Mitochondrial Metabolism in Developing Embryos of *Brassica napus*[^5]^[6]

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The metabolism of developing plant seeds is directed toward transforming primary assimilatory products (sugars and amino acids) into seed storage compounds. To understand the role of mitochondria in this metabolism, metabolic fluxes were determined in developing embryos of *Brassica napus*. After labeling with [1,2-13C2]glucose + [U-13C6]glucose, [U-13C6]alanine, [U-13C6]glutamine, [15N]alanine, (amino)-[15N]glutamine, or (amide)-[15N]glutamine, the resulting labeling patterns in protein amino acids and in fatty acids were analyzed by gas chromatography-mass spectrometry. Fluxes through mitochondrial metabolism were quantified using a steady state flux model. Labeling information from experiments using different labeled substrates was essential for model validation and reliable flux estimation. The resulting flux map shows that mitochondrial metabolism in these developing seeds is very different from that in either heterotrophic or autotrophic plant tissues or in most other organisms: (i) flux around the tricarboxylic acid cycle is absent and the small fluxes through oxidative reactions in the mitochondrion can generate (via oxidative phosphorylation) at most 22% of the ATP needed for biosynthesis; (ii) isocitrate dehydrogenase is reversible in vivo; (iii) about 40% of mitochondrial pyruvate is produced by malic enzyme rather than being imported from the cytosol; (iv) mitochondrial flux is largely devoted to providing precursors for cytosolic fatty acid elongation; and (v) the uptake of amino acids rather than anaplerosis via PEP carboxylase determines carbon flow into storage proteins.

Directly or indirectly plant seeds provide most of the food consumed by humans. Although the metabolism of developing seeds has been extensively studied, quantitative understanding of fluxes through central metabolism is still quite limited. *Brassica napus* (canola, oilseed rape) is a major oil crop and is amenable to detailed quantitative flux analysis under conditions that closely mimic *in planta* seed development (1). The main storage compounds in seeds of *B. napus* are oil (triacylglycerols) and proteins, which are synthesized by the developing embryo from sugars and amino acids taken up from the surrounding endosperm liquid.

Developing seeds of *B. napus* have also been the subject of numerous biochemical studies and are a model for oil accumulating seeds (2–13). In previous studies we have used intact developing embryos to make a quantitative analysis of steady-state metabolic fluxes during the conversion of carbohydrates to fatty acids *in vivo* (1, 14–17). These studies introduced a labeling approach using multiple carbon sources (1), quantified the contribution of the oxidative pentose phosphate pathway to biosynthetic NADPH demands (14), and revealed that ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) operates in a novel context of carbohydrate conversion to fatty acids, bypassing glycolytic reactions and increasing the efficiency of seed carbon metabolism (16). However, a detailed description of fluxes through mitochondrial metabolism in *B. napus* or other developing seeds is lacking.

Several functions of mitochondrial metabolism known in plants could be quantitatively relevant for seed development in *B. napus*. In root tips and cell suspension cultures substantial flux around the mitochondrial tricarboxylic acid cycle results in more than 20% of all the glucose carbon entering catabolism being oxidized to CO2 (calculated from data in Refs. 18 and 19). This suggests that most of the cellular ATP is generated by oxidative phosphorylation driven by the oxidation of acetyl-CoA in the tricarboxylic acid cycle. In developing seeds this metabolism could likewise provide much of the ATP that is needed in substantial quantities for the synthesis of storage proteins and oil. In addition, the cytosolic elongation of oleic acid (C18:1) to C20:1 and C22:1 fatty acids in developing seeds requires mitochondrial citrate as a precursor (1, 20, 21). This demand could be large since in many *Brassica* species erucic acid (C22:1) is the most abundant fatty acid stored in the seed. Furthermore, in leaves and roots mitochondrial conversion of OAA and Pyr to KG is essential to primary nitrogen assimilation. Because developing embryos receive glutamine and other

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[^8]: The abbreviations used are: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; ICDH, isocitrate dehydrogenase; KG, α-ketoglutarate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; TBS, N-0-tert-butylidimethylsilyl; GC-MS, gas chromatography-mass spectrometry; FW, fresh weight.Suffixes: “cyt,” “pl,” and “mit” designate the cytosolic, plastidic, and mitochondrial subcellular compartments, respectively (e.g. Ala_cyt for cytosolic alanine).
amino acids from the mother plant, it is unclear how their mitochondria are involved in amino acid metabolism. Finally, in leaves mitochondria carry large fluxes through the photorespiratory pathway (22). However, photorespiration does not occur in B. napus embryos (1) probably because of high CO₂ and low oxygen concentrations (17, 23).

Building on our earlier studies on central carbon metabolism in B. napus embryos (1, 14, 16), this study focuses on the quantification of fluxes through mitochondrial metabolism and their integration with the major fluxes of de novo fatty acid synthesis in plastids and fatty acid elongation in the cytosol. The resulting map of mitochondrial fluxes shows that mitochondrial metabolism in developing B. napus embryos differs fundamentally from that described for other eukaryotic cells.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—d-[-1,2-13C₂]Glucose, d-[-U-13C₆]glucose, l-[-U-13C₅]Gln, l-(amide)-[15N]Gln and l-(amino)-[15N]Gln (all 99% 13C or 15N abundance) were purchased from Isotec (Miamisburg, OH). l-[-U-13C₅]Ala and l-15N-Ala were from Cambridge Isotope Laboratories (Andover, MA).

**Embryo Culture**—Oilseed rape plants (B. napus L., cv. Reston) were grown as described previously (1, 14). Siliques were harvested 20 days after flowering, and embryos at the early stage of oil accumulation (0.5–1 mg of FW) were immediately dissected under aseptic conditions and transferred into a liquid culture medium with inorganic nutrients (1), 20% polyethylene glycol 4000, and carbon and nitrogen sources sucrose (80 mM), Glc (40 mM), Gln (35 mM), and Ala (10 mM). Embryos were grown for 14 days in 5 ml of growth medium in 250-ml Erlenmeyer flasks closed with cotton plugs under low light conditions (21 °C, continuous light of 50 μmol m⁻² s⁻¹). Embryos of 5 mg of FW doubled in weight in 50 ± 11 h (n = 5). For 13C labeling, isotopes were selected to increase the sensitivity of flux determinations as described by Schwender et al. (15) following principles outlined by Mollney et al. (24). Glucose, Ala, or Gln were replaced with [1,2-13C₂]glucose/[-U-13C₆]glucose (1:1 mol/mol ratio), [U-13C₅]Ala, or [U-13C₅]Gln (1:1 mol/mol mixture with unlabeled Gln), respectively. In addition to 13C labeling, experiments using (amide)-[15N]Gln, (amino)-[15N]Gln, or [15N]Ala were performed to assess the contribution of Gln and Ala to protein synthesis independently from the 13C experimental results as well as to assess the exchange of 15N label between proteinogenic amino acids by transaminase activity (see supplemental text (section 4.2)).

**Extraction of Lipids and Proteins**—Embryos were collected, frozen immediately, and kept at −20 °C until extraction. Embryos were ground in a glass tissue grinder at 4 °C and proteins were extracted in a buffer containing sodium phosphate, pH 7.5 (10 mM), and NaCl (500 mM, 1). Storage lipids were extracted by adding hexane/diethylene (1:1, v/v) during protein extraction. Extracted proteins were precipitated by the addition of 0.10 volume of 50% trichloroacetic acid.

**Derivatization of Lipids and Proteinogenic Amino Acids**—After extraction, lipids were reduced under hydrogen (platinum-IV-oxide), and the saturated fatty acids were transesterified to form methyl esters as described previously (14). Proteins were hydrolyzed in 6 N HCl, and the amino acids were derivatized to N,N,O-tert-butylidemethylsilyl (TBS) derivatives as described previously (14).

**Measurement of Labeling in Lipids and Amino Acids by GC-MS**—Fatty acid methyl esters and TBS amino acids were analyzed with a HP 5890 II (Hewlett-Packard) gas chromatograph–mass spectrometer (HP 5972 quadrupole MS). The carrier gas was helium at a flow rate of 1 ml/min. DB23 and DB1 columns (30 m × 0.25 mm; J&W Scientific, Folsom, CA) were used for analyzing fatty acid methyl esters and TBS amino acids, respectively. The relative abundance of mass isotopomers in selected fragments of each analyzed derivative was measured using selected ion monitoring (14). Correction for the occurrence of 13C in derivative parts of the molecules and for heavy isotopes in heteroatoms (carbon, hydrogen, oxygen, nitrogen, silicon) at their natural abundances was performed as described earlier (14). MS measurements from three injections and analyses from each sample were averaged. For saturated fatty acid methyl esters the McLafferty fragment (m/z 74) was analyzed to determine the fractional labeling in the fragment comprising carbons one and two of each fatty acid (14). Glycerol remaining from the transesterification of lipids was analyzed as the trfluorooracetic acid ester (14). Glucose was released from starch by hydrolysis and analyzed as Glc methoxime penta-acetate (14). For each GC-MS chromatogram of amino acid derivatives 136 mass isotopomer fractions were recorded, whereas for fatty acid methyl esters, glycerol, and glucose derivatives 29 mass isotopomer fractions were monitored.

**Computer-assisted Flux Estimation Using Labeling and Biomass Constraints**—Flux analysis of central metabolism was based on the labeling signatures measured in metabolic end products, considering that the carbon atoms incorporated into amino acids and fatty acids can be traced back to the structures of a number of central intermediates (see supplemental text (section 1.2)). A biochemical reaction network of central carbon metabolism was derived from the literature that describes enzymes of central metabolism in B. napus embryos (see supplemental text (section 1.3)) as well as from the biochemical pathway data base for Arabidopsis (AraCyc). Implementation of the isotopomer model, isotopomer balancing, flux parameter fitting, and statistical analysis were performed using the software package 13CFLUX obtained from Dr. W. Wiechert (Department of Simulation, University of Siegen, Germany; see Ref. 25 and references cited therein). In applying the modeling software the flux values in central metabolism were constrained by: 1) the topology of the metabolic network and its stoichiometric relations at metabolic steady state; 2) assuming irreversibility of particular reactions according to the thermodynamics of the reaction; 3) the biomass composition of the embryos, which defines the net fluxes of metabolites into amino acid and fatty acid synthesis (mass balancing); 4) the results of 15N label experiments, which quantified the reversibilities of Ala and Glu aminotransferases; and 5) the measured 13C labeling patterns in metabolites from independent labeling experiments using [1,2-13C₂]glucose/[U-13C₆]glucose, [U-13C₅]Gln and [U-13C₅]Ala, respectively. By varying the values for the 17 freely variable fluxes in the system a best fit between predicted and measured mass isotopomer abundances was obtained by mini-
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mizing the sum of squared differences. The reliability of the flux estimates was tested by starting the flux parameter fitting process 100 times independently with starting values for the free flux parameters randomly chosen within the feasible flux space. Further details of the flux estimations are described under “Results” and in the supplemental text (section 4).

Statistical Analysis—Using the 13CFlux statistical software tool, the S.D. values for the flux values (see Table 1) were derived from the S.D. values of the biomass and MS measurements. As the MS data from three independent 13C labeling experiments ([1,2-13C2]glucose/[U-13C6]glucose, [U-13C5]Gln, and [U-13C6]Ala) were combined in the model, the biological and analytical variability resulting from triplication of the labeling experiment are present in the data set. However, since the three different labeling patterns cannot be directly averaged, values for the S.D. in the MS measurements were estimated as detailed in the supplemental text (section 5.2), and S.D. in the fluxes calculated on this basis (Table 1).

Based on the range of values for the S.D. observed in the MS measurement data in comparable labeling experiments the χ2 value is >35 (see supplemental text (section 5.2)). The χ2 test for goodness of fit is expected to pass since 131 is the maximal allowed value for χ2 on a 90% significance level. In addition, the robustness of the system was tested by removal of 13 of the 164 MS measurements from the data set, causing 50% of the χ2 error. With the reduced data set flux parameter fitting still consistently converged to the same solution (see supplemental text (section 4.2)). This demonstrates the robustness of the model achieved by overdetermination. Therefore we judge the model predictions (flux values, Table 1) to reasonably explain the experimental data.

Design and Validation of the Flux Model—The following reactions were tested in the model to determine whether they improved the agreement between measured and model-predicted labeling levels or if they led to uncertainties in the routes of metabolic fluxes: 1) isocitrate lyase together with malate synthase, defining a glyoxylate bypass; 2) malic enzyme as a reversible reaction; 3) export of plastidic AcCoA into the cytosol. In each case flux parameter fitting consistently assigned very small fluxes to the added reactions and the quality of the fit was not increased by their inclusion (data not shown). Similarly, flux through reactions of gluconeogenesis (conversion of OAA to PEP) could be excluded based on labeling metabolites made from OAA and PEP. The transformation of glycolate to serine, which carries a very high flux in leaf mitochondria during light respiration (22), is not a significant flux in developing B. napus embryos (1). As outlined in the results section, the reversibility of isocitrate dehydrogenase (ICDH) was included based on fitting and further experiments.

Consideration of Alternative Compartmentation—In some cases there are alternative pathways that would produce identical labeling signatures in end metabolites. In the flux model ICDH was assumed to be mitochondrial, although cytosolic and plastidic isoenzymes exist in plants (26). Similarly, the conversion of KGmit to Succmit is assumed to be performed by mitochondrial KG-DH. However this conversion could also take place via the GABA shunt, as assumed by Sriram et al. (27) for soybean embryos. The labeling of the product succinate via the two alternative routes would be identical and they cannot be distinguished based on the labeling data of this study or of Sriram et al. (27).

RESULTS

Experimental Strategy—Previous studies of in vivo flux in plant central carbon metabolism using steady-state stable isotope labeling have been based almost exclusively on [13C]glucose or [13C]sucrose feeding, generally using a single labeled substrate. Our previous studies showed that because of the complexities introduced by the subcellular compartmentation of metabolism, experiments using only [13C]glucose or [13C]sucrose have limited ability to determine several key mitochondrial fluxes. Therefore, to obtain reliable flux measurements, embryos were labeled in separate experiments with [U-13C6]Alanine, [U-13C5]glutamine, [15N]Alanine, (amino)-[15N]glutamine, or (amide)-[15N]glutamine in addition to [1,2-13C2]glucose + [U-13C6]glucose. After each labeling experiment, protein and oil were extracted, hydrolyzed, and analyzed by GC-MS to determine fractional 13C or 15N enrichments for selected fragment ions. With labeling patterns obtained from the different 13C label experiments, best-fit values for the metabolic fluxes were derived using computer-assisted modeling (see “Experimental Procedures” and supplemental text (section 2)).

The strategy of combining data from experiments with different labeled precursors increased the reliability and accuracy of the flux determinations. Unbiased flux parameter fitting consistently converged to one best fit only if the labeling data derived from the three 13C labeling experiments (using [1,2-13C2]glucose + [U-13C6]glucose, [U-13C6]Alanine, or [U-13C5]glutamine) and 15N labeling experiments were combined. For the reactions of mitochondrial metabolism statistical accuracy was increased by the combined data. For example, if only the data from [13C]glucose labeling is considered, the standard deviations for the net fluxes vME, vICDH, vGAT, vPyr1,2, and vPEPC (Table 1) were determined to be 57, 454, 29, 27, and 38% of the flux value, respectively (see supplemental text (section 4.3)). However, with the combined labeling data these S.D. values were reduced to 14%, 216, 12, 9, and 10%, respectively. Overall 30 out of 31 net fluxes were determined with a confidence of better than ±20% of their value. The reversibility of the isocitrate dehydrogenase reaction (see below) was unambiguously identified only after labeling with [U-13C6]Gln.

In addition, and importantly for model validation, the interconversion of amino acids and their corresponding α-keto acids (e.g. Pyr/Ala, KG/Glu) was assessed by 15N labeling experiments. Flux parameter fitting consistently converged to unique solutions for most free net fluxes. However, for the import of pyruvate into the plastid (vPyr1) more detailed inspection of independent sets of optimized flux values revealed that the isotopomer model explains the MS data equally well with different combinations of the reversible interconversion of Ala, and pyruvate (vAATXCH) and vPyr1 (see Fig. 1) and that these fluxes cannot be resolved by 13C data alone. Therefore, since vAATXCH exchanges amino...
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Values of net and exchange fluxes (± S.D.) for B. napus embryos as derived by flux parameter fitting using the fractional enrichment in amino acids, carbohydrate, and fatty acids of $^{13}$C and $^{15}$N labeling experiments

The fluxes are based on the observed growth rate of $\mu = 0.014$ h$^{-1}$ at 5 mg of embryo FW. For exchange fluxes unsymmetrical 68% confidence intervals are given in parentheses instead of S.D. 3-PGA, 3-phosphoglycerate.

| Flux name | Reaction | Enzyme name(s)/comment | Fluxes
|-----------|----------|------------------------|--------------------------|
| | | | Net flux $\mu$ mol h$^{-1}$ mg FW$^{-1}$ |
| | | | Exchange flux $\mu$ mol h$^{-1}$ mg FW$^{-1}$ |
| vuptGlc | Glc uptake | | 58.3 ± 4.2 |
| vuptAla | Ala uptake | | 7.4 ± 0.7 |
| vuptGln | Gln uptake | | 4.3 ± 0.3 |
| vPGM | 3-PGA → PEP | Phosphoglyceromutase, enolase | 83 ± 8.3 |
| vPK | PEP → Pyr$_{cyt}$ | Cytosolic pyruvate kinase | 17.9 ± 2.0 |
| vPK$_{pl}$ | PEP → Pyr$_{pl}$ | Plastidic pyruvate kinase | 60.0 ± 6.5 |
| vPyr$_c$ | Pyr$_c$ → Pyr$_{cyt}$ | Transport of pyruvate into plastids | 21.0 ± 2.6 |
| vPyr$_{mit}$ | Pyr$_{mit}$ → Pyr$_{cyt}$ | Transport of pyruvate into mitochondria | 3.5 ± 0.3 |
| vPDH$_{mit}$ | Pyr$_{mit}$ → AcCoA$_{mit}$ | Plastidic pyruvate dehydrogenase complex | 77.0 ± 8.9 |
| vAAT | Ala$_{cyt}$ → Pyr$_{cyt}$ | Ala:KG aminotransferase | 6.6 ± 0.7 |
| vGlnGlu | Gln$_{cyt}$ → Glu$_{cyt}$ | Glutamate synthase | 3.0 ± 0.2 |
| vGAT | Glu → KG$_{mit}$ | Transaminases, Glutamate dehydrogenase, dicarboxylate transporter | 1.4 ± 0.2 |
| vPEPC | PEP + CO$_2$ → PEP carboxylase | | 3.6 ± 0.3 |
| vME$_{mit}$ | Malate → PEP + CO$_2$ | Mitochondrial malic enzyme | 2.3 ± 0.3 |
| vCS | OAA + AcCoA$_{mit}$ → CO$_2$, Cit | Citrate synthase | 5.8 ± 0.6 |
| vPDH$_{mit}$ | Pyr$_{mit}$ → OAA + AcCoA$_{mit}$ | Mitochondrial pyruvate dehydrogenase complex | 5.8 ± 0.6 |
| vACLO | Cit$_{cyt}$ → AcCoA$_{cyt}$ + OAA | Cytosolic ATP:Citrate lyase | 5.9 ± 0.6 |
| vC3DH | Cit → KG + AcCoA$_{mit}$ | Aconitase, isocitrate dehydrogenase | −0.1 ± 0.2 |
| vKDH | KG → Fum + CO$_2$ | Ketoglutarate dehydrogenase | 1.3 ± 0.2 |
| vFM | Fum → OAA/ malate | Succinate dehydrogenase | 1.3 ± 0.2 |
| | | Fumarase, malate dehydrogenase | 30 (14, 47) |

Flux values are given as $\mu$ mol h$^{-1}$ mg FW$^{-1}$, except for CO$_2$ fluxes, which are given as $\mu$ mol g FW$^{-1}$. Fluxes were calculated for the fraction of carbon in the labeled amino acid that enters the plastidic pool (see supplemental text section 4.2).

$^{15}$N-Ala labeling experiments (see supplemental text (section 4.2)).

Using Equation 1 and the $^{15}$N measurements shown in supplemental Table S7a, the value for $v_{\text{AAT}_{XCH}}$ was determined as 25.8 ± 11.4 times the input flux of alanine ($v_{\text{uptAla}}$), which corresponds to essentially complete isotopic equilibration between cytosolic pyruvate and alanine. In the $^{13}$C isotopomer model the exchange flux $v_{\text{AAT}_{XCH}}$ was then fixed to be 25.8 times $v_{\text{uptAla}}$. After this determination of $v_{\text{AAT}_{XCH}}$, the optimizer algorithm was started 100 times with randomly assigned starting values for the free fluxes, and the optimization algorithm consistently converged to one solution with the lowest sum of weighted squared deviations (residuum) for the free net fluxes, suggesting that this solution is a global optimum. This approach allowed $v_{\text{Pyr$_c$}}$ to be well determined as being 21.0 ± 2.6 nmol h$^{-1}$ mg FW$^{-1}$ (Table 1).

FIGURE 1. Interconversion of cytosolic Ala and pyruvate by alanine aminotransferase. By using $^{15}$N-labeled Ala and unlabeled Gln in embryo cultures the $^{15}$N enrichment in protein-bound Ala (Ala$_{protein}$) is dependent on the ratio of the uptake of $^{15}$N-Ala (vuptAla) and the conversion of pyruvate to Ala (vAAT$_{XCH}$). Given $^{15}$N enrichment measurements in Ala in the medium, Alaprotein, and Proprotein (see supplemental text (section 4.2)) the exchange flux $v_{\text{AAT}_{XCH}}$ can be determined relative to the rate of alanine uptake (see “Results”).
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Of critical importance for the validity of the tricarboxylic acid cycle fluxes is that label measured in protein-derived Asp represents the label of the mitochondrial OAA pool (Fig. 2). As detailed in the supplemental text (section 6.4) this assumption is justified as extensive symmetric randomization of label was observed in protein-derived Asp, making evident an intensive interconversion between cytosolic OAA and mitochondrial fumarate via mitochondrial transporters, malate dehydrogenase and fumarase. This symmetric randomization has also been observed before in other plant systems (18, 28, 29). In addition, the flux parameter fitting consistently assigned a high exchange flux to the interconversion of fumarate and OAA (Table 1), isotopically equilibrating both pools.

**Fluxes through Mitochondrial Metabolism**—The current study focuses on mitochondrial metabolism as it relates to the production of precursors for the major storage products, oil and protein, and, via oxidative phosphorylation, to ATP production. The flux values obtained are shown in Table 1 and Fig. 2. Several reactions shown in Fig. 2 describe two or more sequential enzyme reactions that are considered together (see Table 1). Although the reactions of glycolysis, the pentose phosphate pathway, and Rubisco were included in the full analysis, Fig. 2 and the discussion focus on the reactions and metabolic pools downstream of phosphoglycerate.

**Isocitrate Dehydrogenase Is Reversible in Vivo**—The ICDH reaction was initially assumed to be irreversible. However, during flux parameter fitting, the labeling observed in Pro (whose labeling represents that in αKG) was much better explained by the model if ICDH was allowed to be reversible (fitting results not shown). To test this possibility, embryos were grown with [U-13C5]Gln (50% 13C enrichment), and free organic acids were extracted and analyzed as TBS derivatives. In the mass spectrum of citrate the molecular ion included a m + 5 peak of >10% abundance (Fig. 3). Based on the mass isotopomer distribution in Asp (representing OAA) and in the C1 + C2 fragment of C22 fatty acids (derived from mitochondrial acetyl-CoA), if citrate was formed from OAA and Ac-CoA, the m5 peak in citrate (1–6) should be <2% (see Fig. 3). Therefore the intensity of this peak cannot be explained by the action of citrate synthase. Instead, the conversion of [U-13C5]KG (the labeling that was determined from the mass isotopomer distribution of Pro) to citrate by reverse flow through ICDH accounts for the detection of this citrate isotopomer containing five 13C atoms.

Several features of mitochondrial metabolic flux shown in Fig. 2 are either novel or highly unusual. These are discussed below.

**DISCUSSION**

**Unconventional Mitochondrial Metabolism in Developing B. napus Embryos**—Studies in plants have assigned mitochondrial metabolism central roles in respiration, photorespiration and biosynthesis as well as in gluconeogenesis, the glyoxylate cycle, and amino acid degradation (22, 30, 31). Several canonical flux patterns are recognized: (a) primarily respiratory in heterotrophic tissues; (b) primarily photorespiratory in leaves and other autotrophic tissues of C3 plants; and (c) primarily gluconeogenic in germinating oilseeds, using the glycolate bypass. Metabolic flux analysis in heterotrophic plant tissues using stable isotope labeling has confirmed the first of these functions (18, 19, 27–29, 32). Our findings (Table 1 and Fig. 2) show that mitochondrial metabolism of developing B. napus embryos differs substantially from that previously described in other plant systems or indeed animal or microbial cells. The following sections discuss these novel features.

**Flux around the Tricarboxylic Acid Cycle Is Absent**—In developing B. napus embryos there is no cyclic flux through the reactions of the tricarboxylic acid cycle (Fig. 2). Although there is a significant forward flux through the other reactions, there is almost no net flux or possibly a small net reverse flux through the ICDH reaction. Furthermore, only 3.7% of the amount of hexose carbon that is catabolized is released as CO2 by B. napus embryo mitochondrial decarboxylase activities (pyruvate dehydrogenase, malic enzyme, ICDH, and ketoglutarate dehydrogenase combined). This is in striking contrast to other plant
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Mitochondrial Substrate Oxidation Contributes Little to ATP Production in B. napus Embryos—Mitochondrial substrate oxidation and biosynthetic ATP demands were calculated from the flux values in Table 1 (supplemental Table S9). For the mitochondrial oxidative phosphorylation all mitochondrial NADH- and FADH-producing reactions were balanced assuming ATP production stoichiometries of 3 for NADH and 2 for FADH. The biosynthetic ATP demands were estimated as follows. During fatty acid synthesis acetyl-CoA carboxylase requires one ATP per acetyl unit incorporated into fatty acids. Protein synthesis needs ATP for the polymerization of amino acids. The cost for protein synthesis was assumed to be 4.3 mol of ATP per amino acid incorporated (based on estimates for protein biosynthesis in microorganisms; Ref. 33). This does not include ATP demands for amino acid biosynthesis.

Based on these assumptions ATP produced by mitochondrial substrate oxidation and oxidative phosphorylation is 35 nmol h\(^{-1}\) mg FW\(^{-1}\), while the demand is 159 nmol h\(^{-1}\) mg FW\(^{-1}\). Since maximal possible phosphate/oxygen ratios were assumed and the biosynthetic ATP demand reflects minimum requirements, the resulting ratio of mitochondrial ATP production to biosynthetic ATP demand of 0.22 is likely to be an overestimate. Therefore we conclude that mitochondrial substrate oxidation with subsequent oxidative phosphorylation produces at most 22% of the ATP required for the biosynthetic needs of developing B. napus embryos. Thus mitochondrial substrate oxidation makes only a small contribution to the energy required for storage product accumulation and other cellular processes in B. napus embryos. The main amount of cellular ATP has to be provided by photosynthetic light reactions (12, 17), mitochondrial oxidative phosphorylation using cytosolic NADH, or substrate-level phosphorylation in glycolysis.

Isocitrate Dehydrogenase Is Reversible in Vivo—The data of Fig. 3 demonstrate the in vivo reversibility of ICDH in developing B. napus embryos, an aspect of tricarboxylic acid metabolism that has not been reported previously for plants. As a consequence of this reversibility there are more options for directing flux through mitochondrial metabolism since reversibility allows net reverse flux (Table 1 and Fig. 2). Reversibility of the ICDH reaction has been reported previously for mammalian liver (34), and it has been shown that failure to consider reversibility for the ICDH reaction can lead to incorrect quantification of mitochondrial fluxes (35). The in vivo reversibility of ICDH in B. napus may in part be attributed to the very high tissue concentration of CO\(_2\) in developing seeds of B. napus (36), which shifts the reaction equilibrium toward carboxylation.

Mitochondrial Malic Enzyme—NAD malic enzyme is active in plant mitochondria (37), but flux distributions reported for other plant tissues describe a relatively low contribution by malic enzyme to mitochondrial pyruvate, almost all of which is imported (see Table 2 and references therein). In contrast, 40% of mitochondrial pyruvate is made via malic enzyme in B. napus embryos (Fig. 2 and Table 2).

Most Mitochondrial Metabolic Flux Is Devoted to Cytosolic Fatty Acid Elongation—Oil represents the largest reserve of carbon in seeds of B. napus and for the Reston variety 60% of its fatty acids are 20 carbons or more in chain length. While de novo synthesis of C16 and C18 fatty acids takes place in the plastids, elongation to 20 and more carbons takes place in the cytosol (38). The cytosolic Ac-CoA used for elongation of C18 is formed mainly by ATP:citrate lyase (1, 20, 21). As shown in Fig. 2, essentially all the citrate formed in the mitochondria is exported and is used for producing cytosolic Ac-CoA; this represents the largest flux of carbon leaving the mitochondrion.

Mitochondrial Metabolism Does Not Provide Precursors for Plastidic Fatty Acid Synthesis—As shown in Fig. 2 (Table 1), about 75% of the pyruvate feeding plastidic fatty acid synthesis is produced by plastidic pyruvate kinase. The remaining 25% of pyruvate in the plastid is imported from the cytosol. Besides these reactions, significant contributions to plastidic fatty acid synthesis from import of cytosolic Ac-CoA or from the generation of pyruvate via plastidic malic enzyme can be excluded (1), and this was confirmed in this study by the labeling signatures in C18(1–2), C22(1–2), and Asp.

Balancing of Carbon/Nitrogen Metabolism—The formation of KG as a precursor for glutamate synthesis is regarded as a central function of mitochondrial metabolism. However, as shown in Fig. 2, in B. napus embryos the mitochondrion is a net consumer of KG. A related common feature of cellular anabolism is OAA synthesis via PEP carboxylase with OAA entry into systems where 19–27% of hexose that enters catabolism is oxidized to CO\(_2\), by these reactions (see Table 2).

Mitochondrial Substrate Oxidation Contributes Little to ATP Production in B. napus Embryos—Mitochondrial substrate oxidation and biosynthetic ATP demands were calculated from the flux values in Table 1 (supplemental Table S9). For the mitochondrial oxidative phosphorylation all mitochondrial NADH- and FADH-producing reactions were balanced assuming ATP production stoichiometries of 3 for NADH and 2 for FADH. The biosynthetic ATP demands were estimated as follows. During fatty acid synthesis acetyl-CoA carboxylase requires one ATP per acetyl unit incorporated into fatty acids. Protein synthesis needs ATP for the polymerization of amino acids. The cost for protein synthesis was assumed to be 4.3 mol of ATP per amino acid incorporated (based on estimates for protein biosynthesis in microorganisms; Ref. 33). This does not include ATP demands for amino acid biosynthesis.

Based on these assumptions ATP produced by mitochondrial substrate oxidation and oxidative phosphorylation is 35 nmol h\(^{-1}\) mg FW\(^{-1}\), while the demand is 159 nmol h\(^{-1}\) mg FW\(^{-1}\). Since maximal possible phosphate/oxygen ratios were assumed and the biosynthetic ATP demand reflects minimum requirements, the resulting ratio of mitochondrial ATP production to biosynthetic ATP demand of 0.22 is likely to be an overestimate. Therefore we conclude that mitochondrial substrate oxidation with subsequent oxidative phosphorylation produces at most 22% of the ATP required for the biosynthetic needs of developing B. napus embryos. Thus mitochondrial substrate oxidation makes only a small contribution to the energy required for storage product accumulation and other cellular processes in B. napus embryos. The main amount of cellular ATP has to be provided by photosynthetic light reactions (12, 17), mitochondrial oxidative phosphorylation using cytosolic NADH, or substrate-level phosphorylation in glycolysis.

Isocitrate Dehydrogenase Is Reversible in Vivo—The data of Fig. 3 demonstrate the in vivo reversibility of ICDH in developing B. napus embryos, an aspect of tricarboxylic acid metabolism that has not been reported previously for plants. As a consequence of this reversibility there are more options for directing flux through mitochondrial metabolism since reversibility allows net reverse flux (Table 1 and Fig. 2). Reversibility of the ICDH reaction has been reported previously for mammalian liver (34), and it has been shown that failure to consider reversibility for the ICDH reaction can lead to incorrect quantification of mitochondrial fluxes (35). The in vivo reversibility of ICDH in B. napus may in part be attributed to the very high tissue concentration of CO\(_2\) in developing seeds of B. napus (36), which shifts the reaction equilibrium toward carboxylation.

Mitochondrial Malic Enzyme—NAD malic enzyme is active in plant mitochondria (37), but flux distributions reported for other plant tissues describe a relatively low contribution by malic enzyme to mitochondrial pyruvate, almost all of which is imported (see Table 2 and references therein). In contrast, 40% of mitochondrial pyruvate is made via malic enzyme in B. napus embryos (Fig. 2 and Table 2).

Most Mitochondrial Metabolic Flux Is Devoted to Cytosolic Fatty Acid Elongation—Oil represents the largest reserve of carbon in seeds of B. napus and for the Reston variety 60% of its fatty acids are 20 carbons or more in chain length. While de novo synthesis of C16 and C18 fatty acids takes place in the plastids, elongation to 20 and more carbons takes place in the cytosol (38). The cytosolic Ac-CoA used for elongation of C18 is formed mainly by ATP:citrate lyase (1, 20, 21). As shown in Fig. 2, essentially all the citrate formed in the mitochondria is exported and is used for producing cytosolic Ac-CoA; this represents the largest flux of carbon leaving the mitochondrion.

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The mitochondrial (anaerobic) flux. In this context, PEP carboxylase is generally considered to exert control over the entry of carbon into tricarboxylic acid-derived amino acid synthesis in leaves (39, 40). However, in B. napus, most of the OAA produced is committed to the synthesis of Asp and amino acids derived from it (Fig. 2), rather than being devoted to replenishing tricarboxylic acid cycle intermediates.

Directing more carbon from sugars into protein synthesis by increasing anaerobically via PEP carboxylase flux has also been proposed as a strategy to increase protein content in seeds (41–43). However, as shown in Fig. 2, anaerobic flux from PEP via OAA to KG does not take place. This is because B. napus embryos import nitrogen as amino acids (primarily Gln and Ala), which are not only used directly for protein synthesis but also provide nitrogen via transamination/deamination for the synthesis of other amino acids. Consequently embryo metabolism is provided with the KG derived from Gln (Fig. 2), while conventionally the converse would be assumed. We suggest therefore that for seeds that rely on Gln as a substantial nitrogen source it is unlikely that anaerobic flux into KG can increase protein content. It rather might reduce the use of Gln as a nitrogen source, thus reducing not increasing protein production. Thus Fig. 2 provides an explanation for the observation that seed protein content depends on the identities and amounts of amino acids supplied by the maternal plant and on the metabolic fate of their carbon skeletons (44, 45).

**Plasticity of Mitochondrial Metabolism**—In summary, based on the flux analysis described above, several aspects of mitochondrial metabolism in B. napus embryos are clearly unconventional compared with flux modes previously described. These differences emphasize the highly plastic nature of plant mitochondrial metabolism. The absence of respiratory tricarboxylic acid cycle flux is at first surprising, considering the high biosynthetic ATP demands in an oilseed. However, to assess the need for mitochondrial oxidation and ATP production, all cellular ATP producing and consuming reactions have to be balanced including photosynthetic ATP and reductant production. Ruuska et al. (12) calculated that light absorbed by embryos can provide sufficient ATP and reductant for all oil synthesis. The lack of tricarboxylic acid cycle flux found in B. napus embryos is also consistent with previously described regulatory mechanisms. For example, it has been reported that in leaves the tricarboxylic acid cycle is inhibited by light (46), and embryos may respond similarly to the low light levels that reach seeds growing in planta (9). The observed absence of cyclic tricarboxylic acid flux might also be attributed to anaerobiosis in the embryos. In this case fermentative processes could provide ATP instead of oxidative phosphorylation. However, we conclude that anaerobiosis is not the case because 1) we could not detect products of fermentative metabolism in the growth media (unpublished results) nor is a high CO₂ evolution rate observed (16, 17); 2) culturing B. napus embryos at increased oxygen levels (60% v/v) did not increase the carbon use efficiency or incorporation of precursors into oil, i.e. oxygen was not limiting the transformation of carbon into storage compounds (17); 3) under the experimental growth conditions photosynthesis is generating oxygen inside the embryo; and 4) although in planta the oxygen tension inside developing seeds of B. napus has been reported to be low, the seeds do not enter anaerobiosis (23, 47).

Using a similar steady-state labeling approach as in this study, Sriram et al. (27) studied developing soybean embryos using U-¹³C-labeled sucrose and unlabeled Gln as sole carbon sources. In comparison with the B. napus embryos of our study, the soybean embryos produce and store much more protein and less oil. Also cytosolic elongation of fatty acids to C20 and C22 chain length is essentially not present in soybean due to the different fatty acid composition of the seed oil. Similar to our results and as a consequence of using Gln as nitrogen source, the soy flux map does not show net anaerobic flux of OAA into the tricarboxylic acid cycle (27). PEP carboxylase appears to produce only the amount of OAA needed for production of Asp and derived amino acids. In addition, there is no indication of significant glyoxylate shunt activity in the developing soy embryos. In contrast to B. napus, the export of mitochondrial acetyl-CoA via citrate synthase/ATP:citrate lyase, attributed to cytosolic fatty acid elongation, was not observed in soy (27). Also the relative amount of carbon oxidized by activities of the tricarboxylic acid cycle enzymes to CO₂ is substantially higher in soy (Table 2), suggesting that in soy embryos the fraction of cellular ATP produced via mitochondrial tricarboxylic acid cycle activity and oxidative phosphorylation is much higher than in the B. napus embryos.

This current study documents the broad plasticity of plant mitochondrial and related metabolism and how this metabolism is tailored toward the oil and protein storage functions that dominate oilseed development. The major differences in flux between B. napus embryos and other plant tissues as shown in Table 2 might reflect different transcript levels of tricarboxylic acid cycle related enzymes. However, a comparison of different

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**Table 2**

| Fluxes Related to Mitochondrial Metabolism | B. napus embryos | Maize root tips | Tomato cell culture | Soy bean embryos | Escherichia coli |
|------------------------------------------|------------------|-----------------|---------------------|-----------------|-----------------|
| CO₂ release by mitochondrial reactions | 3.7%             | 21%             | 27%                 | 19%             | 29%             |
| Malic enzyme flux (vMEmit)               | 40%              | 9%              | 7%                  | 16%             | 8%              |
| PEP carboxylase flux (vPEPC)             | 62%              | 57%             | 37%                 | 38%             | 28%             |

*This study.

†Dieude-Noubhani et al. (18).

‡Exponential phase (Rontein et al. (19)).

§Sriram et al. (27).

Glucose fed in bioreactor (Fisher et al. (49)).

IPercent of total carbon of hexose catabolized (6 × vuptGlc × vST), released as CO₂ by ICDH, KDH, PDHmit, and MEmit together (see Table 1).

JPercent of flux, relative to pyruvate entry into the mitochondrion (vPDHmit)
tissues in *A. thaliana* (leaves, roots, seeds) that have different metabolic functions of mitochondrial metabolism indicated comparatively moderate differences in gene expression (48). Therefore, it is likely that plants achieve substantial plasticity of mitochondrial central metabolism via post transcriptional mechanisms in addition to differences in gene expression.

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