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

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ABSTRACT

^{14}C 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 ^{13}C-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.

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 ^{13}C-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., 2007a, 2010; Allen et al., 2009b).

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

Abbreviations: CCE, carbon conversion efficiency; DAP, days after pollination; GC–MS, gas chromatography–mass spectrometry; MFA, metabolic flux analysis; NMR, nuclear magnetic resonance; OPPP, oxidative pentose-phosphate pathway; -P, -phosphate; TCA cycle, tricarboxylic acid cycle

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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 pyrophophorylase 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 ^{13}C-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-^{13}C]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). In this approach ^{13}C-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 ^{13}C 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-^{14}C]glucose (317 mCi/mmol, 11.7 GBq/mmol) was from Amersham Biosciences (Piscataway, NJ). [1-^{13}C]glucose and [U-^{13}C]glucose were from Isotec (Miamiasburg, 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 μE 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 Gengenbach 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 4.33 g L^{-1}; thiamine hypochloride 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 −20 °C prior to extraction of sugars and amino acids as described by Porter et al. (1985, 1987). The samples were allowed to warm at room temperature for 1 h and then the liquid phase was transferred to a new vial. The residual agar traps were leached for 2 h with 15 mL of MCW followed by a final wash with 10 mL MCW. All washes were combined and the methanol and chloroform were removed by evaporotation at 40 °C.

2.4. Carbon conversion efficiency

Carbon balance and CCE was determined according to the method of Goffman et al. (2005) by providing [U-^{14}C]glucose and determining ^{14}C incorporation into biomass and $^{14}CO_2$. The $^{14}CO_2$ released specifically by the endosperm was estimated by incubating pieces of cob with or without kernels. Kernel precultures were set up from 5DAP to 10DAP as described above. Two pieces of cob with or without kernels, were then placed on 2×2 cm^2 florists' foam soaked with 10 mL of medium containing [U-^{14}C]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 CO_2 was trapped as previously described by Goffman et al. (2005) and $^{14}CO_2$ 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 O_2 consumption and CO_2 production

The CO_2 produced and O_2 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 CO_2 production and a paramagnetic oxygen analyzer to measure total O_2 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 Salon et al. (1988). Ethanol was removed from extracts by evaporation under nitrogen flow and then water by sublimation using a lyoplilizer.

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, α-amylase/amyloglucosidase 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 Salon 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 × 50 mm column with mobile phases A: 90% acetonitrile/10% H_2O/0.1% NH_4OH and B: 0.1% NH_4OH. 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 ($CDCl_3$) and amino acid, organic acid, sugar and starch fractions in deuterium oxide (D_2O). ^1H NMR spectra were obtained at 499.74 MHz with 90° pulses using a recycling time $> 6T_1$. ^{13}C NMR spectra were obtained at 125.66 MHz with 90° pulses using a recycling time $> 6T_1$. The absolute ^{13}C 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 *N*,*O*-*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-^{13}C]glucose and 20% [U-^{13}C]glucose into a single flux estimation procedure each flux and metabolite pool was programmed twice (using subscript U for the [U-^{13}C]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% polyethylenglycol, in the dark at 25 °C. The rates of substrate uptake were caculated according to total radioactivity incorporated by the maize embryos plus the $^{14}CO_2$ released in the headspace. The following net fluxes Vg, Vf, Vhk1, Vald, Vfas2, Vfas1, Vppp3, Vme, 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 whereas Vhk1, Vhk2, Vres, Vglyco, Vpk, Vpyr, Vsts, Vstsp, Valpd, Vpkp, Vpdhp, Vmep, Vppp1, Vpdh, Vcs, Vca, Vsfa, Vfum1, Vme, Vpepc (Table 3) and the fluxes of amino acid synthesis were constrained to 0 (irreversible). Flux values quantified by biomass composition and quantification as well as Vres calculated according to Alonso et al. (2005) were included as measurements in the 13C-FLUX model. The optimization program used was Donlp2 and the optimization was performed with over 300 different starting points. The best fit statistically was also the most common solution (over 68% of the fits) for the fluxes. The results are presented in Fig. 4 and Table 3.

Table 1
Determination of labeling in metabolites of the cob and the agar trap after 10, 14, 18 and 22 days of labeling with [1-^{13}C]glucose.

Labeling in % enrichment		20DAP—10d labeling		24DAP—14d labeling		28DAP—18d labeling		32DAP—22d labeling	
		TRAP	COB	TRAP	COB	TRAP	COB	TRAP	COB
Glucose	**C1**	41.6 ± 0.3	42.9 ± 0.8	50.2 ± 0.7	47.4 ± 0.8	62.7 ± 0.5	47.7 ± 1.1	56.6 ± 2.3	44.0 ± 1.2
	C6	9.8 ± 0.2	3.7 ± 0.2	12.1 ± 0.6	3.9 ± 0.2	13.6 ± 0.3	2.1 ± 0.2	15.0 ± 1.2	4.4 ± 0.6
Sucrose	**Glucosyl C1**	ud	45.6 ± 0.3	ud	51.9 ± 0.2	ud	58.4 ± 0.4	ud	62.8 ± 0.4
	Glucosyl C6	ud	11.2 ± 0.2	ud	13.1 ± 0.1	ud	13.5 ± 0.1	ud	16.8 ± 0.2
	Fructosyl C1	ud	44.7 ± 0.2	ud	50.6 ± 0.1	ud	58.8 ± 0.1	ud	61.6 ± 0.3
	Fructosyl C6	ud	11.1 ± 0.2	ud	13.1 ± 0.0	ud	13.9 ± 0.1	ud	16.1 ± 0.2
Glutamine	**C2**	20.1 ± 0.6	18.9 ± 0.9	nd	24.7 ± 0.5	nd	20.8 ± 0.7	26.3 ± 1.7	27.0 ± 0.8
	C3	22.2 ± 0.4	21.9 ± 0.5	nd	24.4 ± 0.5	nd	24.1 ± 0.8	24.3 ± 1.9	25.9 ± 0.6
	C4	25.9 ± 0.5	24.8 ± 0.9	nd	28.6 ± 1.4	32.2 ± 1.6	27.0 ± 1.5	30.4 ± 3.0	31.1 ± 1.4

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 and 22 days into 100% [1-^{13}C]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 ^{1}H and ^{13}C 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.

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 (R^2=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 ± 0.9% 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-^{14}C]glucose. Label incorporation into biomass pools (starch, cell wall, oil and protein) was determined for the endosperm tissue. The amount of $^{14}CO_2$ released was estimated by the difference between $^{14}CO_2$ produced by the whole kernel culture and by cob tissue alone. Fig. 2 shows the partitioning of the ^{14}C carbon after labeling with [U-^{14}C]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 ^{14}C 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 apoplast 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-^{13}C]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 ^{1}H and ^{13}C NMR (Table 1). The labeling of trapped glutamine in the apoplast was not significantly different from the free glutamine present in the cob (χ^2 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 (χ^2 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

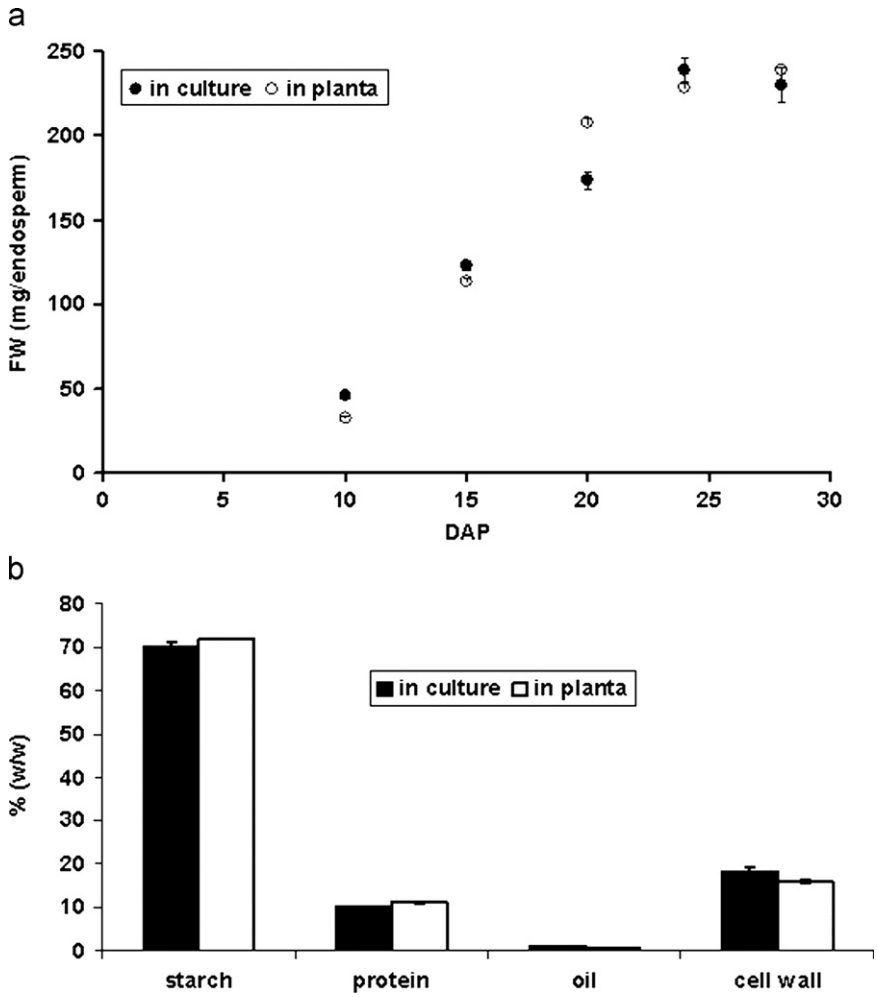

Fig. 1. Endosperm in culture. (a) Fresh weight accumulation. Increase of fresh weight of endosperm tissue isolated from kernels cultured in sterile conditions (solid circles) is compared to endosperm tissue isolated from kernels grown in planta (empty circles). Each point is the average ± standard error 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.

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 ± 6 and 100 ± 12 nmol of glucose equivalents h^{-1} endosperm^{-1}, respectively ($n=3$), 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-^{13}C]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-^{13}C]glucose, or 20% [U-^{13}C]glucose for 18 days. Extracted, oil, starch, free sugars and amino acids were analyzed by ^{1}H NMR and ^{13}C 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-^{13}C]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 ^{1}H NMR and were found to be not significantly different (76.2%, 74.8% and 76.7%, respectively). Thus there were no

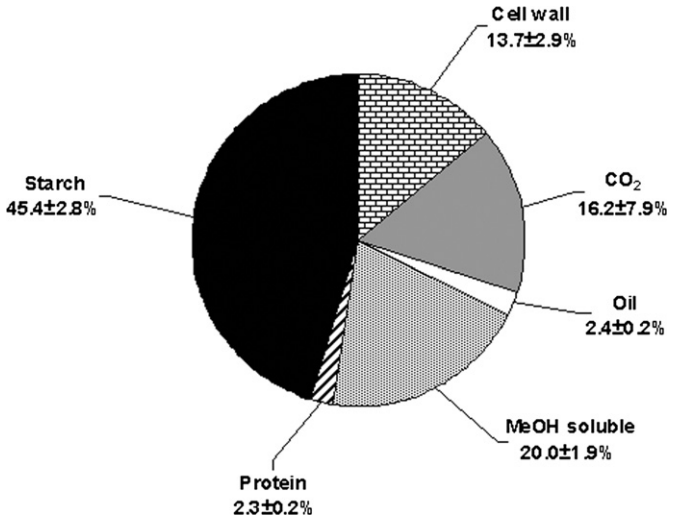

Fig. 2. Distribution of ^{14}C-labeling among biomass fractions and CO_2. Cob tissue was cultured with or without the attached kernel at 27 ^{0}C for 7 days in the presence of [U-^{14}C]glucose. After culture, the $^{14}CO_2$ was collected by flushing the flasks through a trapping system as described by Goffman et al. (2005) and the $^{14}CO_2$ produced by the kernel was calculated by the difference of $^{14}CO_2$ produced by whole kernel culture and the cob alone. Kernels were frozen in liquid N_2 and then dissected under dry ice in order to isolate the endosperm. The analysis of ^{14}C-labeling in biomass components was performed for the endosperm tissue as described in Section 2 and Alonso et al. (2007a).

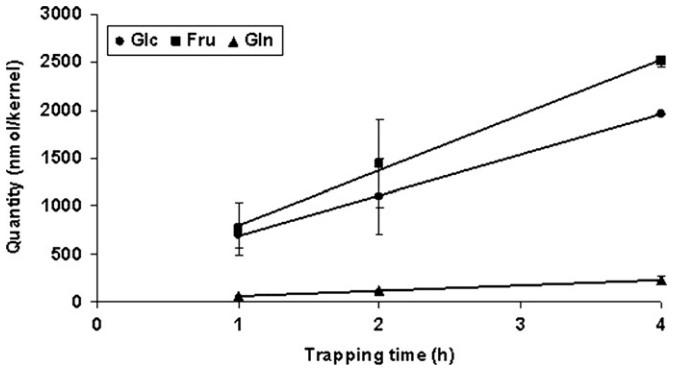

Fig. 3. Assimilate unloading from maternal tissue. Maize kernels were cultured from 5 to 19DAP. As previously described by Porter et al. (1985), the endosperm was scooped out at 19DAP and replaced by an agar drop that was removed after 1, 2 or 4 h. Water soluble molecules trapped in the agar drop were extracted as described by Porter et al. (1985). Sugars and amino acids were quantified by HPLC as described in Section 2.

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; Glawischnig 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-^{13}C]glucose revealed that labeling in C2–C5 of hexose-P was close to zero indicating the absence of gluconeogenic flux (Supplemental Table 3). The glycolytic (Vglyco) and PEPC (Vpepc) fluxes were thus set as irreversible (Table 3). Plastidic glycolysis was set to be irreversible (Valdp, Table 3) because amyloplasts lack fructose 1,6-bisphosphatase (Entwistle and ap Rees, 1988; 1990; Borchert et al., 1993) and other enzymes that convert fructose 1,6-bisP to fructose 6-P. There was no significant difference between the label in the carbon C1 of the fructose uptaken (58.8 $\pm$ 0.1%, Table 1) and the sucrose fructosyl (62.2 $\pm$ 3.6%, Supplemental Table 3), suggesting that there was no OPPP in the cytosol. As previously shown in maize root tips (Dieuaide-Noubhani et al., 1995), and developing sunflower and maize embryos (Alonso et al., 2007a, 2010), the observation of a substantial loss of label in the carbon C1 of starch glucosyl units (42.2 $\pm$ 2.6%, Supplemental Table 3) in comparison with the glucose uptaken (58.4 $\pm$ 0.4%, Table 1) suggested that the OPPP is exclusively located in the plastid of maize endosperm. However, according to Denyer et al. (1996) more than 90% of ADP-glucose pyrophosphorylase activity is localized in the cytosol of maize endosperm. The ADP-glucose synthesized from cytosolic hexose-P crosses the plastidic membrane (Shannon et al., 1998) via brittle-1 protein (BT1) and is a precursor for starch synthesis. We thus included two fluxes for starch synthesis, one (Vsts) from cytosolic hexose-P and the other (Vstsp) from plastidic hexose-P; Vsts was set to represent 90% of the flux of starch synthesis. Different values between 50% and 100% of the total flux of starch synthesis (Vsta) were mathematically simulated for Vstsp (starch synthesis from plastidic hexose-P) and yielded no difference in the goodness of fit. The labeling data did not enable us to assign the relative magnitudes of the cytosolic and plastidic hexose-P fluxes into starch biosynthesis but had no impact on the value of the other fluxes. The net synthesis of sucrose was not included in the model of maize endosperm since it was very small compared with other fluxes from the hexose pools (less than 3%) in maize line LH59. The reactions describing the network of central carbon metabolism are presented in Supplemental Table 5.

The 13C-FLUX® software package (obtained from Prof. Dr. Wiechert, Department of Simulation, University of Siegen, Germany) was used for quantifying flux values (Wiechert and de Graaf, 1997; Wiechert et al., 1997, 1999; Möllney et al., 1999). The following information was entered in the model: (i) the ^{13}C labeling data from two different labeling experiments (100% [1-^{13}C]glucose and 20% [U-^{13}C]glucose; Supplemental Table 3 and 4), (ii) the reactions describing carbon metabolism (one subnetwork is presented in Supplemental Table 5), (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 CO_2 production rate of 558 $\pm$ 44 nmol of CO_2 h^{-1} endosperm^{-1}, which is not significantly different at the $P<0.05$ level (Tukey's studentized range test) from the CO_2 production directly measured by gas analysis (529 $\pm$ 179 nmol CO_2 h^{-1} endosperm^{-1}, $n=3$). The agreement between calculated and measured CO_2 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).

Table 2
Determination of labeling in metabolites of maize endosperm after 10, 14, 18 and 22 days of labeling with [1-^{13}C]glucose.

Labeling in % enrichment		**20DAP 10d labeling**	**24DAP 14d labeling**	**28DAP 18d labeling**	**32DAP 22d labeling**
Glucose	**C1**	34.5 ± 0.3^{a}	46.7 ± 0.8^{b}	48.7 ± 2.9^{b}	50.5 ± 2.4^{b}
	C6	7.6 ± 0.8^{a}	9.6 ± 1.0^{a}	20.4 ± 1.5^{b}	18.8 ± 1.7^{b}
Sucrose glucosyl	**C1**	39.0 ± 0.2^{a}	41.3 ± 0.2^{b}	50.1 ± 3.0^{bc}	47.8 ± 0.3^{c}
	C6	15.2 ± 0.3^{a}	22.0 ± 0.5^{b}	26.1 ± 1.8^{bc}	26.4 ± 0.3^{c}
Alanine	**C2**	1.8 ± 0.9^{a}	nd	1.6 ± 1.6^{a}	3.2 ± 0.2^{a}
	C3	24.3 ± 0.4^{a}	nd	35.7 ± 2.7^{ab}	34.9 ± 0.5^{b}
Starch	**C1**	nd	35.1 ± 0.4^{a}	42.2 ± 2.6^{a}	44.2 ± 1.4^{a}
	C6	nd	20.7 ± 0.3^{a}	23.5 ± 1.7^{ab}	24.7 ± 0.4^{b}
Oil (AcCoA unit)	**C1**	nd	2.2 ± 0.2^{a}	3.2 ± 0.7^{ab}	3.3 ± 0.2^{b}
	C2	nd	18.0 ± 1.2^{a}	22.6 ± 1.6^{ab}	25.6 ± 1.4^{b}

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 and 22 days into media with 100% [1-^{13}C]glucose. Endosperm tissue was dissected from frozen kernels and then water soluble metabolites were extracted as described in Section 2. The labeling in metabolites extracted from endosperm was determined by ^{1}H and ^{13}C 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 for 20, 24 and 32DAP and ± SD calculated as described in Supplemental Table 4 for 28DAP. "nd", non-determined. For each row, means with the same letter are not significantly different at the $P < 0.05$ level (Tukey's studentized range test).

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 O_2 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 998 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 O_2, the rate of oxygen consumption estimated by the model is 326–499 nmol O_2 h^{-1} endosperm^{-1}, which is not significantly different at the $P < 0.05$ level (Tukey's studentized range test) from the rate of O_2 consumption determined experimentally (523 ± 182 nmol O_2 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 reductant 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 O_2 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-bisP 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 distinguish 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 invertase 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,

Table 3
Metabolic fluxes in maize endosperm.

Flux name	Flux or rate description	Flux values nmole h^{-1} endosperm^{-1}	
		Net	Exchange
Vg	Rate of glucose uptake	203 ± 47^{m}	0^{cs}
Vf	Rate of fructose uptake	495 ± 47^{m}	0^{cs}
Va	Rate of glutamine uptake	12 ± 2^{m}	0^{cs}
Vhk1	Flux through hexokinase from glucose to G6P	555 ± 69^{e}	0^{ct}
Vhk2	Flux through hexokinase from fructose to F6P	495 ± 47^{e}	0^{ct}
Vres	Flux from G6P to glucose	353 ± 47^{m}	0^{ct}
Vgf	Fluxes through G6P isomerase	-716 ± 90^{e}	573 ± 113^{e}
Vald	Fluxes through cytosolic aldolase	-221 ± 70^{e}	0 ± 75^{e}
Vglyco	Glycolytic flux	346 ± 13^{e}	0^{cl}
Vfas2	Rate of glycerol incorporation into TAG	2 ± 0^{m}	0^{cs}
Vpk	Flux through pyruvate kinase	155 ± 10^{e}	0^{ct}
Vpyr	Flux of pyruvate from cytosol to mitochondria	137 ± 10^{e}	0^{ct}
Vakg	Flux of glutamate to α-ketoglutarate	-28 ± 3^{e}	high exchange^{e}
Vhcp	Exchange of cytosolic and plastidic hexose-P	465 ± 70^{e}	884 ± 42^{e}
Vsts	Starch synthesis from cytosolic hexose-P	366 ± 5^{ct}	0^{cs}
Vstsp	Starch synthesis from plastidic hexose-P	41 ± 1^{ct}	0^{cs}
Valdp	Flux through plastidic aldolase	391 ± 71^{e}	0^{ct}
Vpkp	Flux through plastidic pyruvate kinase	104 ± 3^{e}	0^{ct}
Vpdhp	Flux through plastidic pyruvate DH	65 ± 1^{e}	0^{ct}
Vfas1	Flux of AcCoA to fatty acid synthesis	51 ± 0^{m}	0^{cs}
Vmep	Flux through plastidic malic enzyme	8 ± 2^{e}	0^{ct}
Vppp1	Flux of the oxidative part of the pentose-P pathway	87 ± 29^{e}	0^{ct}
Vppp2	Fluxes through transketolase	30 ± 10^{e}	132 ± 15^{e}
Vppp3	Fluxes through transaldolase	30 ± 10^{e}	high exchange^{e}
Vppp4	Fluxes through transketolase	23 ± 10^{e}	1289 ± 64^{e}
Vpdh	Flux through pyruvate DH	149 ± 13^{e}	0^{ct}
Vcs	Flux through citrate synthase	146 ± 13^{e}	0^{ct}
Vca	Flux through aconitase	146 ± 13^{e}	0^{ct}
Vsfa	Flux through 2-oxoglutarate DH	115 ± 14^{e}	0^{ct}
Vfum	Fluxes through fumarase	114 ± 14^{e}	520 ± 200^{e}
Vme	Flux through malic enzyme	12 ± 10^{e}	0^{ct}
Vpepc	Anaplerotic flux through PEPC	73 ± 10^{e}	0^{cl}
Vco2	Rate of CO_2 production	558 ± 44^{e}	0^{cs}
Vwall	Rate of wall synthesis	88 ± 8^{m}	0^{cs}
Vsta	Total starch synthesis	407 ± 6^{m}	0^{cs}
Vp5peff	Flux of P5Pp to amino acids for protein synthesis	2 ± 0^{m}	0^{cs}
Vtpeff	Flux of TP to amino acids for protein synthesis	15 ± 1^{m}	0^{cs}
Vpepeff	Flux of PEP to amino acids for protein synthesis	14 ± 1^{m}	0^{cs}
Ve4peff	Flux of E4Pp to amino acids for protein synthesis	7 ± 0^{m}	0^{cs}
Vpyrpeff	Flux of PYRp to amino acids for protein synthesis	47 ± 2^{m}	0^{cs}
VAcCoApef	Flux of AcCoAp to amino acids for protein synthesis	14 ± 1^{m}	0^{cs}
Vpyrceff	Flux of PYRc to amino acids for protein synthesis	18 ± 1^{m}	0^{cs}
Voaaeff	Flux of OAA to amino acids for protein synthesis	29 ± 1^{m}	0^{cs}
Vglueff	Flux of GLU to amino acids for protein synthesis	43 ± 2^{m}	0^{cs}

Fluxes units 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 SD's were reported in the model but set as free for the fitting process. The flux for glucose resynthesis from hexose-P (*Vres*) was calculated using Eq. (4) ($Vres = Vg.(G_{ex}1/Sg6 - G_{in}1/G_{in}6.G_{ex}6/Sg6)/(G_{in}1/G_{in}6 - Sg1/Sg6)$; $G_{ex}1$, Sg1 and $G_{in}1$ represent the enrichment of the carbon 1 of extracellular glucose, the glucosyl moiety of sucrose and intracellular glucose, respectively and $G_{ex}6$, Sg6 and $G_{in}6$ 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-^{13}C]glucose or 20% [U-^{13}C]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-^{13}C]glucose or [U-^{13}C]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 mimick of endosperm metabolism *in planta* (Spielbauer et al., 2006).

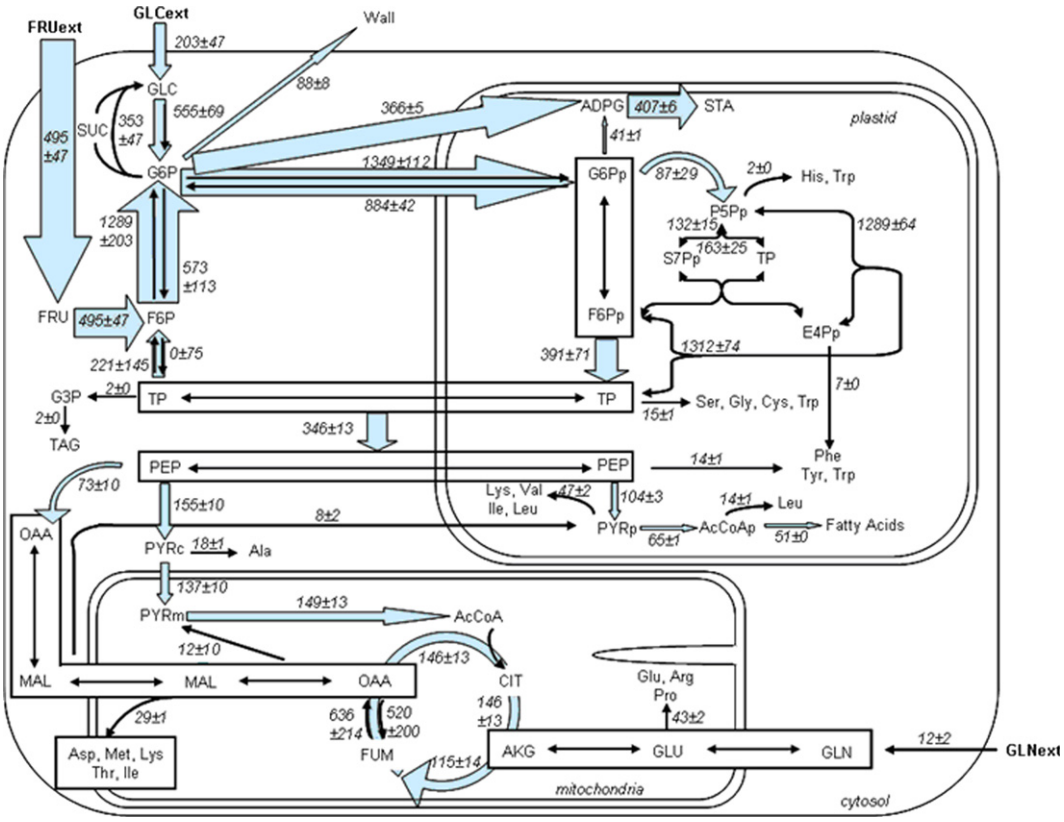

Fig. 4. Metabolic network and fluxes in maize endosperm. The scheme shown is a simplified overview indicating steps for which our model allows calculations of fluxes and their localization. Values reported are the best fit and the ones most commonly obtained by optimized fitting. Units are nmol h^{-1} endosperm^{-1} ± 90% confidence interval. For the net fluxes, gray arrow widths are directly proportional to flux values. The boxes surrounding metabolites indicate that any differences in the labeling between these metabolites cannot be distinguished by our experiments.

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-^{13}C]glucose revealed that free glutamine and the glucosyl moiety of sucrose present in the cob tissue contain the same labeling patterns as the glutamine and glucose 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. 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 ^{13}C-substrate provided in the media, metabolized it and furnished newly labeled substrates to the kernel. For instance, Table I 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 CO_2 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 ^{13}C 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 glyceraldehyde 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%. Futile cycling thus appears to be relatively high in developing maize endosperm. 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 invertase 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 apoplast 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.ymben.2010.10.002.

References

Alexander, D.E., Lambert, R.J., 1968. Relationship of kernel oil content and yield in maize. Crop Sci. 8, 273–274.

Allen, D.K., Ohlrogge, J.B., Shachar-Hill, Y., 2009a. Metabolic flux analysis in plants: coping with complexity. Plant Cell Environ. 32 (9), 1241–1257.

Allen, D.K., Ohlrogge, J.B., Shachar-Hill, Y., 2009b. The role of light in soybean seed filling metabolism. Plant J. 58 (2), 220–234.

Alonso, A.P., Vigeolas, H., Raymond, P., Rolin, D., Dieuaide-Noubhani, M., 2005. A new substrate cycle in plants. Evidence for a high glucose-phosphate-to-glucose turnover from *in vivo* steady-state and pulse-labeling experiments with [C-13] glucose and [C-14] glucose. Plant Physiol. 138, 2220–2232.

Alonso, A.P., Goffman, F.D., Ohlrogge, J.B., Shachar-Hill, Y., 2007a. Carbon conversion efficiency and central metabolic fluxes in developing Sunflower (*Helianthus annuus* L.) embryos. Plant J. 52 (2), 296–308.

Alonso, A.P., Raymond, P., Hernould, M., Rondeau-Mouro, C., de Graaf, A., Chourey, P., Lahaye, M., Shachar-Hill, Y., Rolin, D., Dieuaide-Noubhani, M., 2007b. A metabolic flux analysis to study the role of sucrose synthase in the regulation of the carbon partitioning in central metabolism in maize root tips. Metab. Eng. 9 (5–6), 419–432.

Alonso, A.P., Raymond, P., Rolin, D., Dieuaide-Noubhani, M., 2007c. Substrate cycles in the central metabolism of maize root tips under hypoxia. Phytochemistry 68 (16-18), 2222–2231.

Alonso, A.P., Val, D.L., Shachar-Hill, Y., 2010. Understanding fatty acid synthesis in developing maize embryos using metabolic flux analysis. Metab. Eng. 12 (5), 488–497.

Andre, C., Froehlich, J.E., Moll, M.R., Benning, C., 2007. A heteromeric plastidic pyruvate kinase complex involved in seed oil biosynthesis in Arabidopsis. Plant Cell 19, 2006–2022.

Aoki, N., Hirose, T., Takahashi, S., Ono, K., Ishimaru, K., Ohsugi, R., 1999. Molecular cloning and expression analysis of a gene for sucrose transporter in maize (*Zea mays* L.). Plant Cell Physiol. 40, 1072–1078.

Baud, S., Wuillème, S., Dubreucq, B., de Almeida, A., Vuagnat, C., Lepiniec, L., Miquel, M., Rochat, C., 2007. Function of plastidial pyruvate kinases in seeds of *Arabidopsis thaliana*. Plant J. 52, 405–419.

Borchert, S., Harborth, J., Schunemann, D., Hoferichter, P., Heldt, H.W., 1993. Studies of the enzymic capacities and transport properties of pea root plastids. Plant Physiol. 101, 303–312.

Cheng, W.H., Chourey, P.S., 1999. Genetic evidence that invertase-mediated release of hexoses is critical for appropriate carbon partitioning and normal seed development in maize. Theor. Appl. Genet. 98, 485–495.

Cobb, B.G., Hannah, L.C., 1986. Sugar utilization by developing wild type and shrunken-2 maize kernels. Plant Physiol. 80, 609–611.

Converti, A., Perego, P., 2002. Use of carbon and energy balances in the study of the anaerobic metabolism of *Enterobacter aerogenes* at variable starting glucose concentrations. Appl. Microbiol. Biotechnol. 59, 303–309.

Denyer, K., Dunlap, F., Thorbjørnsen, T., Keeling, P., Smith, A.M., 1996. The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial. Plant Physiol. 112 (2), 779–785.

Dieuaide-Noubhani, M., Raffard, G., Canioni, P., Pradet, A., Raymond, P., 1995. Quantification of compartmented metabolic fluxes in maize root tips using isotope distribution from 13C- or 14C-labeled glucose. J. Biol. Chem. 270, 13147–13159.

Dieuaide-Noubhani, M., Alonso, A.P., Rolin, D., Eisenreich, W., Raymond, P., 2007. Metabolic flux analysis: recent advances in carbon metabolism in plants. EXS 97, 213–243.

Eisenreich, W., Ettenhuber, C., Laupitz, R., Theus, C., Bacher, A., 2004. Isotopolog perturbation techniques for metabolic networks: metabolic recycling of nutritional glucose in *Drosophila melanogaster*. Proc Natl. Acad. Sci. USA 101 (17), 6764–6769.

Ekman, A., Hayden, D.M., Dehesh, K., Bülow, L., Stymne, S., 2008. Carbon partitioning between oil and carbohydrates in developing oat (*Avena sativa* L.) seed. J. Exp. Bot. 59, 4247–4257.

Entwistle, G., ap Ress, T.A., 1988. Enzymic capacities of amyloplasts from wheat (*Triticum aestivum*) endosperm. Biochem. J. 255, 391–396.

Entwistle, G., ap Ress, T.A., 1990. Lack of fructose-1,6-bisphosphatase in a range of higher plants that store starch. Biochem. J. 271, 467–472.

Ettenhuber, C., Spielbauer, G., Margl, L., Hannah, L.C., Gierl, A., Bacher, A., Genschel, U., Eisenreich, W., 2005. Changes in flux pattern of the central carbohydrate metabolism during kernel development in maize. Phytochemistry 66 (22), 2632–2642.

FAOSTAT, 2007. Food and Agriculture Organization of the United Nations. 〈http://faostat.foa.org〉.

Felker, F.C., 1992. Participation of cob tissue in the uptake of medium components by maize kernels cultures *in vitro*. J. Plant Physiol. 139, 647–652.

Flinn, A.M., Atkins, C.A., Pate, J.S., 1977. Significance of photosynthetic and respiratory exchanges in carbon economy of developing pea fruit. Plant Physiol. 60, 412–418.

Garrett, R.H., Grisham, C.M., 1999. Biochemistry Second edn. Fort Worth: Saunders College Publishing.

Gengenbach, B.G., Jones, R.J., 1994. *In vitro* culture of maize kernels. In: Freeling, M., Walbot, V. (Eds.), The Maize Handbook, pp. 705–708.

Glawischnig, E., Tomas, A., Eisenreich, W., Spiteller, P., Bacher, A., Gierl, A., 2000. Auxin biosynthesis in maize kernels. Plant Physiol. 123, 1109–1119.

Glawischnig, E., Gierl, A., Tomas, A., Bacher, A., Eisenreich, W., 2001. Retrobiosynthetic nuclear magnetic resonance analysis of amino acid biosynthesis and intermediary metabolism. Metabolic flux in developing maize kernels. Plant Physiol. 125 (3), 1178–1186.

Glawischnig, E., Gierl, A., Tomas, A., Bacher, A., Eisenreich, W., 2002. Starch biosynthesis and intermediary metabolism in maize kernels. Quantitative analysis of metabolite flux by nuclear magnetic resonance. Plant Physiol. 130 (4), 1717–1727.

Goffman, F.D., Alonso, A.P., Schwender, J., Shachar-Hill, Y., Ohlrogge, J.B., 2005. Light enables a very high efficiency of carbon storage in developing embryos of rapeseed. Plant Physiol. 138, 2269–2279.

Hill, S.A., ap Rees, T., 1994. Fluxes of carbohydrate-metabolism in ripening bananas. Planta 192, 52–60.

IITA, 1996. Agricultural Research for Development in Africa. IITA Research Guide 33: Nutrition and quality of maize. (http://www.iita.org).

Lambert, R.J., Alexander, D.E., Han, Z.J., 1998. A high oil pollinator enhancement of kernel oil and effects on grain yields of maize hybrids. Agron. J. 90, 211–215.

Libourel, I.G.L., Shachar-Hill, Y., 2008. Metabolic flux analysis in plant: from intelligent design to rational engineering. Ann. Rev. Plant Biol. 59, 625–650.

Lyznik, L., Rafalski, A., Raczyńska-Bojanowska, K., 1985. Amino acid metabolism in the pedicel–placenta–chalazal region of the developing maize kernel. Phytochemistry 24 (3), 425–430.

Méchin, V., Thévenot, C., Le Guilloux, M., Prioul, J.-L., Damerval, C., 2007. Molecular and biochemical mechanisms in maize endosperm development: the role of pyruvate-Pi-dikinase and Opaque-2 in the control of C/N ratio. Plant Physiol. 143, 1203–1219.

Möllney, M., Wiechert, W., Kownatzki, D., de Graaf, A.A., 1999. Bidirectional reaction steps in metabolic networks: IV. Optimal design of isotopomer labeling experiments. Biotechnol. Bioeng. 66, 86–103.

Müller-Röber, B., Sonnewald, U., Willmitzer, L., 1992. Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO J. 11, 1229–1238.

Nelson, O., Pan, D., 1995. Starch synthesis in maize endosperms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 475–496.

Novák, L., Loubière, P., 2000. The metabolic network of *Lactococcus lactis*: distribution of C-14-labeled substrates between catabolic and anabolic pathways. J. Bacteriol. 182, 1136–1143.

Pate, J.S., Sharkey, P.J., Atkins, C.A., 1977. Nutrition of a developing legume fruit—functional economy in terms of carbon, nitrogen, water. Plant Physiol. 59, 506–510.

Peoples, M.B., Pate, J.S., Atkins, C.A., Murray, D.R., 1985. Economy of water, carbon, and nitrogen in the developing cowpea fruit. Plant Physiol. 77, 142–147.

Peterson, J.D., Ingram, L.O., 2008. Anaerobic respiration in engineered *Escherichia coli* with an internal electron acceptor to produce fuel ethanol. Ann. NY Acad. Sci. 1125, 363–372.

Porter, G.A., Knievel, D.P., Shannon, J.C., 1985. Sugar Efflux from maize (*Zea mays* L.) pedicel tissue. Plant Physiol. 77 (3), 524–531.

Porter, G.A., Knievel, D.P., Shannon, J.C., 1987. Assimilate unloading from maize (*Zea mays* L.) pedicel tissues. II. Effects of chemical agents on sugar, amino acid, and ^{14}C-assimilate unloading. Plant Physiol. 85, 558–565.

Ratcliffe, R.G., Shachar-Hill, Y., 2005. Revealing metabolic phenotypes in plants: inputs from NMR analysis. Biol. Rev. 80, 27–43.

Ratcliffe, R.G., Shachar-Hill, Y., 2006. Measuring multiple fluxes through plant metabolic networks. Plant J. 45, 490–511.

Ratledge, C., 2002. Regulation of lipid accumulation in oleaginous micro-organisms. Biochem. Soc. Trans. 30, 1047–1050.

Rios-Estepa, R., Lange, B.M., 2007. Experimental and mathematical approaches to modeling plant metabolic networks. Phytochemistry 68, 2351–2374.

Rontein, D., Dieuaide-Noubhani, M., Dufourc, E.J., Raymond, P., Rolin, D., 2002. The metabolic architecture of plant cells. Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. J. Biol. Chem. 277, 43948–43960.

Sáez, J.C., Schell, D.J., Tholudur, A., Farmer, J., Hamilton, J., Colucci, J.A., McMillan, J.D., 2002. Carbon mass balance evaluation of cellulase production on soluble and insoluble substrates. Biotechnol. Prog. 18, 1400–1407.

Salon, C., Raymond, P., Pradet, A., 1988. Quantification of carbon fluxes through the tricarboxylic acid cycle in early germinating lettuce embryos. J. Biol. Chem. 263, 12278–12287.

Schuster, R., 1988. Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and HPLC. J. Chromatogr. 431, 271–284.

Schwender, J., Ohlrogge, J.B., Shachar-Hill, Y., 2003. A flux model of glycolysis and the oxidative pentosephosphate pathway in developing *Brassica napus* embryos. J. Biol. Chem. 278, 29442–29453.

Schwender, J., Goffman, F., Ohlrogge, J.B., Shachar-Hill, Y., 2004. Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds. Nature 432, 779–782.

Schwender, J., Shachar-Hill, Y., Ohlrogge, J.B., 2006. Mitochondrial metabolism in developing embryos of *Brassica napus*. J. Biol. Chem. 281, 34040–34047.

Seebauer, J.R., Moose, S.P., Fabbri, B.J., Crossland, L.D., Below, F.E., 2004. Amino acid metabolism in maize earshoots. Implications for assimilate preconditioning and nitrogen signaling. Plant Physiol. 136, 4326–4334.

Shannon, J.C., 1972. Movement of ^{14}C-labeled assimilated into kernels of *Zea mays* L. I. Pattern and rate of sugar movement. Plant Physiol. 49, 198–202.

Shannon, J.C., Pien, F.M., Cao, H., Liu, K.C., 1998. Brittle-1, an adenylate translocator, facilitates transfer of extraplastidial synthesized ADP-glucose into amyloplasts of maize endosperms. Plant Physiol. 117 (4), 1235–1252.

Spielbauer, G., Margl, L., Hannah, L.C., Römisch, W., Ettenhuber, C., Bacher, A., Gierl, A., Eisenreich, W., Genschel, U., 2006. Robustness of central carbohydrate metabolism in developing maize kernels. Phytochemistry 67 (14), 1460–1475.

Sriram, G., Fulton, D.B., Iyer, V.V., Peterson, J.M., Zhou, R.L., Westgate, M.E., Spalding, M.H., Shanks, J.V., 2004. Quantification of compartmented metabolic fluxes in developing soybean embryos by employing Biosynthetic ally directed fractional C-13 labeling, [C-13, H-1] two-dimensional nuclear magnetic resonance, and comprehensive isotopomer balancing. Plant Physiol. 136, 3043–3057.

Thomas, P.A., Felker, F.C., Crawford, C.G., 1992. Sugar uptake and metabolism in the developing endosperm of Tasselseed-Tu (Ts5-Tu). Plant Physiol. 99, 1540–1546.

US Department of Agriculture, 2008. Oilseeds: World Markets and Trade. Circular Series FOP 12-08. 〈http://www.fas.usda.gov〉.

Wiechert, W., de Graaf, A.A., 1997. Bidirectional reaction steps in metabolic networks .1. Modeling and simulation of carbon isotope labeling experiments. Biotechnol. Bioeng. 55, 101–117.

Wiechert, W., Siefke, C., de Graaf, A.A., Marx, A., 1997. Bidirectional reaction steps in metabolic networks .2. Flux estimation and statistical analysis. Biotechnol. Bioeng. 55, 118–135.

Wiechert, W., Möllney, M., Isermann, N., Wurzel, W., de Graaf, A.A., 1999. Bidirectional reaction steps in metabolic networks: III. Explicit solution and analysis of isotopomer labeling systems. Biotechnol. Bioeng. 66, 69–85.

Wiechert, W., Möllney, M., Petersen, S., de Graaf, A.A., 2001. A universal framework for C-13 metabolic flux analysis. Met. Eng. 3, 265–283.

Wu, L., Birch, R.G., 2007. Doubled sugar content in sugarcane plants modified to produce a sucrose isomer. Plant Biotechnol. J. 5, 109–117.

Wynn, J.P., Hamid, A.B.A., Ratledge, C., 1999. The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. Microbiology—UK 145, 1911–1917.

Zhang, Y., Adams, I.P., Ratledge, C., 2007. Malic enzyme: the controlling activity for lipid production? Overexpression of malic enzyme in *Mucor circinelloides* leads to 2.5-fold increase in lipid accumulation. Microbiology-SGM 153, 2013–2025.