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Efficient isolation of chloroplasts from *in vitro* shoots of *Malus* and *Prunus*

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Abstract

Investigation of cellular subfraction proteomes allows the study of specific changes induced by environmental changes, stress and other conditions. Chloroplasts participate in a huge number of complex biochemical processes in plant cells by retrograde signalling, as well as by sensing and responding to cellular dysfunction. Changes in environmental conditions in a controlled way are easily achieved in *in vitro* model systems. However, growing plants *in vitro* makes it difficult to obtain sufficient material for chloroplast isolation. Therefore, the chloroplast isolation method needs to be optimised for achieving sufficient yield from a small amount of sample. We used three species of Rosaceae family that are of high agricultural interest for breeding programs in Lithuania. The method used for chloroplast isolation from *Arabidopsis thaliana* was optimized for *Malus domestica*, *M. platycarpa* and *Prunus avium*. Homogenisation of 3 g of *in vitro* plant material in sorbitol-based isolation medium with a laboratory blender yielded a sufficient amount of chloroplasts for proteomic analysis. The purity of the fraction was highly increased by additional step of centrifugation at 200× g. The purity of chloroplasts was evaluated visually by microscopy, by immunoblotting with specific antibodies, as well as by using marker proteins and quantitative mass spectrometry. Although microscopy showed negligible amounts of cellular debris in all of the preparations, immunoblotting allowed detection of the presence of cytosolic marker in some of the preparations. Mass spectrometric analysis of marker proteins confirmed the presence of modest amount of non-chloroplast proteins. In conclusion, the presented method for chloroplast isolation for the *Rosacea* plants *in vitro* gives sufficient yield and purity for subcellular proteomic studies.

Key words: mass spectrometry, Percoll, protein abundance index, subcellular markers, Western blot.

Introduction

Chloroplasts are an essential organelle for plant productivity. Besides photosynthesis, chloroplasts are crucial for many other complex biochemical processes. These organelles synthesize fatty acids and lipids, most of the amino acids, vitamins, plant hormones as well as many other essential compounds. Chloroplasts are sensitive to the environmental changes and play a key role in plant cellular signalling (Ensminger et al., 2006; Stael et al., 2011; Crosatti et al., 2013). The importance of chloroplasts in adapting to environmental changes is related to both – sensing of the changes and triggering the response (Pfannschmidt, Yang, 2012; Miura, Furumoto, 2013).

A way to study the plant response to the environmental conditions is by measuring the changes in the proteins caused by environmental changes. However, the number of different proteins present in plants is larger than that of genes because of post-translational modifications, alternative splicing and other processes. To make matters worse, the concentration of different proteins varies by several degrees of magnitude. Thus, investigating the proteome of subcellular fractions is an effective technique for reducing the sample complexity, as well as giving additional insight into cellular processes such as protein sorting and import. Chloroplasts are of particular interest for plant biologists because of their complex biochemical pathways for essential metabolic functions (Baginsky, Gruissem, 2004).

Growing plants in controlled conditions *in vitro* helps to manipulate the environment and reproducibly, as well as follow the changes in plant response. However, working with *in vitro* plants leads to small amounts of plant sample, necessitating the optimization of the harvesting process. Protoplastation prior to chloroplast isolation is a time consuming and expensive method (Aronsson, Jarvis, 2002). There are methods for isolation of tissue specific chloroplasts; however, these procedures include genetic modification (Truernit, Hibberd, 2007). Isolating chloroplasts for further proteomic studies from small amounts of

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sample material should give high yield and good quality. However, the conditions for the best yield and quality vary among species. Most of the studies are performed with model species like *Arabidopsis thaliana*.

Mass spectrometric quantification of marker protein ensembles was proposed by Andreyev et al. (2010) as a method for evaluating organelle purity. We applied this method for quality control of chloroplast isolation. Since the original method was developed with animal cells, we needed to extend it for plant cells, as well as to include chloroplast marker proteins. We compared the performance of the mass spectrometric method with immunoblotting.

Our goal was to achieve good quality and sufficient yield of chloroplasts from agriculturally important species of *Malus* and *Prunus* grown under *in vitro* conditions and to optimize the conditions for chloroplast isolation from orchard plants grown *in vitro*.

Materials and methods

Chloroplast isolation. The experiments were carried out at the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry in 2015–2016. We used three species of Rosaceae family: *Malus domestica*, *M. platycarpa* and *Prunus avium*. Plants were grown *in vitro* on modified MS (Murashige, Skoog, 1962) medium supplemented with 3.2 μM benzylaminopurine, 3% sucrose and 0.8% plant agar. The shoots were grown at 22 ± 3°C under fluorescent lamp illumination 50–150 μmol m^{-2} s^{-1} intensity and 16/8 h photoperiod.

Due to small biomass of *in vitro* shoots, the leaves and stems were not separated. However, when possible, the amount of stems was minimized. Approximately 3 g of plant material was collected for each of chloroplast isolations. Thee repetitions of chloroplast isolation were done for each condition (isolation medium, homogenisation type, differential and Percoll centrifugations). We used two isolation mediums: a sorbitol based medium, as described by Kley et al. (2010), and a high salt medium, as described in Shi et al. (2012). Plant homogenization was done using a laboratory blender (Waring, USA), basic ultra-turrax polytron homogenizer Ika T 10 (IKA, Germany), or liquid nitrogen with mortar and pestle.

The plant material was blended in the medium containing 0.3 M sorbitol, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/KOH (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethyleneglycoltetraacetic acid (EGTA), 1 mM $MgCl_2$, 10 mM $NaHCO_3$, and freshly added 0.5 mM dithiothreitol (DTT) using a small volume Waring laboratory blender at low speed twice for 5 sec. The homogenate was filtrated through double layer of nylon mesh and applied straight to 50% Percoll or centrifuged at 200× g for 3 min and then applied to 50% Percoll. Subsequent centrifugation was performed at 2000× g in a swing-out rotor for 10 min. The green band was collected, re-suspended in the medium without DTT and centrifuged at 1000× g for 5 min. Obtained chloroplasts were used for further investigation.

High salt isolation medium composition was 1.25 M NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA. In contrast to the original method (Shi et al., 2012), bovine serum albumin (BSA) was omitted and β-mercaptoethanol was replaced by DTT.

The yield of isolated chloroplasts was expressed on a unit chlorophyll basis (mg of chlorophyll) measured at 652 nm by Implen NanoPhotomer (Implen, Germany). All solutions, tubes, glassware and equipment were pre-cooled before experiments and kept at 0–4°C during all experiments. Plant removal from the *in vitro* medium and the basal part dissection was performed in room temperature.

The purity of chloroplast fraction was estimated by counting presence of full cells in 0.02 mm^2 area of microscopy slide with chloroplast fraction using microscope Nikon Eclipse 80i (Japan).

Western blot analysis. Total proteins were extracted by phenol method (Isaacson et al., 2006) and solubilised in 2% sodium dodecyl sulphate (SDS). Concentration was determined using Roti-Quant universal reagent (Carl Roth). 5 μg of proteins with 4 × Laemmli sample buffer (BioRad) were analysed on 5/12% SDS polyacrylamide gel electrophoresis and electroblotted onto Hybond-P polyvinylidene fluoride membrane (GE Healthcare, UK).

To test the purity of *Malus* chloroplast fraction, membranes were probed with rabbit anti-UDP-glucose pyrophosphorylase (UGPase, dilution 1:2500; a marker for cytosolic contamination), isocitrate dehydrogenase (IDH, dilution 1:2500; a marker for mitochondrial contamination) antibodies. Antibodies were detected by incubation with goat anti-rabbit Ig horseradish peroxidase-linked polyclonal antibody (dilution 1:5000) using the enhanced chemiluminescence (ECL) system (BioRad) and Clarity Western ECL substrate (BioRad). Films were scanned using scanner Typhoon FLA900 and analysed by program *ImageQuant TL* (GE Healthcare, UK).

Mass spectrometry (MS). The chloroplast proteins were pre-fractioned using SDS-PAGE. The lane with chloroplast proteins was cut to ten equal sized pieces, and the proteins were digested using trypsin (Sevchenko et al., 2007). In-gel digested tryptic peptides were concentrated and washed on a trap column Acclaim PepMap100 C18 (Dionex, USA) and separated by 75 μm × 15 cm Acclaim PepMap RSLC C18 (Dionex), with a 60 minute gradient from 5% to 40% B (A – 0.1% formic acid (FA), B – 0.1% FA in acetonitrile (ACN) using Ultimate 3000 RSLCnano LC system (Dionex). MS and collision induced dissociation (CID) MS/MS spectra were measured with Bruker maXis 4G equipped with CaptiveSpray nano-electrospray source (Bruker Daltonics GmbH, Germany). The mass spectra were internally calibrated using hexakis lock mass standard. Proteins were identified by Mascot (Matrix Science) search with 10 ppm mass tolerance against either *Malus* × *domestica* consensus gene model proteins (Velasco et al., 2010). Data was checked for contaminating peptides by performing a Mascot search against the database Swiss-Prot (Boutet et al., 2016). The peptide matches were visualized and validated using the software *Proteinscape* (Bruker Daltonics). The exponentially modified protein abundance index (emPAI) was employed for quantitation of the proteins. This index is calculated from the number

of observed peptides, normalized by the number of observable peptides per protein, and has been previously shown to be proportional to the protein concentration in a protein mixture (Ishihama et al., 2005).

Bioinformatics. The functional annotation of gene model proteins was done using a Blast search against the non-redundant sequence database at NCBI (Pruitt et al., 2005) and by Argot (Falda et al., 2012). The criteria for marker proteins used for purity analysis were the following: (1) they had a high confidence annotation in the database UniProt (UniProt Consortium, 2015), (2) previously described in literature (Andreyev et al., 2010; Ferro et al., 2010) and (3) availability of antibodies (Table 1). Proteins that are exclusively found in a specific organelle were prioritized.

Table 1. Organelle marker proteins quantified using mass spectrometry

Location	Malus ID	emPAI	UniProt ID	Description
Chloroplast	MDP0000294406	2.06	O81439	Plastoglobulin 35
Chloroplast	MDP0000198078	8.12	P10933	Ferredoxin-NADP reductase
Chloroplast	MDP0000602932	0.66	Q9M591	MPE-cyclase
Chloroplast	MDP0000708928	1.61	O82425	Chlorophyll a-b binding protein
Chloroplast	MDP0000149416	0.48	D7TWA1	Ferredoxin-plastoquinone reductase 2
Chloroplast	MDP0000597996	2.22	B2MZZ1	Rubisco large subunit
Chloroplast	MDP0000706975	3.7	O24500	Glycolate oxidase
Chloroplast	MDP0000624350	2.98	A5AEB4	Photosystem I reaction centre subunit II
Chloroplast	MDP0000464827	1.75	E3W0J3	Photosystem I P700 chlorophyll a apoprotein A1
Chloroplast	MDP0000233921	1.61	B9S1T5	O2 evolving complex 33kD family protein
Mitochondria	MDP0000295277	0.06	B9RZW7	Dihydrolipoyl dehydrogenase
Mitochondria	MDP0000874020	0.0	Q39219	Ubiquinol oxidase 1a
Mitochondria	MDP0000216734	0.28	Q9T070	Cytochrome c oxidase subunit 6a
Mitochondria	MDP0000309512	0.12	Q945K7	Isocitrate dehydrogenase
Mitochondria	MDP0000332597	0.0	Q9SZJ5	Serine hydroxymethyltransferase 1
ER	MDP0000327191	0.4	P33157	Beta-1,3-endoglucanase (PR-2)
ER	MDP0000322220	0.5	Q9LKR3	Heat shock protein 70-11
ER	MDP0000122791	0.0	Q9LM02	Sterol methytransferase
Peroxisome	MDP0000279170	0.0	Q8VZD4	Glyoxysomal processing protease
Peroxisome	MDP0000132452	1.73	F6I0K4	Catalase
Peroxisome	MDP0000172852	0.78	Q9C9W5	Glycerate dehydrogenase HPR
Peroxisome	MDP0000145531	0.0	Q9FXT6	Peroxisomal membrane protein PEX14
Nucleus	MDP0000624481	0.0	Q9FF75	Nucleoskeleton linkage protein
Nucleus	MDP0000464636	0.0	Q8RWK8	Coilin
Nucleus	MDP0000024484	1.25	Q2XPW1	Histone H2
Cytosol	MDP0000251810	0.0	Q9MA79	Fructose-1,6-bisphosphatase
Cytosol	MDP0000171559	0.23	B7FH28	Large ribosomal subunit
Cytosol	MDP0000306580	0.0	Q94BT0	Sucrose-phosphate synthase 1
Cytosol	MDP0000323036	0.0	Q9M9P3	UDP-glucose pyrophosphorylase 2
Cell wall	MDP0000209964	0.0	P93046	XTH-31
Cell wall	MDP0000605874	0.21	B9RG92	Aspartic-type endopeptidase
Cell wall	MDP0000470441	0.0	Q9SWW6	Cellulose synthase A subunit
Cell wall	MDP0000740981	0.0	K4ELD8	Endoglucanase

ER – endoplasmic reticulum, emPAI – protein abundance index

Results and discussion

Although chloroplast isolation can be preceded by the use of the cell wall-degrading enzymes (cellulase and pectinase) for protoplastation (Aronsson, Jarvis, 2002), this additional step adds considerable cost and complexity to the method. On the other hand, mechanical rupturing of cell wall and membrane, leading to the release of chloroplasts to the isolation medium is relatively easy and inexpensive. However, this step is very important for the yield and quality – too soft homogenization decreases the yield, whereas too intensive homogenization would lead to rupture of not only the cell membrane, but also the chloroplast envelope.

Effect of homogenization. Plant tissue grinding with mortar and pestle is a very common method for disruption of biological samples. Homogenization with polytron was described in Aronsson and Jarvis (2002) work as leading to the best results, whereas Kley et al. (2010) preferred a mini blender. We compared these three methods for isolating chloroplast form *Malus* species. Table 2 summarizes the results. In our experience, homogenization with liquid nitrogen did not lead to high yields and was slightly more time consuming, whereas polytron homogenization yielded the least amount of chloroplasts. Laboratory blender was chosen for further experiments since yield was highest and it was the fastest of the three methods.

In vitro plants for chloroplast isolation should be at least 6 weeks old to allow time for the leaves to reach sufficient mass compared to the stem. The exception is *Prunus avium*, which forms short shoots with relatively large leaves. High proportion of mass from the leaves in this species leads to higher yield of chloroplast fraction.

Table 2. Chlorophyll concentration in chloroplast fractions using different homogenization methods

Homogenization method	Chlorophyll concentration mg 3 g^{-1} fresh plant material
Liquid nitrogen	0.105 ± 0.061
Polytron homogenizer	0.085 ± 0.059
Waring laboratory blender	0.125 ± 0.012

Effect of the isolation buffer. There are many reports of successful isolation of intact chloroplasts with varying composition of the isolation medium (Kley et al., 2010; Grabsztunowicz, Jackowski 2012; Shi et al., 2012; Vieira et al., 2014). This suggests that the choice of buffer, osmoticum, salts, reducing agents and other additives is not critical. The addition of bovine serum albumin (BSA) to the isolation buffer might reduce the damage caused by proteolytic enzymes, but it would not be compatible with proteomic analysis due to the significant interference with the downstream measurements. Isolation medium with sorbitol and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) is very commonly used. However, some authors found that a high salt method gives better yield of chloroplast compared to a sucrose gradient method (Shi etal., 2012; Vieira etal., 2014). Presumably, using the high salt method for chloroplast isolation has the advantage that hypertonic conditions will prevent chloroplasts from rupturing during isolation. In our experiments, the high salt method did not have significant effect either on yield or on purity. Therefore, the sorbitol based medium, which is close to isotonic was used in further experiments.

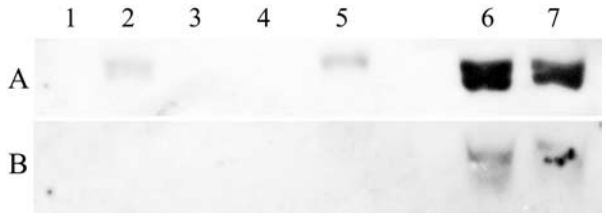
Purity of the chloroplast fraction. Chloroplast fraction was investigated by microscopy for evaluating the purity and integrity of the chloroplasts. Unbroken or partially broken cells were present in the chloroplast fraction if the homogenate was applied on Percoll directly. The presence of cell debris was significantly reduced if there was an extra centrifugation step at 200× g before applying the filtrate to the Percoll. The pellet was investigated by microscopy and in addition to some unbroken cells; there was a large amount of chloroplasts that sedimented at low speed. This fraction was considered as the cellular fraction and investigated by immunoblotting. Extra centrifugation at low speed increased the purity, but also highly reduced the amount of chlorophyll (Table 3). This amount, however, is still comparable to that obtained in previous studies (Aronsson, Jarvis, 2002; Ferro et al., 2010) and corresponds to 200–300 μg of protein, which is sufficient for proteomic analysis (Kley et al., 2010).

Table 3. The purity of chloroplast fractions estimated by microscopy and the concentration of chlorophyll

Full cells present in microscope slide		Chlorophyll concentration mg 3 g^{-1} fresh material	
Percoll	differential and Percoll	Percoll	differential and Percoll
4.8 ± 5.60	0.3 ± 0.46	0.17 ± 0.264	0.08 ± 0.043

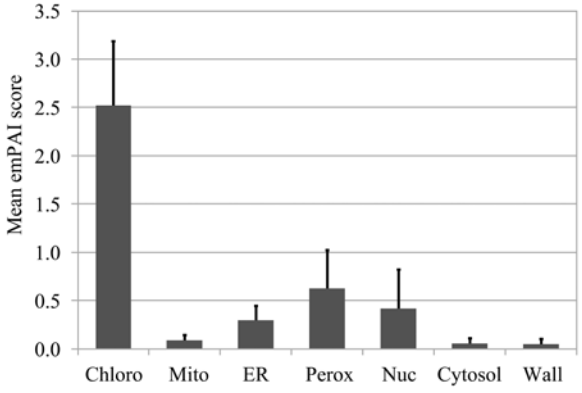
Immunoblotting with cytosolic marker protein (anti-UGPase / UDP-glucose phosphorylase) produced a strong band in the positive control (total protein extract) and a weak band in the cellular fraction, as well as in one of the chloroplast fractions). Cellular or mitochondrial contaminations are common in chloroplast fraction and cytosolic marker enzyme activity was reported to account for 2–6% of the activity in the total leaf (Kley et al., 2010; Grabsztunowicz, Jackowski, 2012). However, most of our chloroplast preparations did not have a detectable cytosolic marker band. The mitochondrial marker (anti-IDH / isocitrate dehydrogenase) was only detectable in the positive control (Fig. 1).

Mass spectrometric quantification of marker proteins revealed varying amounts of organelle-specific proteins (Table 1). In general, the chloroplast marker proteins had the highest protein abundance index (emPAI) value that is proportional to protein content in a protein mixture, although there were some exceptions such as the peroxisomal catalase and nuclear histone H2 protein, which had high emPAI values (1.73 and 1.25, respectively). The average emPAI values for each organelle were calculated to estimate the contribution of the proteins originating from each organelle (Fig. 2),



Note. The primary antibody used was cytosolic marker anti-UGPase / UDP-glucose phosphorylase (A) and mitochondrial marker anti-IDH / isocitrate dehydrogenase (B).

Figure 1. Immunoblot of purified chloroplasts (1, 2, 3, 4), cell fraction (pellet after 200× g) (5) and total (non-fractioned) phenol-extracted proteins (6, 7)



Note. Compartments labelled: Chloro – chloroplasts, Mito – mitochondria, ER – endoplasmic reticulum, Perox – peroxisomes, Nuc – nucleus, Cytosol – cytosole, Wall – cell wall; marker amount expressed as protein abundance index error bars indicate the 95% confidence interval of the mean.

Figure 2. Mean marker protein amount per subcellular location

corresponding to 62, 2, 7, 15, 10, 1 and 1 percentage points for chloroplast, mitochondrial, endoplasmic reticulum, peroxisomal, nuclear, cytosolic and cell wall proteins, respectively. Thus, the main contaminants appear to originate from peroxisomes and nuclei.

Conclusions

1. We present an efficient chloroplast isolation method for small *in vitro* samples for *Malus* and *Prunus* species. The method is suitable and provides sufficient yield for proteomic studies. The purity of the chloroplasts was evaluated by immunoblotting as well as by quantitative mass spectrometry of marker proteins.

2. In our experience, the sensitivity of detection is better for the marker protein isocitrate dehydrogenase in mass spectrometry. However, the marker enzyme UDP-glucose pyrophosphorylase could not be detected with either method. This likely reflects the low amount of cytosolic contamination in our preparations.

3. The use of quantitative mass spectrometry for the assay of marker enzymes appears to be a sensitive and reliable method for the quality control of subcellular fractions.

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Efektyvus chloroplastų išskyrimas iš *Malus* ir *Prunus* mikroūglių

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Santrauka

Subląstelinių frakcijų proteomikos tyrimai leidžia nustatyti specifinius aplinkos kitimo, streso ar kitų sąlygų nulemtus pokyčius. Chloroplastai dalyvauja daugelyje sudėtingų biocheminių procesų, sąlygojančių ląstelės grįžtamojo signalo perdavimą. Jausdami ląstelės funkcijų sutrikimus chloroplastai atitinkamai į juos reaguoja. Kintamas kontroliuojamas sąlygas lengviausia sukurti *in vitro* sistemoje. Tačiau sodo augalus auginant *in vitro* yra sudėtinga išauginti didelį kiekį žalios masės, reikalingos išskirti chloroplastams. Todėl chloroplastų išskyrimo metodą reikia optimizuoti taip, kad išeiga būtų pakankama ir iš nedidelio kiekio medžiagos. Tyrimo metu naudotos trys Rosaceae šeimos rūšys, turinčios svarią agronominę vertę Lietuvos sodo augalų selekcijos programoje. *Arabidopsis thaliana* chloroplastams skirti tinkantis metodas buvo optimizuotas *Malus domestica*, *M. platycarpa* ir *Prunus avium* rūšims. Proteomikos tyrimams pakankama chloroplastų išeiga buvo gauta homogenizuojant 3 g *in vitro* augalų laboratoriniu smulkintuvu ir naudojant izoliavimo terpę sorbitolio pagrindu. Frakcijos grynumas reikšmingai padidėjo taikant papildomą lėto (200× g) centrifugavimo žingsnį. Chloroplastų grynumas vertintas mikroskopu vizualiai, imunopernaša su specifiniais antikūnais ir kiekybine masių spektrometrija – pagal baltymus žymeklius. Nors mikroskopavimo metu visose chloroplastų frakcijų pavyzdžiuose nustatyti nereikšmingi kiekiai ląstelių priemaišų, imunopernašos metodu kai kuriuose pavyzdžiuose nustatyti ir citozoliniai žymekliai. Masių spektrometrijos analizė patvirtino nedidelius kiekius ne chloroplastams būdingų baltymų.

Reikšminiai žodžiai: baltymo gausumo indeksas, imunobloto metodas, masių spektrometrija, Percoll, subląsteliniai žymekliai.