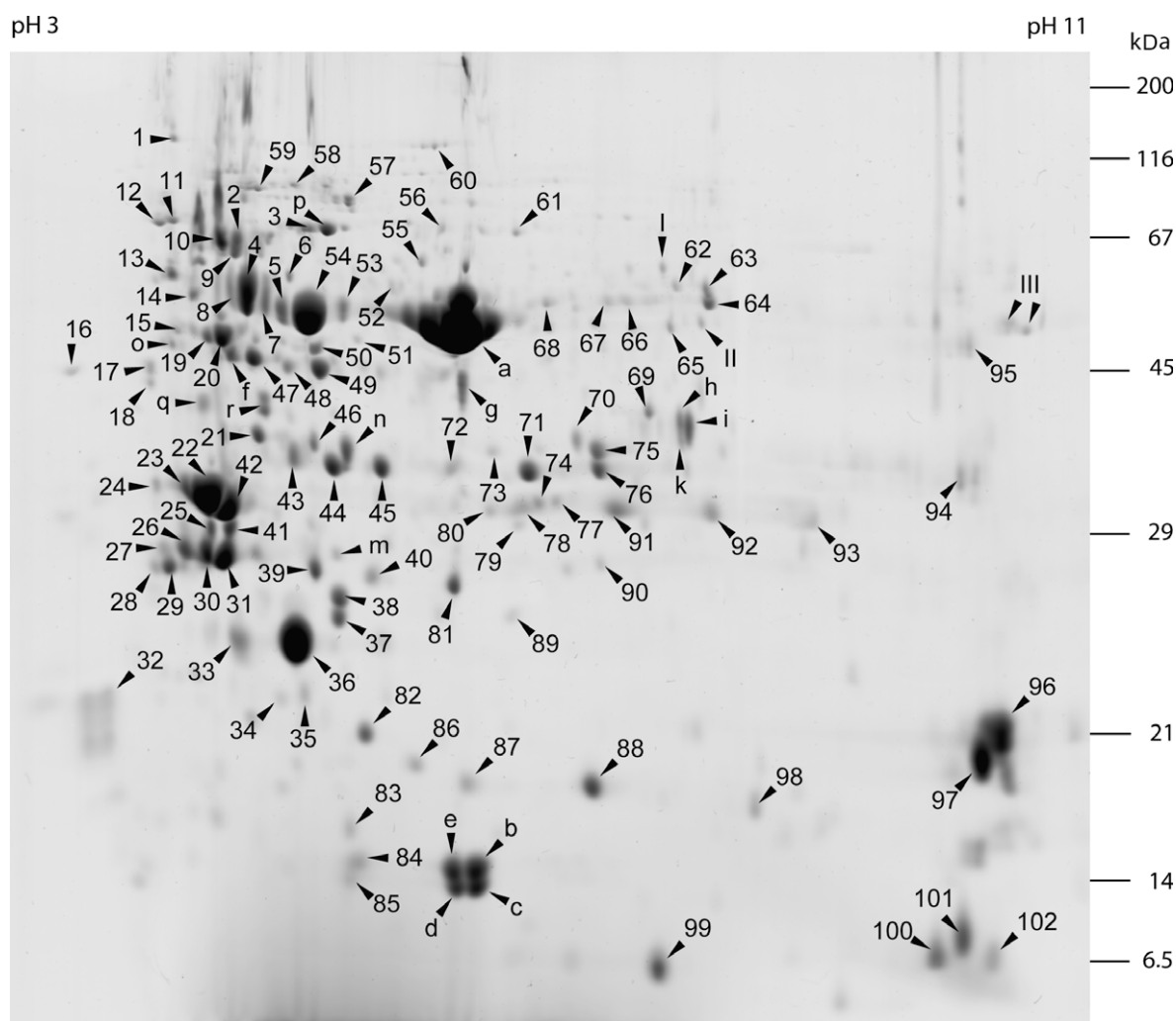


Fig. 4. Chloroplast proteome of *A. thaliana*. Protein (150 μ g) was applied to isoelectric focusing (IEF) strips (pH 3–11) in the first dimension followed by 12% SDS-PAGE and colloidal Coomassie staining in the second dimension. Numbers (1–102) refer to chloroplast proteins, letters (a–r) refer to enzymes of the Calvin cycle, and Roman numerals (I–III) refer to potential contaminants. See Supplemental Table 1 in supplementary material for protein identifications.

Even when considering these three proteins as nonplastidial protein contaminants, the overall purity of our plastidial proteome was more than 96%, which is in good agreement with Peltier and coworkers [26] and Ferro and coworkers [36], who detected 4 and 6 potential contaminants, respectively, from other compartments. These results demonstrate that the chloroplast protein fraction obtained with our method is comparable to the fractions gained with previously published methods. Regarding the high yield that can be obtained from small samples, we consider the novel protocol as a highly efficient purification method to study the chloroplast proteome of *A. thaliana*.

Concluding remarks

Our protocol allows a fast and high-yield separation of chloroplasts from leaf homogenates of *A. thaliana* by using a 50% Percoll layer for separation of organelles in crude filtrates instead of the classical gradient centrifugation. The procedure reduces the exposure to harmful secondary metabolites during initial centrifugation and does not require resuspension of chloroplasts, which also may cause damage. Thus, the procedure of using a Percoll layer reduces the loss of separated chloroplasts and strongly supports the efficiency of the extraction and purification process.

We could not find any, or could find only marginal, protein contamination from mitochondria or cytosol, respectively. Chloroplast integrity was also confirmed by gel electrophoresis. The novel isolation protocol allows working with a few grams of leaf material, which is essential to study the effect of biotic stress factors. In addition to the enrichment of plastidial proteins, this procedure is also suitable for the investigation of plastidial lipids, pigments, or other metabolites.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.11.016.

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