

RESEARCH ARTICLE

Purification and proteomic characterization of plastids from *Brassica napus* developing embryos

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Plastids are functionally and structurally diverse organelles responsible for numerous biosynthetic reactions within the plant cell. Plastids from embryos have a range of properties depending upon the plant source but compared to other plastid types are poorly understood and therefore, we term them embryoplasts. Isolating intact plastids from developing embryos is challenging due to large starch granules within the stroma and the prevalence of nonplastid, storage organelles (oil bodies and protein storage vacuoles) which compromise plastid integrity and purity, respectively. To characterize rapeseed embryoplasts it was necessary to develop an improved isolation procedure. A new method is presented for the isolation of intact plastids from developing embryos of *Brassica napus* seeds. Intactness and purity of embryoplast preparations was determined using phase-contrast and transmission electron microscopy, immunoblotting, and multidimensional protein identification technology (MudPIT) MS/MS. Eighty nonredundant proteins were identified by MudPIT analysis of embryoplast preparations. Approximately 53% of these proteins were components of photosystem, light harvesting, cytochrome *b/f*, and ATP synthase complexes, suggesting ATP and NADPH production are important functions for this plastid type.

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1 Introduction

Oilseed rape (*Brassica napus*) seed store up to 50% triacylglycerol primarily within the embryo, which dominates the seed structure [1]. Acyl chains for triacylglycerol originate from the plastid, the site for *de novo* fatty acid synthesis in plants [2]. Plastids are envelope membrane-bound

organelles found in the cells of all higher plants and are the site for many processes including photosynthesis, starch, fatty acid, isoprenoid, and amino acid biosynthesis [3]. All plastids develop from proplastids, to beget the wide diversity of plastid types including chloroplasts, etioplasts, chromoplasts, amyloplasts, or leucoplasts depending on the cell or tissue [4]. Previous characterization of rapeseed embryo plastids suggested they were similar but also different than the closest plastid type, chloroplasts [5]. One difference being the ability to uptake many different carbon precursors including glucose-6-phosphate, dihydroxyacetone phosphate, malate, pyruvate, acetate [5], and phosphoenolpyruvate [6]. Moreover, the context and function of some enzymes in *B. napus* embryo plastids may be slightly differ-

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Abbreviations: **BCCP**, biotin carboxylase carrier protein; **FA**, formic acid; **MudPIT**, multidimensional protein identification technology; **PIM**, plastid isolation media; **PSVs**, protein storage vacuoles; **TEM**, transmission electron microscopy; **SSP**, seed storage protein; **SUS**, sucrose synthase; **WAF**, weeks after flowering

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ent than in chloroplasts. For example, in embryo plastids a partial Calvin cycle pathway functions primarily to refix carbon dioxide released upon production of acetyl-CoA from pyruvate to maximize the efficiency of *de novo* fatty acid synthesis [7], as opposed to the traditional role in leaf chloroplasts of fixing atmospheric carbon dioxide. A redundant glycolytic pathway in plastids, prominent in developing seed [8], may also have unique functions such as enabling a more cooperative distribution of glycolytic intermediates between the cytosol and the plastid. Due to these and other differences between chloroplasts we use the term “embryoplast” to describe plastids isolated from plant embryos that contain chlorophyll yet are also heterotrophic. Embryoplasts have properties of both chloroplasts and leucoplasts, non-photosynthetic plastids specialized for bulk storage of starch lipid and protein. However, it is unclear how distinct *B. napus* embryoplasts are from chloroplasts and leucoplasts.

To begin characterizing rapeseed embryoplasts at the proteome level it was essential to develop and employ an isolation procedure avoiding mechanical shearing, which could lyse the starch-dense plastids. We present a new method for embryoplast isolation from *B. napus* embryos that begins with protoplast isolation. Plastids isolated using this method were verified for intactness and purity using various techniques. Multidimensional protein identification technology (MudPIT) analysis of these organelle preparations followed by stringent database searches resulted in the assignment of 80 nonredundant proteins with a high level of confidence. The prominence of light harvesting and photosystem complex proteins amongst these assigned proteins indicates ATP and NADPH production is essential and strongly suggests these plastids are more closely related to chloroplasts than leucoplasts or amyloplasts.

2 Materials and methods

2.1 Plant growth conditions

Developing *B. napus* cv. Reston seed were harvested from plants grown in a greenhouse maintained at 23°C. Plants were fertilized at 2-wk intervals using an all-purpose fertilizer (15–30–15; nitrogen–phosphorous–potassium). Flowers were tagged immediately prior to opening of floral buds. Seeds were harvested at three weeks after flowering (WAF).

2.2 Isolation of embryoplast from developing embryos of *B. napus* seed

Developing embryos were dissected by scoring the seed coat with a fresh razor blade and squeezing the embryo (and liquid endosperm) from the seed coat. Dissected embryos (*ca.* 0.5 g) were rinsed in 10 mL cold (4°C) holding solution (0.5 M sorbitol, 5 mM CaCl₂, 5 mM HEPES–KOH, pH 6.0) and filtered through Miracloth (Calbiochem–Novabiochem, Darmstadt, Germany) to remove starch and endosperm.

Washed embryos were then transferred into a glass Petri dish containing 1.7 mL cold holding solution and the embryos were minced with sterile blades into pieces smaller than 0.5 mm. Enzyme digestion solution (3.0 mL, 2% w/v cellulysin, 0.1% w/v pectolyase, 0.6 M mannitol, 0.1 M DTT, 5 mM 2-(4-morpholino)-ethane sulfonic acid (MES)-KOH, pH 5.5) was added and the media was vacuum infiltrated into the minced embryos for 30 s inside a vacuum chamber. Embryos were then incubated for 1.5 h at RT on an orbital shaker. After cell wall digestion, 50 mL cold plastid isolation media (PIM, 0.5 M sorbitol, 20 mM HEPES–NaOH, pH 7.4, 10 mM KCl, 1 mM MgCl₂, EDTA, 5 mM DTT, 10% v/v ethanediol) was added and then filtered three consecutive times through one layer of 150 µm nylon mesh into a fresh tube on ice to separate nonmacerated plant material from protoplasts. The 150 µm nylon mesh flow through was then passed three consecutive times through one layer of a 60-µm nylon mesh into a fresh tube on ice. This step was performed to gently lyse the protoplasts and was assisted by gently forcing the protoplasts through the mesh with a soft bristle paint brush. The 60 µm-flow through was then centrifuged for 5 min at 750 × g (Sorvall, HB-6 rotor) to collect intact plastids. Pellets were gently resuspended in 2 mL of cold PIM, layered on top of 10 mL of 35% Percoll™ (GE Healthcare, Piscataway, NJ) in PIM plus 3% w/v PEG-4000, and centrifuged 8 min at 1000 × g in a swinging bucket rotor (Sorvall HB-6) prechilled to 4°C. The upper band (approximately 1 cm down from the surface) and 35% Percoll pellet was retrieved with a wide-bore pipette and added to 10 mL of PIM. Washed plastids were then filtered through two layers of 10 µm nylon mesh into a fresh tube on ice. The flow-through was centrifuged at 750 × g for 5 min and the embryoplast pellet was resuspended in 200 µL PIM for all further analyses (Fig. 1).

2.3 Chlorophyll determination

For total chlorophyll determinations, samples were extracted in 80% v/v acetone and quantified in a spectrophotometer as described previously [9].

2.4 Phase contrast and transmission electron microscopy (TEM)

Embryoplast preparations were observed directly by bright field and phase contrast microscopy using a 100X oil immersion objective lens on a Leica DM 4000B microscope. Developing seed and embryoplast samples for TEM were prepared and analyzed as described previously [10].

2.5 SDS-PAGE and immunoblot analyses

Fractions from embryoplast isolation including crude homogenate, 750 × g pellet, and final embryoplast pellet were resuspended in 0.5 × SDS-PAGE sample buffer (1 × sample

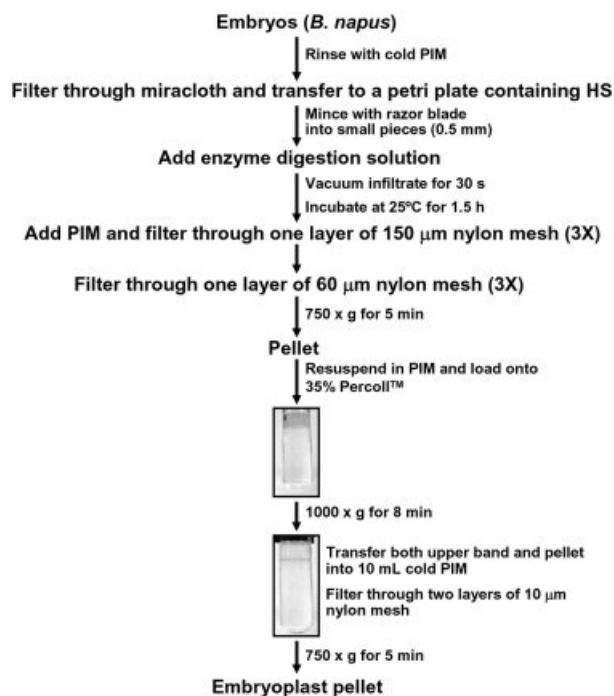


Figure 1. Flow diagram for embryoplast isolation procedure from *B. napus* developing embryos. PIM, plastid isolation media.

buffer, 60 mM Tris-HCl, pH 6.8, 60 mM SDS, 5% w/v glycerol, 100 mM DTT, and 30 mM bromophenol blue), heated at 65°C for 10 min, and centrifuged for 15 min at 13 000 × g to collect insoluble debris. Supernatants were removed and protein quantified in triplicate using Rediplate EZQ protein quantitation kit (Invitrogen, Carlsbad, CA) with ovalbumin as a standard. Equal amounts of protein (20 μg) from each fraction were loaded into individual lanes on 12% SDS-PAGE gels and a wide range marker (Sigma Chemical, St. Louis, MO; Product M4038) was used as a protein standard. For immunoblot analyses proteins were electroblotted to NC membrane for 16 h at 100 mA in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Antibody probing and colorimetric development was performed as described previously [11]. Digital images of blots and gels (12-bit TIFF, 300 dpi) were acquired using a Microtek 800 scanning densitometer. Protein bands were quantified using Image Quant TL (GE Healthcare). Immunoblotting was performed using polyclonal antibodies raised against biotin (KPL Labs, Gaithersburg, MD) for detecting biotin carboxylase carrier protein (BCCP) a marker for plastid organelles [11], mAb to maize mitochondrial pyruvate dehydrogenase alpha subunit [12], mAb to luminal binding protein (BiP, ER marker, Assay Designs, Ann Arbor, MI), mAb heat shock cognate 70 (cytosolic marker, Assay Designs), and a mAb to oleosin (oil body marker, SemBioSys Genetics, Calgary, Canada). Immunoblots were incubated with either antimouse or rabbit IgG alkaline phosphatase secondary antibody (Sigma) for colorimetric detection.

2.6 Semicontinuous MudPIT

Embryoplast pellets obtained from six biological replicates of developing seed of *B. napus* at 3 WAF were individually resuspended in IEF extraction media (8 M urea, 2 M thiourea, 2% w/v CHAPS, 2% v/v Triton X-100, 50 mM DTT) plus 2% w/v SDS, vortexed at low speed for 30 min at RT, and centrifuged at 14 000 rpm for 15 min to remove insoluble material. Supernatant was used to measure protein concentration in triplicate using RediPlate EZQ protein quantification kit (ovalbumin as standard). Fifty micrograms of total protein was added to a 40% stock of acrylamide (39% w/v acrylamide, 1% w/v bis-acrylamide), 5 μL of 10% w/v ammonium persulfate, 3 μL *N,N,N',N'*-tetramethylethylenediamine (TEMED), and adjusted to a final volume of 150 μL with water to obtain a 12% polyacrylamide–protein gel plug. Under these conditions for polymerization no detectable acrylamide modification of Cys residues was observed, based upon mass spectral sequence querying with +71.07790 (propionamide) as the modification. Polymerized gel plugs were minced into approximately 1-mm cubes and transferred into fresh 1.5-mL microcentrifuge tubes. Gel pieces were washed with water, reduced with 10 mM DTT at RT for 1 h, alkylated with 40 mM iodoacetamide in the dark for 1 h at RT, and in-gel digested with sequencing grade modified trypsin (Promega, Madison, WI; resuspended at 0.02 μg/μL in 50 mM ammonium bicarbonate) for 20 h at 37°C.

All samples were reconstituted in 25 μL of 0.1% v/v formic acid (FA) in water. Peptides (40 μg) were analyzed on a ProteomeX LTQ workstation (Thermo Fisher, San Jose, CA) using the semicontinuous MudPIT configuration according to the manufacturer's instructions. Peptides were loaded onto a strong cation exchange resin (BioBasic SCX, 100 × 0.32 mm², 300 Å, 5 μm) and eluted with 12 semicontinuous salt (ammonium chloride) solution gradients (10, 25, 35, 50, 65, 75, 90, 110, 130, 175, 250, and 500 mM, respectively) onto peptide traps (C18, 5 × 1 mm²) for concentrating and desalting prior to final separation by RP capillary column (BioBasic C18, 100 × 0.18 mm², 300 Å, 5 μm) using an ACN gradient (0–90% v/v solvent B in solvent A for a duration of 75 min, Solvent A = 0.1% (v/v) FA in water; Solvent B = 100% ACN containing 0.1% v/v FA). Eluted peptides were ionized with a fused-silica PicoTip emitter (12 cm, 360 μm od, 75 μm id, 30 μm tip; New Objective, Woburn, MA) at ion spray 3.0 kV and a flow rate of 250 nL/min. Five data-dependent MS/MS scans (isolation width 2 amu, 35% normalized collision energy, minimum signal threshold 500 counts, dynamic exclusion (repeat count, 2; repeat duration, 30 s; exclusion duration 180 s) of the five most intense parent ions were acquired in a positive acquisition mode following each full scan (mass range *m/z* 400–2000).

For protein identification, acquired MS/MS spectra were searched against the nonredundant protein National Center for Biotechnology Information (NCBI; ftp://ftp.ncbi.nih.

gov/blast/) database (as of June 2006) using the SEQUEST algorithm within the BioWorks 3.2 software package (Thermo Fisher). Peptide mass tolerance was 1.5 Da and one missed trypsin cleavage was allowed for the database search. Additional criteria were applied to SEQUEST searches to obtain high-confidence protein assignments including: (i) a minimum of two unique, nonoverlapping peptides for protein assignment; (ii) minimum crosscorrelation score (Xcorr) versus charge state of 1.5, 2.0, and 2.5 for +1, +2, and +3 charged peptides, respectively; and (iii) a minimum peptide probability of 0.05% were required for each peptide match. All peptide assignments were also manually inspected to ensure apparent protein isoforms (based upon sequence annotation and molecular weight) contained distinguishing peptides.

2.7 Bioinformatic analyses

Protein sequences from MudPIT analyses were queried against a plastid proteome database (<https://www.plprotblast.ethz.ch/blast/>) to determine if previous investigations also assigned the accession number as a plastidial protein [13]. Additionally, three different organelle targeting prediction programs were also queried with each of the proteins identified by MudPIT including: Target P (<http://www.cbs.dtu.dk/services/TargetP>) version 1.1 [14] using plant group with no cutoffs; Predotar version 1.03 (<http://urgi.inbio.gen.fr/predotar/predotar.html>) version 1.03 [15]; and PCLR chloroplast localization prediction (<http://andrewscheine.com/pclr/index.html>) [16].

3 Results and discussion

3.1 Staging of embryos for plastid isolation

One of the challenges of isolating intact plastids from plant tissues is the presence of starch granules within the stroma which upon tissue disruption results in a high degree of plastid lysis, particular with mechanical disruption methods. Most leaf chloroplast isolation procedures begin with tissue harvested after a long dark cycle when transient starch granules are small or nonexistent. However, in heterotrophic or photoheterotrophic tissues such as embryos starch granules persist throughout the day/light cycle. Additionally, plant embryos also contain specific organelles including protein storage vacuoles (PSVs) and oil bodies which can interfere with plastid isolation methods. These organelles and the products they store change dramatically with embryo development; storage proteins and oil (*i.e.*, PSVs and oil bodies) rapidly increase in expression between 3 and 4 WAF [17]. For this reason, 3 WAF was the most suitable stage of embryo development for plastid isolation. At this stage the embryo is fully developed and represents 10–20% of the seed mass (Fig. 2, [18]). TEM analyses of 3 WAF *B. napus* embryonic cells (Fig. 3) confirm a higher ratio of plastids to PSVs and oil

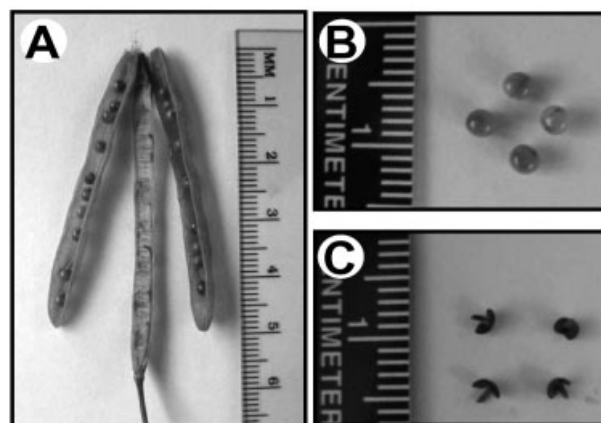


Figure 2. Three week after flowering (3 WAF) *B. napus* cv. Reston (A) opened silique with two halves and septum (in the middle), (B) seed, and (C) embryos.

bodies. In contrast, 6 WAF *B. napus* embryos are densely packed with PSVs and oil bodies, collectively comprising greater than 95% of the cell area on TEM micrographs [10].

3.2 Embryoplastid isolation from 3 WAF embryos

Previously published methods [5, 6] for embryoplastid isolation from rapeseed rely on mechanical disruption of tissue in osmotically balanced media to release intact plastids. Our initial efforts to isolate rapeseed embryoplasts focused on these procedures although SDS-PAGE and MudPIT analysis of these plastid preparations indicated a high level of contamination with PSV and mitochondria proteins (data not shown). Protoplast isolation followed by cell lysis is reportedly milder than mechanical methods and was previously employed to isolate recalcitrant organelles including amyloplasts from maize endosperm [19], protein bodies from mung bean [20], and plastids from castor bean endosperm [21]. We present a modified procedure here for rapeseed embryos.

With any protoplast isolation method it is necessary to physically abrade the plant material initially to increase the surface area for cell wall digestion. Several methods were tested including pressing between glass plates and mincing with razor blades as well as more rigorous techniques such as grinding in a mortar and pestle and shearing in a Waring blender or Polytron homogenizer. For rapid monitoring of plastid enrichment and yield, protein and chlorophyll (plastid marker) were quantified from total homogenates and 750 × g pellets for specific activity measurements. From this comparison, the optimal tissue abrasion technique was mincing with razor blades (data not shown). After mincing of 3 WAF embryos into small (>0.5 mm²) pieces using fresh razor blades, the cell wall matrix was digested with a combination of cellulases and pectinases. Released protoplasts were then separated and lysed by filtering through nylon mesh of 150 and 60 μm, respectively, followed by cen-

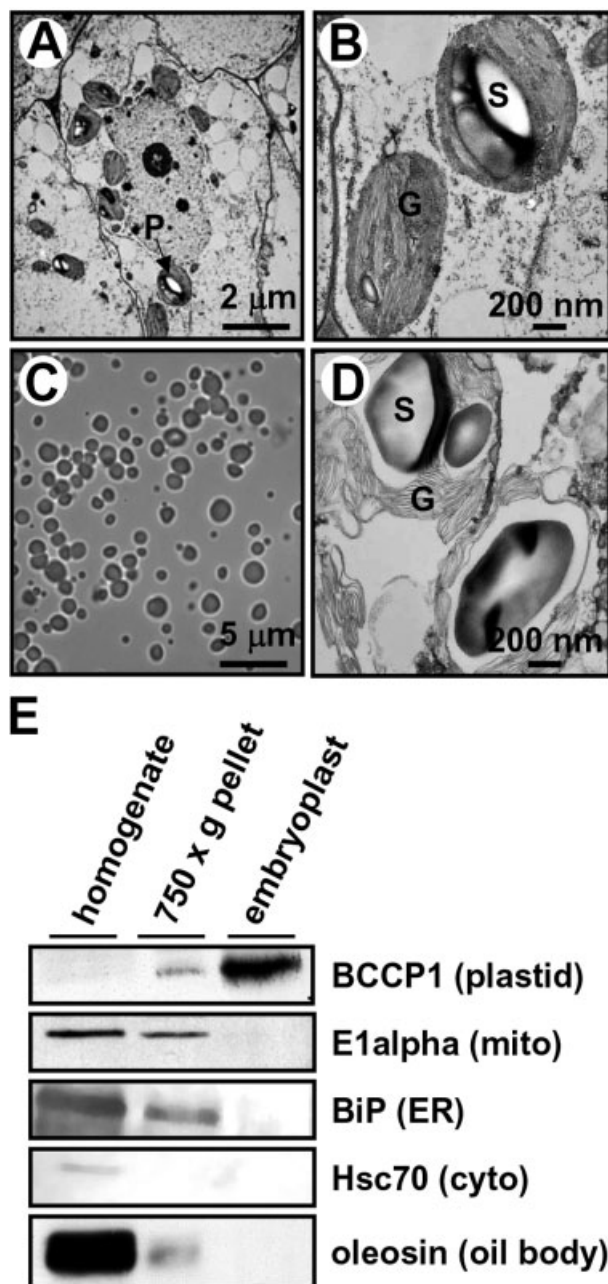


Figure 3. Plastid purification and TEM analysis. (A) Transmission electron microscopic image of *B. napus* embryonic cells 3 WAF, (P) plastid, bar equals 2 μm ; (B) TEM image of plastids from panel A under higher magnification (G) grana, (S) starch, bar equals 200 nm; (C) purified embryoplast in brightfield microscopic view bar equals 5 μm ; (D) transmission electron microscopic image of purified embryoplast, bar equals 200 nm; (E) immunoblot analyses of embryoplastid preparations isolated from embryos dissected from 4 WAF *B. napus* cv. Reston seeds 20 μg protein was loaded in each lane total crude homogenate (CH), 750 g pellet (P) and final embryoplastid preparation (E). The blots were probed with antibodies raised against biotin for detecting BCCP (plastid marker); pyruvate dehydrogenase α subunit (mitochondrial marker); luminal binding protein (BiP, ER marker); heat shock cognate 70 (Hsc70, cytosolic marker) and oleosin (oil body marker).

trifugation to collect embryoplasts. The crude embryoplast pellet obtained was further purified by Percoll to remove nonplastid organelles. Comparison of Percoll step gradients and various isocratic gradients resulted in optimal separation with a 35% isocratic Percoll pad. A final passage through two layers of 10 μm nylon mesh helped to remove larger, non-plastid organelles with similar buoyant densities and was followed by centrifugation to collect intact plastids. On average, final embryoplast pellets from 0.5 g embryos yielded 0.3 mg of protein and 0.01 mg chlorophyll, which is a five-fold increase in chlorophyll specific activity when compared to the total homogenate (Table 1).

3.3 Integrity and properties of isolated embryoplasts

Purified embryoplasts were viewed under brightfield and phase-contrast microscopy to examine plastid size and also monitor intactness. As seen under brightfield microscopy, embryoplasts were spherical in shape with a varied range of diameters averaging $0.87 \pm 1.7 \mu\text{m}$ (SD, $n = 87$; Fig. 3C). The diversity of size likely reflects a mixed developmental series of embryonic cells. Plastid preparations were generally greater than 95% intact as determined by the characteristic “halo” surrounding the envelope under phase-contrast microscopy (data not shown). TEM of phase-contrast microscopy at 20 000 \times magnification (Fig. 3D) shows thylakoid membranes but limited grana stacking. There are also one or two large starch granules, occupying a majority of the plastid stroma.

3.4 Immunoblot analyses indicate embryoplast preparations are highly purified

To determine the purity of embryoplast preparations, immunoblots were performed using antibodies specific to known proteins in plastids, mitochondria, ER, cytosol, and oil bodies (Fig. 3E). Anti-biotin antibodies which reacted with a 35 kDa BCCP indicated an approximate 20-fold enrichment in the final 750 \times g embryoplast pellet (embryoplast lane) compared to the total homogenate. mAb against the mitochondrial pyruvate dehydrogenase α subunit detected a prominent 43 kDa band in embryo homogenates and 750 \times g pellets containing crude embryoplasts, but was not detected in final embryoplast preparations. There was minimal presence of ER and soluble cytosolic proteins as observed with antibodies to BiP (70 kDa) and Hsc70 (70 kDa), respectively. An intense 19 kDa band was observed in crude homogenate fractions when probed with *Arabidopsis* oleosin antibodies, and this band was absent in final embryoplast preparations. These Western blots indicate that embryoplast preparations were highly enriched and largely free of mitochondria, ER, cytosol, and oil body organelles.

3.5 MudPIT analysis of embryoplast preparations

Light and TEM microscopy, chlorophyll quantitation, and immunoblot analyses are all traditional approaches to validate organelle purity, and indeed each of these techniques

Table 1. Purification of *B. napus* embryoplasts

Fractions	Protein (mg/g FW)	Chlorophyll (mg/g FW)	Chlorophyll mg/mg protein	Chlorophyll yield (%)	Fold purification
Homogenate	90.5 (2.60)	0.60 (0.07)	0.0066	100	1.0
750 × g pellet	10.1 (1.34)	0.092 (0.01)	0.0091	15.3	1.4
Embryoplast	0.63 (0.14)	0.021 (0.01)	0.033	3.5	5.0

Total protein and chlorophyll content of 3 WAF embryoplast isolation steps from *B. napus* embryos including: unfiltered protoplast fraction (homogenate); initial 750 × g pellet (750 × g pellet); and final 750 × g pellet (embryoplast). Values are average of eight replicates. Values within parentheses are SDs.

support the purity of embryoplasts in the current investigation. A more recent technique to verify organelle purity is large-scale protein identification, or proteomics. Numerous proteomics strategies are available, each with advantages and limitations. It is well known that 2-DE is biased against “extreme” proteins with respect to hydrophobicity, molecular weight, and *pI*. In contrast, MudPIT avoids polyacrylamide gels and the inherent problems with separating bulky proteins by immediately producing tryptic peptides for chromatographic separation. For these reasons MudPIT offers a broader, more accurate representation of protein expression. We decided to employ MudPIT to characterize embryoplast preparations with some modifications on the traditional in-solution digestion procedure. Proteins were resuspended in media containing SDS and polymerized into a polyacrylamide gel plug for in-gel digestions. This ensured membrane proteins were resuspended and that trypsin digestion was thorough [22].

Six embryoplast preparations (40 µg protein each) were analyzed by MudPIT on a linear IT mass spectrometer resulting in the identification of 80 nonredundant proteins (Supporting Information Table 1). Each of these proteins was confirmed from at least two embryoplast preparations. Of these 80 proteins, 15 (19%) are plastid-encoded, while 40 (50%) proteins are predicted to be plastid-localized by at least one of the three major organelle prediction programs (TargetP, Predotar, PCLR; Supporting Information Table 1) or are previously characterized plastid proteins (light harvesting chlorophyll *a/b* binding protein LHCA3). Of the remaining 25 (31%) proteins, seven (8%) are cruciferin and napin seed storage proteins (SSP). Interestingly, querying each of the 80 identified proteins against plprot, a plastid protein database compiled from empirical proteomic analysis of multiple plastid types [13] revealed 71 (89%) of the 80 identified proteins were present in this database with low *e*-value scores (*i.e.*, high similarity; Supporting Information Table 1). However, since the plprot database is a collection of plastid proteins based upon experimental proteomics data, it is conceivable that some of the entries are contaminants. Despite this caveat, proteomics is considered to be the most comprehensive approach to determine the protein composition of organelles [23]. Besides

contaminants, proteins that are not predicted to be plastid localized based upon target prediction algorithms but are present in the plprot database are candidates for either association with the outside of the plastid envelope or alternative plastid targeting. The nine proteins with high *e*-value scores (*i.e.*, low similarity) from the plprot database include the seven SSPs and two hypothetical proteins. It was previously shown that cruciferin and napin are prominent components of the seed proteome even at 3 WAF [17]. Given the abundance of SSPs in developing embryos these are most likely contaminating proteins. However, it was shown that an ER-mediated targeting pathway could be an alternative route for plastid proteins, although at present this has been shown for only two proteins [24, 25]. Since cruciferin and napin mostly reside in PSVs each must transiently proceed through the ER towards their final destination.

The apparent success of the plprot program at predicting plastid proteins in this study is no doubt due to the source of data, experimental evidence, which is blind to the mechanism of import (or association) unlike the prediction algorithms which rely solely on known principles for plastid import. This becomes clear when comparing the frequency of assignment for these four prediction programs. Out of the 65 nonplastid encoded proteins targetP, predotar, and PCLR identified 52, 52, and 55% of the proteins as plastid-localized, respectively.

3.6 Classification of identified embryoplast proteins

The 80 identified embryoplast proteins belong to many different functional classes including light reactions of photosynthesis, protein stability, plastid biogenesis, and biosynthetic pathways for fatty acids, starch, amino acids, and chlorophyll (Fig. 4). Proteins involved in light harvesting and phosphorylation accounted for 53% of the total proteins indicating this is an important function of 3 WAF embryoplasts. Additionally, proteins related to plastid development and biogenesis represented 8%. Plastid-encoded proteins represented 19% of the embryoplast proteins suggesting plastid transcription and translation must also be active.

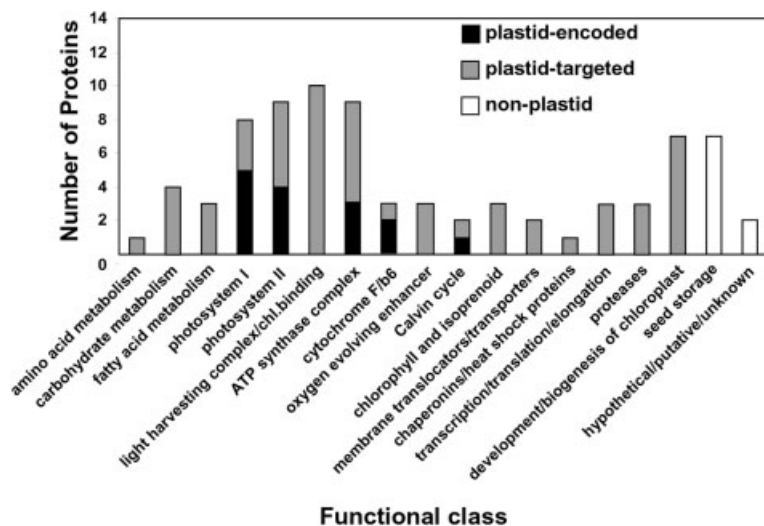


Figure 4. Distribution of identified embryoplast proteins classified according to protein function. The data represent MudPIT analyses from six independent embryoplast preparations, number of proteins in each functional class that are plastid-encoded are shown in black bars, proteins targeted to plastids (as determined by plprot <https://www.plprot-blast.ethz.ch/blast/>) are denoted by the gray bars and nonplastidial proteins are shown in white bars. See Supporting Information Table 1 for additional information about these proteins.

3.7 Light reactions of photosynthesis are prominent in rapeseed embryoplasts

Plastids of developing embryos of rapeseed contain thylakoid membranes and chlorophyll. In this respect, they are similar to leaf chloroplasts. Chloroplasts contain four protein complexes photosystem I (PSI), photosystem II (PSII), cytochrome *b₆/f*, and ATP synthase collectively involved in light harvesting and energy conversion. Chloroplasts also need several other proteins that are important for assembly, maintenance, and regulation of photosynthetic machinery [26]. We have identified eight and nine proteins involved in PSI and PSII reaction centers, respectively, as well as ten light harvesting complex proteins, and three oxygen evolving enhancer proteins (Fig. 4). Additionally, subunits to the plastid ATP synthase complex (F1 alpha, beta, delta, gamma and CF0, CF1 alpha, and beta subunits) were identified, as well as cytochromes *f* and *b*. Identification of each of these proteins clearly indicates light harvesting and noncyclic/cyclic phosphorylation for ATP and NADPH production are critical for embryoplast function. This is not surprising since *de novo* fatty acid synthesis is an energy expensive process; ATP and NADPH are stoichiometrically required for every two carbon condensation. The presumed importance of these processes to fatty acid synthesis is supported by the observation that maximum oil production in rapeseed requires light harvesting by the developing embryo [27].

Although many of the components necessary for light reactions of photosynthesis were identified from developing embryoplasts, enzymes for dark reactions (*i.e.*, Calvin cycle) were not, or at least not as prominent. The large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and phosphoglycerate kinase were the only two Calvin cycle proteins identified from 3 WAF embryoplasts. However, a complete cycle was not expected based upon the findings of Schwender *et al.* [7]. Developing rapeseed embryos employ

enzymes of the Calvin cycle to retain lost carbon dioxide upon oxidative decarboxylation of pyruvate to acetyl-CoA, the precursor for *de novo* fatty acid synthesis. This carbon economy model also contributes to the oil yield efficiency of rapeseed and does not require all activities of the cycle.

3.8 Starch and fatty acid synthesis

Sucrose synthase (SUS) was previously shown to be prominently expressed throughout seed development in *B. napus* [17]. Proteomic analysis of isolated embryoplasts revealed two forms of SUS, most likely associated with the outside of the plastid envelope. Since embryoplasts contain large starch granules (Fig. 3) produced from leaf-derived sucrose, it is possible that an association would allow for more rapid uptake of hexoses for starch synthesis. In addition to SUS, starch synthase and glucan phosphorylase were identified, the former involved in starch synthesis.

De novo fatty acid synthesis from pyruvate to oleic acid requires at least eight different activities [1]. Subunits to the pyruvate dehydrogenase (dihydrolipoamide acetyltransferase) and acetyl-CoA carboxylase (α -carboxyltransferase) multienzyme complexes, as well as ketoacyl-ACP synthase (KAS) I were identified from 3 WAF embryoplasts. The inability to identify all fatty acid synthesis activities is possibly due to the early developmental stage of the embryos for this study. Indeed, proteomic profiling of *B. napus* seed filling revealed most of the activities peaked at 4 or 5 WAF [17]. Alternatively, the preponderance of proteins involved in light reactions of photosynthesis may have masked lower abundance proteins due to column capacity on the SCX and C18 columns or column traps. It is likely that an alternative, off-line pre-fractionation approach such as SDS-PAGE coupled to on-line C18 nanochromatography would improve embryoplast proteome coverage.

4 Concluding remarks

A new method for the isolation of intact and pure plastids from developing *B. napus* embryos was developed. Complementary approaches support the high purity of these plastids including chlorophyll specific activity, light, and TEM microscopy, Western blot, and proteomic analyses. MudPIT analysis of these preparations revealed 80 nonredundant proteins, over half of which mapped to various complexes involved in the light reactions of photosynthesis. Complete or nearly complete PSI and PSII, cytochrome *b₆/f*, and ATP synthase complexes, compared to a fractional Calvin cycle, indicate these plastids are capable of light harvesting and associated light reactions of photosynthesis, and could therefore be considered chloroplast-like. In this respect the term often used to describe these plastids, photoheterotrophic, seems appropriate as these organelles harvest light for ATP and NADPH production but obtain carbon (sucrose) principally from the source leaves. Ostensibly, association of SUS with rapeseed embryoplasts may facilitate the flow of monosaccharides into starch. Indeed, multiple ¹⁴C-labeled hexoses and hexose phosphates, including fructose, were readily incorporated into a methanol-insoluble fraction when incubated with developing *B. napus* embryo plastids [5].

As a first proteomic characterization of rapeseed embryoplasts this collection of proteins is not comprehensive based upon the lack of complete pathways such as *de novo* fatty acid synthesis. We attribute this not to the analysis of a nonmodel plant (from a genomics perspective) but rather to the proteomics method that was chosen, to obtain an unbiased representation of proteins regardless of abundance, molecular weight, or hydrophobicity. We believe this was achieved and the results are consistent with a recent discovery about the importance of light reactions of photosynthesis during embryo development for seed oil content yield [27].

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