Central metabolic fluxes in the endosperm of developing maize seeds and their implications for metabolic engineering

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14C labeling experiments performed with kernel cultures showed that developing maize endosperm is more efficient than other non-photosynthetic tissues such as sunflower and maize embryos at converting maternally supplied substrates into biomass. To characterize the metabolic fluxes in endosperm, maize kernels were labeled to isotopic steady state using 13C-labeled glucose. The resultant labeling in free metabolites and biomass was analyzed by NMR and GC–MS. After taking into account the labeling of substrates supplied by the metabolically active cob, the fluxes through central metabolism were quantified by computer-aided modeling. The flux map indicates that 51–69% of the ATP produced is used for biomass synthesis and up to 47% is expended in substrate cycling. These findings point to potential engineering targets for improving yield and increasing oil contents by, respectively, reducing substrate cycling and increasing the commitment of plastidic carbon into fatty acid synthesis at the level of pyruvate kinase.

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1. Introduction

Maize (Zea mays L) is one of the most productive cereal crops in the world. Maize kernels provide nutrients for humans and animals and material for producing starch, oil, protein, syrup, and biofuel. In maize kernels, starch synthesis and accumulation mainly occur in the endosperm tissue whereas fatty acids production takes place predominantly in the embryo. Because of a negative correlation between oil levels in maize kernels and grain yield (Alexander and Lambert, 1968), efforts to develop high oil maize hybrids have been relatively ineffective and resulted in a small increase in germ weight, an increased concentration of oil in the germ and a reduction in the percentage of endosperm (Lambert et al., 1998). In oat (Avena sativa L) most of the oil in kernels is deposited in the same endosperm cells that accumulate starch, reaching 10% of the oat endosperm dry weight (Ekman et al., 2008). This shows that it may be possible to engineer maize endosperm to produce more oil. If maize endosperm made 10% oil by weight instead of 0.8% it would increase vegetable oil production significantly (by 19% in the US alone; IITA, 1996; USDA, 2008; FAOSTAT, 2007). But to advance towards this goal, a quantitative understanding of central metabolism in this tissue is needed.

Determining the fluxes through central metabolism is important for understanding the biochemistry involved in storage product accumulation, has directly led to improved yields in microbial fermentation (Peterson and Ingram, 2008), and can pave the way for rational genetic engineering of maize and other cereal crops (Nelson and Pan, 1995). There are several approaches to quantifying multiple metabolic fluxes in plant systems (Ratcliffe and Shachar-Hill, 2005, 2006; Dieuaide-Noubhani et al., 2007; Rios-Estepa and Lange, 2007; Libourel and Shachar-Hill, 2008; Allen et al., 2009a). The most well suited and frequently used method for quantifying multiple central metabolic fluxes is steady-state metabolic flux analysis (MFA) using 13C-labeling with nuclear magnetic resonance (NMR) and/or gas chromatography–mass spectrometry (GC–MS) analysis of labeled metabolites combined with computer-aided modeling. This approach has already yielded considerable insight into the metabolism of developing oil seeds (Schwender et al., 2003, 2004, 2006; Sriram et al., 2004; Alonso et al., 2007, 2010; Allen et al., 2009).

The development of maize kernels depends on the import of sucrose transferred from phloem across pedicel and basal cells (Porter et al., 1985; Thomas et al., 1992; Aoki et al., 1999) to the endosperm and embryo (Shannon, 1972). Porter et al. (1985) studied sugar release from the pedicel tissue of maize kernels by removing the distal portion of the kernel and the lower endosperm, followed by...
replacement of the endosperm by an agar trap. They concluded that passive efflux of sucrose from the maize pedicel symplast is followed by extracellular hydrolysis to hexoses (glucose and fructose). Thus sugar assimilates may be imported as hexoses into the cytosol of maize endosperm and embryo. Once in the cytosol, these hexoses can be phosphorylated and serve as precursors of structural and storage compounds. In endosperm, the major site of starch storage, glucose 1-phosphate (glucose 1-P) is converted into ADP-glucose by an ADP-glucose pyrophosphorylase mainly located (90%) in the cytosol (Denyer et al., 1996) and then transported into the plastid as the starch precursor (Shannon et al., 1998).

Hexose-P also furnishes the substrates for respiratory metabolism needed to synthesize ATP. Substantial proportions of the ATP produced (45–70%) in non-photosynthetic tissues, such as maize root tips and tomato cells in culture, were reported to be consumed by futile cycling (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002; Alonso et al., 2007b, 2007c, 2005). However, in developing sunflower embryos, Alonso et al. (2007a) found that substrates cycles utilize less than 20% of the ATP produced, which is in agreement with the relatively high efficiency (50%) of carbon conversion from substrates into products. The conversion of carbohydrate to seed storage oil and protein can exceed 80% in developing Brassica napus embryos (Schwender et al., 2004, Goffman et al., 2005) due to light-driven provision of ATP and reductant which allows the operation of the “RuBiSCO bypass” and eliminates the need for tricarboxylic acid cycle (TCA cycle) flux (Schwender et al., 2006).

Previous investigations have been performed on starch biosynthesis and intermediary metabolism in maize kernels using different 13C-labeled substrates (glucose, sucrose or acetate) on maize kernel cultures (Glawischnig et al., 2001, 2002; Ettenhuber et al., 2005; Spielbauer et al., 2006). Applying [U-13C]glucose to maize kernel in culture, Glawischnig et al. (2001, 2002) measured the isotopomer composition of amino acids, triglyceride and glucosyl units from starch hydrolysis. Eisenreich et al. (2004) improved the method for the determination of glucose isotopologue abundances, which together with certain assumptions allowed the estimation of the relative contributions of glycolysis, oxidative pentose-P pathway (OPPP) and TCA cycle to glucose turnover in developing maize kernels (Ettenhuber et al., 2005). This approach 13C-starch was hydrolyzed and then relative fluxes through alternative hexose turnover routes was estimated by a retrobiosynthetic approach based on glucose isotopologues that were determined by quantitative 13C NMR and/or GC–MS. Ettenhuber et al. (2005) and Spielbauer et al. (2006) reported that glycolysis/gluconeogenesis and OPPP are the dominant metabolic processes participating in hexose recycling in developing maize kernels.

By contrast with previous seed flux analyses that have focused on storage in the embryo cotyledons (Schwender et al., 2003, 2004, 2006; Alonso et al., 2007a; Allen et al., 2009b), we have quantified the fluxes through primary metabolism in the developing endosperm during seed filling. The results reveal metabolic differences from other systems in terms of source of carbon assimilate, carbon conversion efficiency (CCE), the extent of futile cycling and suggest targets for future metabolic engineering. The present work also extends the scope of metabolic flux analysis to multiple tissue systems where the composition and labeling of substrates supplied to the tissue of interest are altered by another tissue.

2. Material and methods

2.1. Labeled isotopes

[U-13C]glucose (317 mCi/mmml, 11.7 GBq/mmml) was from Amersham Biosciences (Piscataway, NJ). [1-13C]glucose and [U-13C]glucose were from Isotec (Miamisburg, OH).

2.2. Plant and kernel culture

Maize plants (Zea mays L.) line LH59 were grown in 30-cm pots in a greenhouse maintained at 30–31 °C/20–21 °C day/night temperature and with supplemental lighting to provide irradiance of ~900–1400 μM m−2 s−1 and a 18:06-h day/night photoperiod. Plants were hand-pollinated and the ears were detached at 5 days after pollination (DAP). The ear was sprayed with isopropanol and flamed. After aseptically removing the inner husks and silks, as described by Ingenben and Jones (1994), cobs were sliced longitudinally into double rows, the white central pith was removed, and the cob strips were cut into 6 kernel segments. Five kernels were excised from each segment, leaving a cob:kernel ratio of 6:1, which is the standard ratio for in vitro cultures because kernel development is more uniform and similar to in planta (Felker, 1992). Explants were then incubated at 27 °C in the dark in sterile plant cell culture tray (Phytatray™ from Sigma-Aldrich, St Louis, MO) as previously described by Glawischnig et al. (2000) in sterile-filtered medium containing: glucose 80 g L−1, Murashige and Skoog basal salts 43.3 g L−1; thiamine hydrochloride 400 μg L−1; gentamycin 20 mg L−1 and 10 streptomycin sulphate mg L−1 [pH 5.8].

2.3. Apoplastic metabolite trapping experiments

Trapping experiments were performed on maize kernel cultures as previously described by Porter et al. (1985, 1987) under sterile conditions. Briefly, the distal (top) half of several kernels was cut off and the remaining endosperm was removed from the kernel base. Altered kernels were immediately rinsed with deionized water after removal of the basal transfer cells. The excess water was removed from the cup with a micropipette. The pedicel cups were filled for 10 min with 70 μL potassium phosphate 20 mM (pH 7.0) which was then replaced with 70 μL of warm (38 °C) 1% agar prepared in potassium phosphate 20 mM (pH 7.0). It quickly solidified, forming a trap in contact with the pedicel cells. Sugars and amino acids unloaded from the pedicel tissue were allowed to accumulate in the solidified agar trap for 1–4 h. The agar was then removed from the cup and placed in 15 mL of methanol:chloroform:water (MCW, 13:4:3, v/v/v) and stored in MCW at 4 °C.

Immediately after culture, the flasks were placed in an ice bath and evaporotation at 40 °C was used for 5DAP to 10DAP as described above. Two pieces of cob with or without kernels, were then placed on 2 × 2 cm2 florists’ foam soaked with 10 mL of medium containing [U-13C]glucose and cultured for 7 days at 27 °C in the dark in a 250 mL screw-cap Erlenmeyer flask sealed with a septum closure. Immediately after culture, the flasks were placed in an ice bath and 1 mL 0.2 N HCl was injected through the septum to stop metabolism and to release inorganic carbon. The CO2 was trapped as previously described by Goffman et al. (2005) and 14CO2 in the trapping solution was counted. The kernels were removed from the
cob and rinsed three times each with 10 mL water. Kernels were frozen in liquid nitrogen and dissected on dry ice to isolate the endosperm tissue which was then lyophilized.

2.5. Determination of O2 consumption and CO2 production

The CO2 produced and O2 consumed specifically by the endosperm were estimated by incubating pieces of cob with or without kernels. Kernel precultures were set as described above (Section 2.4). Immediately after culture, the flasks were placed in an ice bath and 1 mL 0.2 N hydrochloric acid was injected through the septum to stop metabolism and to release inorganic carbon to the flask headspace. Duplicate 200 μL gas samples were withdrawn from the flask headspace using a 1 mL syringe, and injected into an InfraRed Gas Analyzer (IRGA) to determine total CO2 production and a paramagnetic oxygen analyzer to measure total O2 consumption as previously described (Goffman et al., 2005; Allen et al., 2009b).

2.6. Metabolite extraction from cob tissue

The cob tissue from maize kernel cultures was rinsed with deionized water, frozen in liquid nitrogen, lyophilized and finely ground. 100 mg of cob powder were extracted using boiling aqueous ethanol as described by Salom et al. (1988). Ethanol was removed from extracts by evaporation under nitrogen flow and then water by sublimation using a lyophilizer.

2.7. Separation and determination of biomass compounds

Oil, protein, starch and cell wall were extracted from dissected endosperm tissue as described by Goffman et al. (2005) and Alonso et al. (2007a) with the following modifications: (i) soluble components with 1 mL methanol 70%, repeated once; (ii) zein protein extraction was performed with 1 mL ethanol 80%, repeated once and then 1 mL deionised water; (iii) starch extraction and analysis was performed using a Total Starch Assay Kit, ß-amylosel/amyloligosidase method from Megazyme International Ireland LTD. Oil content was determined by gas chromatography (GC) with flame ionization detection of fatty acid methyl esters (FAMES) as described by Goffman et al. (2005).

2.8. HPLC analyses

For HPLC analyses, soluble components extracted from endosperms and agar traps were separated into neutral, acidic and cationic fractions containing sugars, organic acids and amino acids respectively as described by Salom et al. (1988). Free sugars were quantified by electrospray LC-MS/MS using multiple ion monitoring with a Waters Micromass Quattro Ultima system and MassLynx 4.0 software. Samples were dissolved in 80% methanol. LC separation was with a Shodex Asahipack NH2P-54B 4 x 50 mm column with mobile phases A: 90% acetonitrile/10% H2O/0.1% NH4OH and B: 0.1% NH4OH. Run time was 6.3 min at 1.5 mL/min with gradients of: 100% A, 0 min; 90% A, 1.7 min; 75% A, 4 min; 20% A, 4.1 min; 20% A, 5.1 min; 100% A, 5.2 min; 10% A, 6.3 min. Free amino acids and amino acids from protein hydrolysis were derivatized with o-phthalaldehyde, separated on a reverse phase system and quantified as described by Schuster (1988).

2.9. NMR analyses

Soluble components extracted from endosperm, agar traps and cob were fractionated as above. Spectra were performed at 25 °C with a Varian (Palo Alto, CA) 500 MHz spectrometer equipped with a 5-mm probe. The oil extracts were resuspended in 600 μL deuterochloroform (CDCl3) and amino acid, organic acid, sugar and starch fractions in deuterium oxide (D2O). 1H NMR spectra were obtained at 499.74 MHz with 90° pulses using a recycling time > 6T1. 13C NMR spectra were obtained at 125.66 MHz with 90° pulses using a recycling time > 6T1. The absolute 13C enrichments were determined as previously described (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002).

2.10. GC–MS analyses of protein labeling

Proteins extracted from endosperm tissue were hydrolyzed in 6 N HCl and the amino acids were derivatized to N0-tert-butyldimethylsilyl (TBDMs) derivatives as described previously (Schwender et al., 2003). TBDMs-amino acids were analyzed by GC–MS using Agilent Technologies (Santa Clara, CA) 5973 N inert mass selective detector instruments, an Agilent Technologies G2578A system with a 30 m × 0.25 mm HP5 column and an Agilent Technologies G2589A system with a 60 m × 0.25 mm DB5MS column. The carrier gas was helium at a flow rate of 1 mL min−1. The relative abundances of mass isotopomers in selected fragments of each analyzed derivative were measured using selected ion monitoring followed by correction for naturally occurring heavy isotopes as previously described (Schwender et al., 2003).

2.11. Modeling metabolic pathways

The metabolic model (Fig. 4) was implemented using the 13C-FLUX software (Wiechert et al., 2001) and is described in Supplement 6. In order to include data from the two separate experiments with 100% [1-13C]glucose and 20% [U-13C]glucose into a single flux estimation procedure each flux and metabolite pool was programmed twice (using subscript U for the [U-13C]glucose experiment). Equality of identical fluxes in the separate metabolic networks thus defined was set by using equality equations. The labeling of assimilate unloaded from the maternal tissue (glucose, fructose and glutamine) was found to be the same as the labeling of free sucrose and free glutamine from the cob tissue (Table 1) and entered as labeling input in the model. The rates of substrate uptake by the endosperm (Vg, Vf and Va) were estimated by difference between the rates of assimilates unloaded from the maternal tissue (Fig. 3) and the rates of substrate uptake by the embryo determined independently. For this purpose, maize embryos were dissected aseptically at 15DAP and cultivated for 7 days in septum-sealed flasks with radiolabeled substrates (200 mM glucose, 200 mM fructose, 40 mM glutamine) and 10% polyethyleneglycol, in the dark at 25 °C. The rates of substrate uptake were calculated according to total radioactivity incorporated by the maize embryos plus the 14CO2 released in the headspace. The following net fluxes Vg, VvH, VvF, VdF, VdS, Vpp3, Vm, Vpepc, Vwall and Vsta (Table 3) were set as free fluxes in the software as well as the fluxes through amino acid synthesis. The exchange fluxes named Vgf, Vald, Vhcp, Vppp2, Vppp3, Vppp4 and Vfum1 (Table 3) were set as free fluxes in the software as well as the fluxes through amino acid synthesis. The exchange fluxes named Vgf, VdF, Vppp2, Vppp3, Vppp4 and Vfum1 (Table 3) were set as free fluxes in the software as well as the fluxes through amino acid synthesis. The exchange fluxes named Vgf, Vald, Vhcp, Vppp2, Vppp3, Vppp4 and Vfum1 (Table 3) were set as free fluxes in the software as well as the fluxes through amino acid synthesis.

The results are presented in Fig. 4 and Table 3.
3. Results

3.1. Maize endosperm growth in culture

Kernels from maize line LH59 cultured with cob tissue develop normally (Felker, 1992; Glawischnig et al., 2000; Spielbauer et al., 2006) with the endosperm fresh weight accumulation being the same as in planta (Fig. 1a) and slowing after 24 days after pollination (DAP). However, we found that the dry weight of the endosperm from kernel cultures increased linearly until at least 32DAP (R²=0.9847, data not shown). Biomass composition was calculated by measuring biomass compounds in maize endosperm from kernel cultures and in planta at 28DAP (Fig. 1b). Starch, protein, oil and cell wall represent, respectively, 70.2 ± 0.8%, 10.3 ± 0.1%, 1.2 ± 0.0% and 18.3 ± 0.3% of the endosperm dry weight at the end of the culture period (n=3), which is comparable to 28DAP endosperm in planta (72.0 ± 0.1%, 11.1 ± 0.2%, 0.8 ± 0.0% and 16.1 ± 0.3%, respectively; n=3). Together these findings indicate that the culture conditions provide a good model for endosperm metabolism in planta.

3.2. Carbon conversion efficiency of endosperm tissue

Carbon balancing is used in order to estimate the efficiency with which substrates are transformed into biomass products in microorganisms (Novák and Loubiere, 2000; Converti and Perego, 2002; Sáez et al., 2002) as well as in plants (Flinn et al., 1977; Pate et al., 1977; Peoples et al., 1985; Schwender et al., 2004; Goffman et al., 2005; Alonso et al., 2007a, 2010). Kernel cultures were grown for five days with unlabeled glucose and then for seven days with [U-14C]glucose. Label incorporation into biomass pools (starch, cell wall, oil and protein) was determined for the endosperm tissue. The amount of 14CO2 released was estimated by the difference between 14C02 produced by the whole kernel culture and by cob tissue alone. Fig. 2 shows the partitioning of the 14C carbon after labeling with [U-14C]glucose for seven days (from 10 to 17DAP). This shows that 45.4 ± 2.8%, 13.7 ± 2.9%, 2.4 ± 0.2% and 2.3 ± 0.2% of the 14C was incorporated into starch, cell walls, oil and proteins, respectively, which represents a CCE of between 76% and 92% (if we consider the methanol soluble fraction as a part of biomass). This CCE is high for a non-photosynthetic tissue. In comparison, the CCE was found to be 50% for developing sunflower embryos (Alonso et al., 2007a).

3.3. Metabolic fluxes in developing maize endosperm

3.3.1. The cob tissue furnishes substrates to the endosperm and modifies their labeling

In order to determine metabolic fluxes in maize endosperm tissue it is necessary to know the nature of the substrates received by this tissue, their uptake rates and their labeling pattern. For this purpose, maize ears were harvested and dissected at 5DAP and maize kernels attached to cob pieces were cultured from 5 to 19DAP using glucose as sole source of carbon. We used the technique of the empty seed coat, commonly used to analyze the photosynthates released from maternal seed tissues in corn (Porter et al., 1985, 1987), in which the top of the kernel was cut off and the embryo (germ) and endosperm tissues were removed leaving a cup which was filled with a drop of agar for 1, 2 or 4 h. HPLC analyses of the water soluble metabolites trapped showed that glucose and fructose are the main sugars exported by the pedicel whereas glutamine represents more than 50% of the amino acids exported (Supplemental Tables 1 and 2). These findings are in agreement with previous studies performed in planta (Porter et al., 1985, 1987; LYZnik et al., 1985) and confirm that developing endosperm receives the same substrates in culture as in planta. The different trapping times allowed us to verify that the method accurately quantifies the rates of delivery of these three compounds into the apoplasm by verifying that transfer is linear (Fig. 3). Since glucose is provided as sole carbon source in the culture medium, fructose and glutamine imported by the endosperm must be made by the cob tissue.

Maize ears were harvested at 5DAP, maize kernels attached to cob segments were precultured for 5 days with glucose as sole carbon source and then grown for 10, 14, 18 or 22 days with 100% [1-13C]glucose. 4 h trapping experiments were performed to determine the labeling in substrates imported by the endosperm. The labeling in metabolites extracted from cob and agar trap were determined by 1H and 13C NMR (Table 1). The labeling of trapped glutamine in the apoplastic was not significantly different from the free glutamine present in the cob (χ² test, P<0.05), showing that the glutamine received by the endosperm is synthesized in the cob tissue. During the labeling time course, the enrichments of trapped apoplastic glucose were significantly different from free glucose but not from the sucrose glucosyl unit of the cob (χ² test, P<0.05). Sucrose is exported from the cob, cleaved into glucose and fructose that are then imported by the endosperm. Thus the cob is metabolically active and the labeling in metabolites it supplies

### Table 1

<table>
<thead>
<tr>
<th>Labeling in % enrichment</th>
<th>20DAP – 10d labeling</th>
<th>24DAP – 14d labeling</th>
<th>28DAP – 18d labeling</th>
<th>32DAP – 22d labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (as C1)</td>
<td>TRAP</td>
<td>COB</td>
<td>TRAP</td>
<td>COB</td>
</tr>
<tr>
<td>C1</td>
<td>41.6 ± 0.3</td>
<td>42.9 ± 0.8</td>
<td>50.2 ± 0.7</td>
<td>47.4 ± 0.8</td>
</tr>
<tr>
<td>C6</td>
<td>9.8 ± 0.2</td>
<td>3.7 ± 0.2</td>
<td>12.1 ± 0.6</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Sucrose (as C6)</td>
<td>COB</td>
<td>COB</td>
<td>COB</td>
<td>COB</td>
</tr>
<tr>
<td>Glucosyl C1</td>
<td>ud</td>
<td>45.6 ± 0.3</td>
<td>ud</td>
<td>51.9 ± 0.2</td>
</tr>
<tr>
<td>Glucosyl C6</td>
<td>ud</td>
<td>11.2 ± 0.2</td>
<td>ud</td>
<td>13.1 ± 0.1</td>
</tr>
<tr>
<td>Fructosyl C1</td>
<td>ud</td>
<td>44.7 ± 0.2</td>
<td>ud</td>
<td>50.6 ± 0.1</td>
</tr>
<tr>
<td>Fructosyl C6</td>
<td>ud</td>
<td>11.1 ± 0.2</td>
<td>ud</td>
<td>13.1 ± 0.0</td>
</tr>
<tr>
<td>Glutamine (as C2)</td>
<td>C2</td>
<td>20.1 ± 0.6</td>
<td>18.9 ± 0.9</td>
<td>nd</td>
</tr>
<tr>
<td>C3</td>
<td>22.2 ± 0.4</td>
<td>21.9 ± 0.5</td>
<td>nd</td>
<td>24.4 ± 0.5</td>
</tr>
<tr>
<td>C4</td>
<td>25.9 ± 0.5</td>
<td>24.8 ± 0.9</td>
<td>nd</td>
<td>28.6 ± 1.4</td>
</tr>
</tbody>
</table>

Maize ears were harvested at 5DAP. Maize kernels with attached cob pieces were precultured for 5 days in media with unlabeled glucose as the sole carbon source and then transferred for 10, 14, 18 or 22 days into 100% [1-13C]glucose media. Trapping experiments consisting in 4 h of trapping in agar plugs were performed as previously described by Porter et al. (1985). The labeling in metabolites extracted from cob and agar trap were determined by 1H and 13C NMR as described by Alonso et al. (2007a). The values reported here are percentages of labeling for a specified carbon ± error calculated according to the signal to noise ratio in the NMR spectra. “nd”, non-determined; “ud”, undetectable.
to the developing kernels is substantially different from the labeling in substrates provided in the medium.

3.3.2. Biomass accumulation in maize endosperm

The rates of starch and oil synthesis in developing maize endosperm were estimated by measuring the biomass accumulated at 20DAP. The rate of cell wall synthesis was calculated according to the percent of w/w (biomass composition). Starch and cell walls have a rate of synthesis of 412 ± 7 nmol of glucose equivalents h⁻¹ endosperm⁻¹ (n = 3), and oil synthesis occurs at a rate of 51 ± 0 nmol equivalent acetyl-CoA h⁻¹ endosperm⁻¹ (n = 3). Amino acid composition of proteins was determined by HPLC after extraction and hydrolysis of proteins from maize endosperm at 20DAP.

3.3.3. Isotopic steady state

To ensure that the fractional enrichment labeling data can be correctly interpreted as representing steady state fluxes, conditions of metabolic and isotopic steady state must hold. We performed [1-¹³C]glucose labeling time course experiments to determine the time taken to reach isotopic steady state in maize endosperm tissue. The results presented in Table 2 indicate that labeling in metabolites of maize endosperm are stable after 18 days of labeling (Tukey’s studentized range test, P < 0.05).

In two separate experiments, developing endosperms were labeled with 100% [1-¹³C]glucose, or 20% [U-¹³C]glucose for 18 days. Extracted, oil, starch, free sugars and amino acids were analyzed by ¹H NMR and ¹³C NMR as described by Alonso et al. (2007a). Proteins extracts were hydrolyzed, derivatized and analyzed by GC–MS as described by Schwender et al. (2003). NMR and GC–MS data are in Supplemental Table 3 and 4, respectively.

3.3.4. Absence of diluting sugar pools in endosperm

Plant cells frequently contain internal pools of sugars and other metabolites which are relatively inactive metabolically. A large unlabeled pool of sugars present at the start of the labeling experiment can complicate the interpretation of the labeling in metabolic flux analysis (Alonso et al., 2005, 2007b, 2007c).

To quantify the impact of any unlabeled pools of sugars, kernel cultures were labeled for 18 days with 100% [U-¹³C]glucose. The substrates received by the endosperm were collected in a 4 h trapping experiment. The percentage enrichment of the C1 of glucose in the trap, glucose and starch in the endosperm were determined by ¹H NMR and were found to be not significantly different (76.2%, 74.8% and 76.7%, respectively). Thus there were no

Fig. 1. Endosperm in culture. (a) Fresh weight accumulation. Increase of fresh weight of endosperm tissue isolated from kernels cultured in sterile conditions (solid circles) is compared to endosperm tissue isolated from kernels grown in planta (empty circles). Each point is the average ± standard error of 34 independent measurements. (b) Biomass composition. Biomass composition (% w/w) of the endosperm tissue dissected from kernels cultures at 28DAP (white bars) is compared to endosperm tissue dissected from 28DAP kernels grown in planta (black bars). Error bars represent standard error of three measurements.
detectable effects on labeling patterns due to diluting pools of sugars in maize endosperm.

3.3.5. Building a model of central metabolism

The metabolic network was defined from information in the literature on maize kernels (Nelson and Pan, 1995; Denyer et al., 1996; Glawischning et al., 2001, 2002; Ettenhuber et al., 2005; Spielbauer et al., 2006) and other heterotrophic tissues (Rontein et al., 2002; Dieuaide-Noubhani et al., 1995; Alonso et al., 2005, 2007a, 2007b, 2007c) and from the labeling data (Supplemental Table 3 and 4). Labeling experiments with 100% [1-13C]glucose and from the labeling data (Supplemental Table 3 and 4), (ii) the reactions describing carbon metabolism (one subnetwork is presented in Supplemental Table 3 and 4), (iii) rates of glucose, fructose and glutamine uptake determined as described in Section 2, and (iv) rates of accumulation for each biomass component. Data from the two separate experiments were included in the flux estimation procedure as described previously (Schwender et al., 2006; Alonso et al., 2007a). 13C-FLUX® evaluates a set of stoichiometrically feasible fluxes that best account for the measured data. The flux optimization was repeated (Monte Carlo optimization) over 300 times from different starting points and over 68% of the optimization runs converged to a very similar set of best fit flux values. Single parameter 90% confidence intervals were calculated as described by Wiechert et al. (1997). The resulting flux map and the values for net and exchange fluxes are shown in Fig. 4 and Table 3.

3.3.6. Validations

The model calculates a CO2 production rate of 558 ± 44 nmol of CO2 h−1 endosperm−1, which is not significantly different at the P < 0.05 level (Tukey's studentized range test) from the CO2 production directly measured by gas analysis (529 ± 179 nmol CO2 h−1 endosperm−1, n = 3). The agreement between calculated and measured CO2 release supports the validity of the empty seed coat experimental approach (Fig. 2) since the rates of metabolite trapping were used as the rates of substrate input into endosperm, and the measured growth and composition rates as the metabolite output rates (to biomass products).
Supplemental Fig. 1 shows simulated versus measured labeling data. The fact that this distribution is close to a 1:1 linear relationship indicates that the mathematical flux model adequately describes the experimentally observed data. The validity of the modeling results was further tested by comparing the rate of O₂ consumption predicted by the model to the experimentally determined value. The flux through reactions producing NADH in the cytosol and mitochondria was estimated by the flux modeling to produce between 652 and 928 nmol of NADH h⁻¹ endosperm⁻¹ depending on glyceraldehyde 3-P dehydrogenase localization. Since two moles of NADH are required to reduce one mole of O₂, the rate of oxygen consumption estimated by the model is 326–499 nmol O₂ h⁻¹ endosperm⁻¹, which is not significantly different at the P < 0.05 level (Tukey’s studentized range test) from the rate of O₂ consumption determined experimentally (523 ± 182 nmol O₂ h⁻¹ endosperm⁻¹, n = 3).

### 3.4. Metabolism in maize endosperm

#### 3.4.1. Precursors for biomass biosynthesis

Mathematical models based on the inclusion of a partial or entire cytosolic pentose-P pathway simulated no fluxes through this pathway, and our labeling data does not support the occurrence of the OPPP in the cytosol (Section 3.3.5). We concluded that the OPPP was localized in the plastid and thus may furnish the redundant used for oil production. The calculated OPPP flux can produce 174 ± 58 nmol NADPH h⁻¹ endosperm⁻¹ whereas the measured rate of fatty acid synthesis required 51 ± 0 nmol NADPH h⁻¹ endosperm⁻¹ (Fig. 4; Table 3). Thus the OPPP generates enough NADPH for fatty acid production without the need for flux through plastidic NADP-dependent malic enzyme. Furthermore, the calculated NADH synthetic flux from plastidic pyruvate dehydrogenase (65 ± 1 nmol NADH h⁻¹ endosperm⁻¹) was also greater than the 51 ± 0 nmol NADH h⁻¹ endosperm⁻¹ required to support fatty acid synthesis without considering other sources of NADH, such as glycolysis (Fig. 4; Table 3). Also, 91–95% of the carbon for fatty acid synthesis comes from triose-P produced from hexoses imported into the plastid and only 5–9% from the import of malate (Fig. 4; Table 3). Thus malate does not appear to be a major source of carbon or of reductant for plastidic fatty acid synthesis during maize endosperm development. The mathematical simulation of a net uptake of pyruvate into the plastid was also not supported by our labeling measurements, alanine and acetyl-CoA units having significantly different labeling (Supplemental Table 3).

#### 3.4.2. Relatively high fluxes through substrate cycles

The rate of oxygen consumption was estimated by the model to be between 326 and 499 nmol O₂ h⁻¹ endosperm⁻¹. Assuming that oxygen is consumed to produce ATP equivalents per hour per endosperm via oxidative phosphorylation, and assuming a maximum feasible rate of substrate-level phosphorylation (Garret and Grisham, 1999; Rontein et al., 2002; Alonso et al., 2005, 2007a; Allen et al., 2009b), we estimate that maximum ATP production from catabolism of sugars to be between 1956 and 2994 nmol ATP h⁻¹ endosperm⁻¹.

Similar to our findings with developing sunflower embryos (Alonso et al., 2007a), triose-P/hexose-P cycling occurs between the cytosol and the plastid compartments in maize endosperm (Fig. 4) and consumes a maximum of 221 nmol ATP h⁻¹ endosperm⁻¹ depending on the enzymes involved in the conversion of fructose 1,6-bisphosphate to fructose 6-P. The equivalent of one ATP is consumed if this reaction is catalyzed by fructose 1,6-bisphosphatase but none if it is the PPI dependent phosphofructokinase. Thus, the maximum consumption of ATP by triose-P/hexose-P cycle is 11% of the ATP produced. The synthesis and breakdown of sucrose and/or glucose 6-P phosphatase activity cannot be distinguished because of the labeling and other data used to determine the fluxes but were considered in our model and named Vres (fluxes from glucose 6-P and glucose; Alonso et al., 2005). In the case of glucose 6-P phosphatase activity, a single ATP equivalent is used by the cycling whereas sucrose synthesis by sucrose-P synthase and degradation by invertebrate requires two ATP if we consider that PPI is a donor of phosphate like ATP (Hill and ap Rees, 1994). Thus, hexose-P/glucose cycling consumes between 353 and 706 nmol ATP h⁻¹ endosperm⁻¹, which represents 18–36% of the ATP produced. Therefore, maize endosperm expends between 18% and 47% of ATP produced in substrate cycles.

#### 3.4.3. ATP required for biomass synthesis in developing maize endosperm

Fatty acid, starch, and cell wall syntheses require one mole of ATP per mole of acetyl-CoA, ADP-glucose and UDP-glucose added respectively, while 4.3 moles of ATP equivalents are necessary per amino acid for protein synthesis (Schwender et al., 2006). Thus, fatty acid, starch, protein and cell wall syntheses consume, respectively,
transported from the leaves through the phloem and then unloaded into the cob tissue. Several studies have shown that glucose or fructose also support in vitro culture of maize kernels (Cobb and Hannah, 1986; Cheng and Chourey, 1999; Glawischnig et al., 2002). Previous labeled studies on maize kernel cultures using either [U-13C]glucose or [U-13C]sucrose have shown no significant difference in growth or isotopologue distribution in glucose units from starch hydrolysis (Spielbauer et al., 2006). In our culture conditions, the fresh weight accumulation, biomass composition and nature of substrates were not significantly different from the ones determined in planta (Fig. 1, Supplemental Tables 1 and 2; Porter et al., 1985, 1987). Together these findings indicate that the culture conditions provide a reasonable mimic of endosperm metabolism in planta (Spielbauer et al., 2006).

### Table 3

<table>
<thead>
<tr>
<th>Flux name</th>
<th>Flux or rate description</th>
<th>Flux values nmol h⁻¹ endosperm⁻¹</th>
<th>Exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vg</td>
<td>Rate of glucose uptake</td>
<td>203 ± 47n</td>
<td>0%</td>
</tr>
<tr>
<td>Vf</td>
<td>Rate of fructose uptake</td>
<td>495 ± 47n</td>
<td>0%</td>
</tr>
<tr>
<td>Vv</td>
<td>Rate of glutamine uptake</td>
<td>12 ± 2n</td>
<td>0%</td>
</tr>
<tr>
<td>Vh1k</td>
<td>Flux through hexokinase from glucose to G6P</td>
<td>555 ± 69°</td>
<td>0%</td>
</tr>
<tr>
<td>Vh2k</td>
<td>Flux through hexokinase from fructose to F6P</td>
<td>495 ± 47°</td>
<td>0%</td>
</tr>
<tr>
<td>Vres</td>
<td>Flux from G6P to glucose</td>
<td>353 ± 47°</td>
<td>0%</td>
</tr>
<tr>
<td>Vgg</td>
<td>Fluxes through G6P isomerase</td>
<td>-716 ± 90°</td>
<td>0%</td>
</tr>
<tr>
<td>Vald</td>
<td>Fluxes through cytosolic aldolase</td>
<td>-221 ± 70°</td>
<td>0% ± 75%</td>
</tr>
<tr>
<td>Vglyco</td>
<td>Glycolytic flux</td>
<td>36 ± 13°</td>
<td>0%</td>
</tr>
<tr>
<td>Vfns2</td>
<td>Rate of glycerol incorporation into TAG</td>
<td>2 ± 0°</td>
<td>0%</td>
</tr>
<tr>
<td>Vpk</td>
<td>Flux through pyruvate kinase</td>
<td>155 ± 10°</td>
<td>0%</td>
</tr>
<tr>
<td>Vpyp</td>
<td>Flux of pyruvate from cytosol to mitochondria</td>
<td>137 ± 10°</td>
<td>0%</td>
</tr>
<tr>
<td>Vakg</td>
<td>Flux of glutamate to α-ketoglutarate</td>
<td>-28 ± 3°</td>
<td>0%</td>
</tr>
<tr>
<td>Vhcpp</td>
<td>Exchange of cytosolic and plastidic hexose-P</td>
<td>465 ± 70°</td>
<td>884 ± 42°</td>
</tr>
<tr>
<td>Vsts</td>
<td>Starch synthesis from cytosolic hexose-P</td>
<td>366 ± 5°</td>
<td>0%</td>
</tr>
<tr>
<td>Vstsp</td>
<td>Starch synthesis from plastidic hexose-P</td>
<td>41 ± 1°</td>
<td>0%</td>
</tr>
<tr>
<td>Vvaldp</td>
<td>Flux through plastidic aldolase</td>
<td>391 ± 71°</td>
<td>0%</td>
</tr>
<tr>
<td>Vvpk</td>
<td>Flux through plastidic pyruvate kinase</td>
<td>104 ± 3°</td>
<td>0%</td>
</tr>
<tr>
<td>Vpdhp</td>
<td>Flux through plastidic pyruvate DH</td>
<td>65 ± 1°</td>
<td>0%</td>
</tr>
<tr>
<td>Vfas1</td>
<td>Flux of AcCoA to fatty acid synthesis</td>
<td>51 ± 0°</td>
<td>0%</td>
</tr>
<tr>
<td>Vnep</td>
<td>Flux through plastidic malic enzyme</td>
<td>8 ± 2°</td>
<td>0%</td>
</tr>
<tr>
<td>Vpp1</td>
<td>Flux of the oxidative part of the pentose-P pathway</td>
<td>87 ± 29°</td>
<td>0% ± 24°</td>
</tr>
<tr>
<td>Vppp2</td>
<td>Fluxes through transketolase</td>
<td>30 ± 1°</td>
<td>132 ± 15°</td>
</tr>
<tr>
<td>Vppp3</td>
<td>Fluxes through transaldolase</td>
<td>30 ± 1°</td>
<td>0%</td>
</tr>
<tr>
<td>Vppp4</td>
<td>Fluxes through transketolase</td>
<td>23 ± 10°</td>
<td>1289 ± 64°</td>
</tr>
<tr>
<td>Vpdh</td>
<td>Flux through pyruvate DH</td>
<td>149 ± 13°</td>
<td>0%</td>
</tr>
<tr>
<td>Vcs</td>
<td>Flux through citrate synthase</td>
<td>146 ± 13°</td>
<td>0%</td>
</tr>
<tr>
<td>Vca</td>
<td>Flux through α-citrate synthase</td>
<td>146 ± 13°</td>
<td>0%</td>
</tr>
<tr>
<td>Vfjo</td>
<td>Flux through 2-oxoglutarate DH</td>
<td>115 ± 14°</td>
<td>0%</td>
</tr>
<tr>
<td>Vfum</td>
<td>Fluxes through fumarase</td>
<td>114 ± 14°</td>
<td>520 ± 200°</td>
</tr>
<tr>
<td>Vme</td>
<td>Flux through malic enzyme</td>
<td>12 ± 10°</td>
<td>0%</td>
</tr>
<tr>
<td>Vppec</td>
<td>Anaplerotic flux through PEPC</td>
<td>73 ± 10°</td>
<td>0%</td>
</tr>
<tr>
<td>Vcs2</td>
<td>Rate of CO₂ production</td>
<td>58 ± 10°</td>
<td>0%</td>
</tr>
<tr>
<td>Vwall</td>
<td>Rate of wall synthesis</td>
<td>88 ± 8°</td>
<td>0%</td>
</tr>
<tr>
<td>Vsta</td>
<td>Total starch synthesis</td>
<td>407 ± 6°</td>
<td>0%</td>
</tr>
<tr>
<td>VpSpeff</td>
<td>Flux of P5P to amino acids for protein synthesis</td>
<td>2 ± 0°</td>
<td>0%</td>
</tr>
<tr>
<td>VpPeff</td>
<td>Flux of TP to amino acids for protein synthesis</td>
<td>15 ± 1°</td>
<td>0%</td>
</tr>
<tr>
<td>VpepEff</td>
<td>Flux of PEP to amino acids for protein synthesis</td>
<td>14 ± 1°</td>
<td>0%</td>
</tr>
<tr>
<td>Vep4peff</td>
<td>Flux of E4P to amino acids for protein synthesis</td>
<td>7 ± 0°</td>
<td>0%</td>
</tr>
<tr>
<td>Vperypeff</td>
<td>Flux of PYR to amino acids for protein synthesis</td>
<td>47 ± 2°</td>
<td>0%</td>
</tr>
<tr>
<td>VAcCoApef</td>
<td>Flux of AcCoA to amino acids for protein synthesis</td>
<td>14 ± 1°</td>
<td>0%</td>
</tr>
<tr>
<td>VpYRCpef</td>
<td>Flux of PYRc to amino acids for protein synthesis</td>
<td>18 ± 1°</td>
<td>0%</td>
</tr>
<tr>
<td>Vaoaef</td>
<td>Flux of OAA to amino acids for protein synthesis</td>
<td>29 ± 1°</td>
<td>0%</td>
</tr>
<tr>
<td>Vgheff</td>
<td>Flux of GLUT to amino acids for protein synthesis</td>
<td>43 ± 2°</td>
<td>0%</td>
</tr>
</tbody>
</table>

Fluxes are nmol h⁻¹ endosperm⁻¹. The rates of glucose (Vg), fructose (Vf) and glutamine (Va) uptake were determined as described in Section 2. The rates of starch (Vsta), cell wall (Vwall), fatty acids synthesis (Vfas1 and Vfas2) were quantified by measuring their accumulation during the incubation period. All these fluxes values and their SDs were reported in the model but set as free for the fitting process. The flux for glucose resynthesis from hexasaccharides (Vres) was calculated using Eq. (4) [Vres = Vg(Gex1/Sg6–G6mG1+S6mG6–Sg6)/(G6mG1+S1+Sg1+S6mG6) + Vstsp]. G6mG1+S6mG6 represent the enrichment of the carbon 1 of extracellular glucose, the glucosyl moiety of sucrose and intracellular glucose, respectively and G6mG1+S6mG6 represent the enrichments of carbon 6 in the same molecules) in Alonso et al. (2005) and then entered as a measured flux in the model. Starch synthesis was constrained to come 90% from cytosolic hexose-P (Vsts) and 10% from plastidic hexose-P (Vstsp). The fluxes for amino acid synthesis were estimated by measuring amino acid accumulation in stored proteins during the incubation period and reported as measured values in the model. The other flux values were calculated from the model based upon carbon enrichment measured after steady-state labeling with 100% [1-13C]glucose or 20% [U-13C]glucose. The values are the best fit and also the most frequently determined optimized flux values ± 90% confidence range. "m" experimentally measured; "cs; ct; cl" constrained according to 13C-FLUX software, thermodynamics and literature (http://pmn.plantcyc.org), and labeling information, respectively. "e" estimated by label modeling.

51, 407, 813 and 88 nmol ATP h⁻¹ endosperm⁻¹. Consequently, about 69% of the ATP produced is used for biomass synthesis.

### 4. Discussion

#### 4.1. Culture conditions and endosperm assimilates supplied by the cob tissue

Maize kernel cultures were grown using glucose as the carbon source, which allows differently labeled forms to be used. The natural precursor would have been photo-assimilated sucrose, transported from the leaves through the phloem and then unloaded into the cob tissue. Several studies have shown that glucose or fructose also support in vitro culture of maize kernels (Cobb and Hannah, 1986; Cheng and Chourey, 1999; Glawischnig et al., 2002). Previous labeled studies on maize kernel cultures using either [U-13C]glucose or [U-13C]sucrose have shown no significant difference in growth or isotopologue distribution in glucose units from starch hydrolysis (Spielbauer et al., 2006). In our culture conditions, the fresh weight accumulation, biomass composition and nature of substrates were not significantly different from the ones determined in planta (Fig. 1, Supplemental Tables 1 and 2; Porter et al., 1985, 1987). Together these findings indicate that the culture conditions provide a reasonable mimic of endosperm metabolism in planta (Spielbauer et al., 2006).
We confirmed that glucose and fructose are the main sugars imported by the endosperm and embryo tissues (Supplemental Table 1; Porter et al., 1985). Amino acids are also unloaded from the maternal tissue, glutamine representing more than 50% of the total (Supplemental Table 2), which is in agreement with previous studies (Lyznik et al., 1985; Porter et al., 1987). Kernel culturing experiments performed with [1-13C]glucose revealed that free glutamine and the glucosyl moiety of sucrose present in the cob tissue contain the same labeling patterns as the glucose and glutamine imported by the endosperm (captured in the apoplastic agar trap, Table 1). These results show that cob tissue actively modifies the C and N substrates supplied to the developing endosperm, as concluded for N by Seebauer et al. (2004) who found that cob tissue has substantial amino acid metabolizing capabilities. This may explain why previous attempts to reduce the cob:kernel ratio in maize kernel cultures were unsuccessful and underlines the active role of cob tissue in absorption of compounds into endosperm (Felker, 1992). The differences measured in the labeling and composition of metabolites in the kernel apoplast (Table 1) compared to the culture medium show that the cob tissue actively modifies the labeling of substrates supplied to the endosperm (Felker, 1992). The differences measured in the labeling and composition of metabolites in the kernel apoplast (Table 1) compared to the culture medium show that the cob tissue actively modifies the labeling of substrates supplied to the endosperm. Failure to account for this in previous studies means that at least some conclusions made about endosperm metabolism are likely to be wrong (Glawischnig et al., 2001, 2002; Ettenhuber et al., 2005; and Spielbauer et al., 2006). Indeed, the rearrangement of label from the forms supplied in culture to the forms found in starch glucose units were assumed to have taken place in the kernel. Our trapping experiments show that much of this rearrangement took place in the cob, before reaching the kernel (Supplements 1 and 2, Fig. 3 and Table 1). Thus the cob tissue assimilated the 13C-substrate provided in the media, metabolized it and furnished newly labeled substrates to the kernel. For instance, Table 1 showed that the labeling of glucose received by the endosperm (glucose from the trap), in comparison to one from the media (C1 = 99% and C6 = 1.1%), was lower in its carbon 1 (62.7% at 28DAP) and higher its carbon 6 (13.6% at 28DAP), which is representative of a glycolysis/gluconeogenesis in the cob. Furthermore the total enrichment C1+C6 of the glucose trapped at 28DAP (76.3%) was much lower than the one from the media (100%), which reveals the action of the OPPP in the cob tissue. These results all together show that omitting the real composition and labeling of the substrates would lead to an overestimation of the contribution of the glycolysis/gluconeogenesis and the OPPP in the endosperm, since actually part of these processes occurred in the cob tissue.

4.2. High carbon conversion efficiency in developing maize endosperm

Developing maize endosperms convert metabolic substrates into reserves with an efficiency of between 76% and 92% (if we consider the methanol soluble fraction as part of the biomass). This CCE is very high for a heterotrophic tissue, contrasting with an efficiency of 50% previously observed in developing sunflower embryos (Alonso et al., 2007a) and 57–71% in maize embryos (Alonso et al., 2010). From the stoichiometry of the biosynthetic pathways we can estimate the theoretical CCE for fatty acid, carbohydrate and protein syntheses to be 66%, > 95%, and around 80%, respectively (depending on amino acid composition and the nature of the precursors). Sunflower embryos which mainly accumulate fatty acids have a CCE of 50% whereas maize endosperm, which stores starch, has a much higher CCE. The CCE...
we determined for maize endosperm is comparable to that of green embryos of *Brassica napus* which have a CCE over 80% (Goffman et al., 2005). In green seeds the light reactions of photosynthesis can provide both ATP and reductant and allow the operation of a RubisCo bypass flux that leads to more efficient conversion of hexose to oil (Schwender et al., 2004; Goffman et al., 2005). Non-green sunflower embryos must produce ATP and reductant by metabolic processes that emit CO₂ thus lowering the CCE (Alonso et al., 2007a). Other predominantly carbohydrate accumulating heterotrophic tissues, albeit having lower total storage product accumulation levels, such as cultured maize root tips and tomato cells, have CCE values of close to 50% and 60%, respectively (Alonso et al., 2007b; Rontein et al., 2002).

4.3. Implications for engineering maize endosperm metabolism

As a major crop plant in many parts of the world, maize is an attractive target for metabolic engineering. One metabolic feature influencing yield is the efficiency of the metabolic network in converting substrates in the seed tissue to storage products. Estimating the metabolic fluxes involved in primary metabolism of developing maize endosperm, we determined for maize endosperm is comparable to that of green embryos of *Brassica napus* which have a CCE over 80% (Goffman et al., 2005). In green seeds the light reactions of photosynthesis can provide both ATP and reductant and allow the operation of a RubisCo bypass flux that leads to more efficient conversion of hexose to oil (Schwender et al., 2004; Goffman et al., 2005). Non-green sunflower embryos must produce ATP and reductant by metabolic processes that emit CO₂ thus lowering the CCE (Alonso et al., 2007a). Other predominantly carbohydrate accumulating heterotrophic tissues, albeit having lower total storage product accumulation levels, such as cultured maize root tips and tomato cells, have CCE values of close to 50% and 60%, respectively (Alonso et al., 2007b; Rontein et al., 2002).

4.3. Implications for engineering maize endosperm metabolism

As a major crop plant in many parts of the world, maize is an attractive target for metabolic engineering. One metabolic feature influencing yield is the efficiency of the metabolic network in converting substrates in the seed tissue to storage products. Estimates of futile cycling based on ¹³C labeling experiments and metabolic flux analysis have led to the discovery of unsuspected mechanisms of ATP dissipation (Alonso et al., 2005) and have suggested that this may account for a surprisingly high fraction of cellular ATP. Depending on the subcellular location of gyceraldehyde-3-P and the identity of the enzymes involved, futile cycling processes consume between 18% and 47% of the ATP produced in developing endosperm tissue, whereas biomass synthesis requires between 45% and 69%. In high yielding maize endosperm tissue, inhibition of these futile cycles would reduce the amount of photosynthate expended in respiration which could potentially increase the productivity of this crop by making more of it available for biomass synthesis. We estimate that yield increases of up to 6% might be realized by reducing substrate cycling. Further enzymatic analysis and more detailed time course experiments are needed to identify targets for such engineering efforts (glucose 6-P phosphatase or inveratase or fructose 1,6-bisphosphatase are of particular interest).

In addition to yield, the endosperm flux map has implications for engineering compositional changes in maize seeds. We found a lower flux through the OPPP in developing maize endosperm than has previously been reported for other heterotrophic plant cells and tissues. Since maize endosperm stores mainly starch it might be expected to have lower NADPH needs than for example developing sunflower seeds. Previous work in oil producing fungi (Wynn et al., 1999; Ratledge, 2002; Zhang et al., 2007) and maize embryo (Alonso et al., 2010) shows that NADPH production may limit oil production. Such observations and the presence of malic enzyme in plastids make it possible that plastidic malic enzyme plays an important role in NADPH supply for oil synthesis. However, the flux estimates obtained here indicate that the rate of NADPH production in developing maize endosperm is enough to support the synthesis of 3 times more oil than is found in this tissue. Furthermore, the metabolic capacities of glucose 6-P dehydrogenase and 6-phosphogluconate dehydrogenase were determined by measuring their activities in vitro and found to be at least 20 times higher than the oxidative flux through the pentose-P pathway (Alonso et al., 2010), showing that neither the capacity for NADPH production nor the plastidic reductant production rate in vivo limits oil biosynthesis.

According to the flux estimates (Fig. 4; Table 3) the entry of carbon into the plastid exceeds the requirements for starch or other biosyntheses so, as was the case with the reductant supply, this is also not likely to limit plastidic oil or protein amino acid synthesis. These findings point to the possibility that the commitment of carbon into plastidic fatty acid synthesis may be important in determining oil production in maize endosperm, consistent with findings in *Brassica napus* and *Arabidopsis thaliana* seeds regarding the importance of plastidial pyruvate kinase for oil synthesis. For example, Schwender et al. (2003) have shown that pyruvate kinase is the main source of plastidic pyruvate for oil synthesis in the oil rich developing embryos of *Brassica napus*, and recent studies with the similarly oil rich developing seeds of *Arabidopsis thaliana* have shown that plastidic pyruvate kinase plays an important role in committing sufficient carbon to support fatty acid synthesis (Andre et al., 2007; Baud et al., 2007). Proteomic analyses of maize endosperm have demonstrated that there are subsequent amounts of plastidic pyruvate dehydrogenase but the quantity of other enzymes leading to fatty acid synthesis, such as plastidic pyruvate kinase and acetyl-CoA carboxylase is extremely low and even undetectable (Méchin et al., 2007), suggesting that they might be a good target for metabolic engineering. However, the flux map reveals that 71% of the carbon entering maize endosperm is required for cell wall and starch synthesis, 12% enters the OPPP and only 25% continues to glycolysis. So, to engineer large increases in maize endosperm oil or protein levels starch synthesis may need to be reduced as part of redirecting the carbon flux through glycolysis or amino acid production. A block of ADP-glucose pyrophosphorylase activity in maize kernels (mutants bt2 or sh2) has been reported to cause an increase in hexose cycling (Spielbauer et al., 2006) and inhibiting ADP-glucose pyrophosphorylase in potato tubers leads to a strong decrease in starch and dry weight and increases in the levels of soluble sugars but not protein or oil (Müller-Röber et al., 1992). Thus blocking starch synthesis in itself does not increase oil or protein synthesis in starch storage tissues and may or may not be needed in combination with engineering increased metabolic demands. For instance an increased metabolic demand for carbohydrate in sugar cane was sufficient to increase total sugar storage without reducing sucrose storage (Wu and Birch, 2007).

5. Conclusions

The major aim of this study was to gain a quantitative insight into the efficiency of endosperm development and the fluxes through primary metabolism that underlie it. The differences measured in the labeling and composition of metabolites in the kernel apoplasm compared to the culture medium (Table 1) show that the cob tissue not only furnishes substrates to the endosperm but also modifies their labeling pattern, which needs to be considered when studying metabolism in multi-tissue systems using labeling experiments. The results show that developing endosperm converts its carbon supply with high efficiency, even though futile cycles appear to be substantial. Estimates for around 50 fluxes involved in primary metabolism of developing maize endosperm were obtained (Fig. 4; Table 3), validated and may be used to guide engineering in maize endosperm metabolism.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jymbem.2010.09.002.

References

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