A Combined Metabolomics and Fluxomics Analysis Identifies Steps Limiting Oil Synthesis in Maize Embryos

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Enhancing fatty acid synthesis (FAS) in maize (Zea mays) has tremendous potential nutritional and economic benefits due to the rapidly growing demand for vegetable oil. In maize kernels, the endosperm and the embryo are the main sites for synthesis and accumulation of starch and oil, respectively. So far, breeding efforts to achieve elevated oil content in maize have resulted in smaller endosperms and therefore lower yield. Directly changing their carbon metabolism may be the key to increasing oil content in maize kernels without affecting yield. To test this hypothesis, the intracellular metabolite levels were compared in maize embryos from two different maize lines, ALEXHO S K SYNTHETIC (Alex) and LH59, which accumulate 48% and 34% of oil, respectively. Comparative metabolomics highlighted the metabolites and pathways that were active in the embryos and important for oil production. The contribution of each pathway to FAS in terms of carbon, reductant, and energy provision was assessed by measuring the carbon flow through the metabolic network (13C-metabolic flux analysis) in developing Alex embryos to build a map of carbon flow through the central metabolism. This approach combined mathematical modeling with biochemical quantification to identify metabolic bottlenecks in FAS in maize embryos. This study describes a combination of innovative tools that will pave the way for controlling seed composition in important food crops.

To meet growing food and energy demands, it is imperative to continue improving crop production. Maize (Zea mays) has important economic and nutritional values in many parts of the world. In 2017, 47% of the maize consumed in the United States was used for ethanol, food, and industrial processing, 36% for feeding animals, and 16% for export (USDA, 2017). The demand for seeds rich in oil has increased considerably due to the rapid growth of human consumption, animal feed, and biofuel production. High-oil maize kernels also show higher levels of protein, free Lys, and carotenoids, enhancing their nutritional value (Han et al., 1987; Song, 2000). Understanding the biochemical basis for enhanced seed oil will provide the “roadmap” to further increase oil levels in maize kernels to ultimately meet food and energy demands in the future.

In maize kernels, the endosperm and embryo are the main sites for synthesis and accumulation of starch and oil, respectively. Thus far, breeding efforts to achieve elevated oil content have resulted in a smaller endosperm, which is the main component of the kernel, therefore producing up to 50% smaller grain size and lower yields (Alexander and Lambert, 1968; Lambert et al., 1998, 2010; Moose et al., 2004). The key to increasing oil content in corn kernels without affecting the grain yield may rely on directly altering embryo metabolism by redirecting carbon toward fatty acid (FA) synthesis (FAS). To achieve this goal, a deep understanding of the metabolic pathways leading to triacylglycerol (TAG) production in the maize embryo is required.

In plants, de novo FAS takes place in the plastid, and requires carbon (acetyl-CoA), energy (ATP), and reducing power (NADH and NADPH). It is important to note that in nonphotosynthetic embryos, such as maize embryos, the aforementioned requirements must be provided by the central metabolism. The sole source carbon backbone for the acetyl-CoA comes from the import of substrates produced by the mother plant. Analysis of the metabolites delivered to the kernel showed that Suc and Gln are, respectively, the main sugar and amino acid received by the endosperm and the embryo (Shannon, 1972; Porter et al., 1985, 1987; Thomas et al., 1992; Aoki et al., 1999; Alonso et al., 2010a). Extracellular hydrolysis of Suc into hexoses (Glc and Fru) occurs before reaching embryo cells (Porter et al., 1987; Alonso et al., 2011). Once in the...
cytosol, hexoses can be phosphorylated and serve as carbon precursors for structural and storage compounds.

With regard to FAS in nonphotosynthetic embryos, it has been previously shown that plastidic glycolysis is the major source of carbon for FAS, producing between 71% and 93% of the acetyl-CoA in the plastids of maize embryos and sunflower (*Helianthus annuus*), respectively (Alonso et al., 2007a, 2010a). Plastidic glycolysis also plays an important role in providing ATP for FAS (Boyle et al., 1990; Kleppinger-Sparace et al., 1992; Qi et al., 1994; Kang and Rawsthorne, 1996). Additionally, the import of ATP (produced by mitochondrial oxidative phosphorylation) into the plastid was identified as a significant contributor to FAS (Fuhrmann et al., 1994; Qi et al., 1994; Möhlmann et al., 1994). In terms of reducing power, NADH necessary for FAS is produced by the plastidic pyruvate dehydrogenase. In isolated plastids from heterotrophic embryos, NADPH was shown to be provided by the oxidative reactions of the pentose-P pathways and/or the activity of the plastidic malic enzyme (Smith et al., 1992; Kang and Rawsthorne, 1996; Pleite et al., 2005).

13C-metabolic flux analyses (13C-MFAs) performed on developing heterotrophic embryos revealed that 97% and 72% of the total plastidic NADPH was produced by the oxidative pentose-P pathway (OPPP) in sunflower and maize, respectively (Alonso et al., 2007a, 2010a). Interestingly, it was previously highlighted that the synthesis of NADPH by the plastidic malic enzyme limited FAS in the embryos of a maize inbred line, LH59, low in oil (34% [w/w]; Alonso et al., 2010a).

In regular corn kernels, such as those of LH59, the total oil content is usually ∼3% to 4% (w/w). Corn with FA levels >6% is considered a “high-oil” corn (Rajendran et al., 2017). We hypothesize that maize kernels accumulating more oil may have changed their carbon metabolism to circumvent the limitation in NADPH provision. To test this hypothesis, the intracellular metabolite levels (comparative metabolomics) were compared between maize embryos from two different maize lines, ALEXHO S K SYNTHETIC (Alex) and LH59, which accumulate 48% and 34% of oil, respectively. The comparative metabolomics highlighted the metabolites and pathways that were active in maize embryos and important for oil production. The contribution of each pathway to FAS in terms of carbon, reductant, and energy provision was assessed by measuring the carbon flow through the metabolic network. For this purpose, a 13C-MFA was performed in developing Alex to build a map of carbon flow through central metabolism that was then compared to the one previously published for LH59 embryos (Alonso et al., 2010a). This study identified and quantified the rerouting of carbon through specific metabolic pathways in order to achieve more oil production. Although the activity of the plastidic malic enzyme was strongly enhanced in Alex embryos accumulating more oil, it still limited the flow of carbon and provision of NADPH to sustain FAS.

### RESULTS

#### Comparative Metabolic Analyses

##### Biomass Composition

LH59 and Alex were selected based upon their divergence in oil content. Indeed, LH59 and Alex mature kernels accumulate ∼4% and 13% of oil, respectively. This study did not include the starchy endosperm, focusing instead on the embryo, which is the major site of FAS and accumulation. Kernels from Alex and LH59 were harvested at 22 d after pollination (DAP). Embryos were dissected and their biomass composition was assessed (Fig. 1). FAs were 1.4 times higher in Alex embryos (48% ± 0.4% [w/w]) in comparison to LH59 ones (34% ± 2.2% [w/w]), which is a significant difference (*P* < 0.05). Additionally, Alex embryos accumulated two times more proteins than LH59 (13% ± 0.7% versus 6% ± 0.8% [w/w]; *P* < 0.05). However, starch quantity was 2.7 times lower in Alex embryos (12% ± 2.9% for Alex versus 31.8 ± 1.0% for LH59; *P* < 0.05). Finally, the content in the cell wall was not significantly different between the two lines. Taken together, these divergences in biomass composition underline strong differences in carbon partitioning between Alex and LH59 embryos.

##### Intermediary Metabolite Levels

Metabolomics quantify key intermediary compounds involved in primary pathways (Cocuron et al., 2014;
De Souza et al., 2015; Tsogtbaatar et al., 2015). Alex and LH59 kernels were harvested at 15, 22, and 28 DAP, the embryos were dissected, and their intracellular metabolite were extracted in boiling water as previously described (Alonso et al., 2010b; Cocuron and Alonso, 2014). Intermediary metabolites and precursors for biomass synthesis (amino acids, sugars/sugar alcohols, phosphorylated compounds, and organic acids) were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), and their content was determined in picomoles per milligram dry weight (DW; Supplemental Table S1) and in picomoles per embryo (Supplemental Table S2). Principal component (PC) analysis (PCA) was performed for the intracellular metabolites at different stages of development (Fig. 2). PC1 and PC2 accounted for 68.6% and 13.3% of the variance, respectively. PC1 clearly separated the different stages of development (15, 22, and 28 DAP), whereas PC2 dissociated the two maize lines (Alex and LH59). Additionally, the PCA revealed that as embryos grew, the metabolic changes became more important between the two lines.

Metabolite contents (picomoles per milligram DW) were compared between Alex and LH59 embryos harvested at 22 DAP (Fig. 3). First, significantly lower sugars (Suc and Fru, \( P < 0.05 \)) and higher hexose Ps (Fru-6-P [F6P] and Glc-6-P [G6P], \( P < 0.05 \)) were found in Alex, which may reflect higher glycolysis. Second, the levels in the intermediaries and products (6-phosphogluconic acid, pentose-5-Ps, and His) of the OPPP were not significantly different between Alex and LH59 embryos, and starch was 2.7 times lower in Alex. Knowing that these two processes occur in the plastid of maize embryos (Alonso et al., 2010a), these results may indicate a lower commitment of carbon toward the plastid. Third, although glycolytic intermediaries (F1, Fru 1,6-bisphosphate, phosphoglycerate, and phosphoenolpyruvate [PEP]) were unchanged, the total protein content was twice as high in Alex embryos (\( P < 0.05 \)). Hence, the flow through the glycolysis and anaplerotic pathway should be higher in Alex embryos to sustain the production of amino acids for protein synthesis. Finally, malate was found to be 1.5 times higher in Alex (\( P < 0.05 \)). Interestingly, malate has been shown to be a precursor for FAS in maize embryos (Alonso et al., 2010a). These results suggest a higher flow through the plastidic malic enzyme for FAS in Alex embryos.

Flux Analyses in Developing Alex Embryos

Development of Culture Conditions for Alex Embryos

Each biomass component (oil, proteins, starch, and cell wall) was found to linearly accumulate between 15 and 28 DAP for Alex embryos in planta, which assumes a metabolic steady state within these developmental stages. For the establishment of culture conditions, Alex embryos were incubated for 7 d between 15 and 22 DAP. Previous studies have shown that Glc, Fru, and Gln were the main sources of carbon and nitrogen received by the developing kernel (Porter et al., 1985, 1987; Alonso et al., 2011). Culture conditions (200 mM of

![Figure 1. Biomass composition (percent total DW) in developing Alex and LH59 embryos. The composition was determined in maize embryos harvested at 22 DAP. A sequential extraction was performed in one lyophilized embryo; oil was first extracted and FA methyl esters were prepared and analyzed with GC-MS. The remaining pellet was used for protein and then starch extractions, quantified spectrophotometrically. The cell wall fraction is considered as the remaining pellet after the three extractions. Error bars represent the standard deviations of three independent experiments, the asterisks represent significant differences between the two lines (\( P < 0.05 \)) according to Student’s t-test. LH59 data were from Alonso et al. (2010a).

![Figure 2. PCA of the intracellular metabolites for Alex and LH59 embryos at different stages of development. The shaded yellow, pink, cyan, red, green, and blue regions in the PCA plots represent 95% confidence intervals for the embryos collected from LH59 and Alex at 15, 22, and 28 DAP.](image-url)
Figure 3. Central metabolic map and biomass composition (picomoles per milligram DW) in developing Alex and LH59 embryos. The quantification was performed in multiple reaction monitoring mode using LC-MS/MS on maize embryos aged 22 DAP. Peak areas were corrected according to internal standards (13C-Glc, 13C-Gly, and 13C-fumarate) added before the extraction. Error bars represent standard deviations from three independent experiments and asterisks represent significant differences between.
Glc, 200 mM of Fru, 40 mM of Gln, and 10% [w/v] polyethylene glycol), specifically developed and validated for maize LH59 embryos (Alonso et al., 2010a), were tested on the Alex line. Unfortunately, the biomass accumulation and composition of developing Alex embryos were substantially different from the ones in planta. Optimal culture conditions that mimic the growth of Alex embryos in planta were obtained with 200 mM of Glc, 200 mM of Fru, 5 mM of Gln, and 15% (w/v) polyethylene glycol (Supplemental Fig. S1). Indeed, in these conditions, the biomass composition of cultured embryos was not significantly different from that of the ones grown in planta. Substrate uptake rates were determined by analyzing the composition of the media with and without embryos after 7 d incubation. Glc, Fru, and Gln were consumed at 335.0 ± 8.5, 393.5 ± 104.6, and 30.8 ± 3.5 nmol h⁻¹ embryo⁻¹, respectively (n = 3).

**13C-Labeling of Alex Embryos**

Isotopic steady state was confirmed by incubating Alex embryos for 7 d with 20% [U-13C₆]-Glc, 20% [U-13C₆]-Fru, and 20% [U-13C₁₅]-Gln. Intracellular metabolites were extracted, and their labeling was determined as described in “Materials and Methods.” For each compound, the average carbon labeling (%) was calculated as a percentage, using the equation Average carbon labeling (%) = (AbundanceM⁺1 × 1 + AbundanceM+2 × 2 + ... + AbundanceM+n × n)/n, where n is the total number of carbon atoms that can be labeled in the molecule. The resulting data showed that of 30 intracellular metabolites, 26 reached isotopic steady state with an average 13C-labeling of 20% ± 2% (Supplemental Table S3), and their labeling was directly included in our model. The one above this range (22%, i.e. Leu) was excluded. For Met, whose fractional labeling was only 14%, we considered that the dilution factor was outside the acceptable range and would be generating large standard deviations. Additionally, Met would only provide redundant labeling information with Asp and Asn, which are already incorporated in the model. Hence, Met labeling was not considered. These discrepancies in the labeling of Leu and Met may be due to peak contamination in the LC-MS/MS run or to the presence of different pools of these amino acids in plant cells (Allen et al., 2012). Finally, His and starch glucosyl were between 16% and 18% and did not completely reach isotopic steady state, because there were unlabeled pools. A dilution factor was therefore applied to these two metabolites as follows.

A corrected value of $M + 0$ was calculated, considering the dilution using Equation 1:

$\frac{(\text{M} + 0)_{\text{corrected}}}{{\text{FL}_{\text{corrected}}}} = \frac{(\text{M} + 0)_{\text{measured}} \times \text{FL}_{\text{measured}}}{\text{FL}_{\text{corrected}}}$

(1)

where $(\text{M} + 0)_{\text{corrected}}$ is the corrected abundance of unlabeled metabolite; $(\text{M} + 0)_{\text{measured}}$ is the measured abundance of unlabeled metabolite; $\text{FL}_{\text{measured}}$ is the measured fractional labeling (Supplemental Table S3); and $\text{FL}_{\text{corrected}}$ is the corrected fractional labeling (here 20%).

Corrected values of each mass isotopomer other than $M + 0$ were calculated using Equation 2:

$\frac{(\text{M} + i)_{\text{corrected}}}{{\text{FL}_{\text{corrected}}}} = \frac{(\text{M} + i)_{\text{measured}} \times [1 - (\text{M} + 0)_{\text{corrected}}]}{[1 - (\text{M} + 0)_{\text{measured}}]}$

(2)

where $(\text{M} + i)_{\text{corrected}}$ is the corrected abundance of a given mass isotopomer other than $M + 0$; $(\text{M} + i)_{\text{measured}}$ is the measured abundance of a given mass isotopomer other than $M + 0$; $(\text{M} + 0)_{\text{corrected}}$ is the corrected abundance of unlabeled metabolite; and $(\text{M} + 0)_{\text{measured}}$ is the measured abundance of unlabeled metabolite.

In this study, we performed two parallel labeling experiments using different 13C substrates and then combined these into a single mathematical model, as described previously (Alonso et al., 2007a, 2007b, 2010a, 2011). This approach is commonly used to achieve better coverage of the metabolic network and more data constraints (Antoniewicz, 2015, 2018; Crown et al., 2016). Once the isotopic steady state was confirmed, Alex embryos were grown in the following media for seven days: (1) 80% [1,2-¹³C₂]-Glc, 20% [¹³C₆]-Glc, and unlabeled Fru and Gln; and (2) unlabeled Glc and Fru, and [¹³C₃]-Gln. These two labeling experiments were performed to ensure good coverage of the metabolic network (Schwender et al., 2006; Alonso et al., 2007a, 2010a, 2011; Allen et al., 2009; Allen and Young, 2013). Indeed, the ¹³C-labeled Glc experiment provides information on the carbon rearrangements in the glycolysis pathway and OPPP (Liboure et al., 2007), whereas the ¹³C-labeled Gln experiment covers organic acids and tricarboxylic acid cycle-derived amino acids. After completion of the labeling experiments, intracellular metabolites and biomass were extracted, and their labeling was measured.

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**Figure 3.** (Continued.)

the two lines ($P < 0.05$) according to Student’s t test. The blue arrows depict the biochemical steps in central metabolism. INO, inositol; Ery/Thr, erythritol/threitol; OHPro, Hyp; Citru, citrulline; T6P, trehalose-6-P; UDPG, UDP-Glc; SUCP, Suc-6-P; Gal1P, Gal-1-P; M1P/G1P, Man-1-P/Glc-1-P; 6PG, 6-phosphogluconic acid; P5P, pentose 5-P; S7P, sedoheptulose-7-P; E4P, erythrose-4-P; F1,6bP, Fru 1,6-bisphosphate; GLYP, glycerol-3-P; PGA, phosphoglycerate; SHI, shikimate; PYR, pyruvate; AcCoA, acetyl-CoA; CIT, citrate; cisACCO, cis-aconitate; isoCIT, isocitrate; AKG, a-ketoglutarate; SUCC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate.

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by NMR (Supplemental Table S4) or mass spectrometry (Supplemental Table S5).

**Metabolic Model and Validation**

The metabolic model used to compile the labeling information for developing Alex embryos was based upon information available in the literature about other heterotrophic plant tissues (Dieuaidé-noubhani et al., 1995; Rontel et al., 2002; Alonso et al., 2007b, 2007c, 2011; Williams et al., 2008), the model previously published for maize embryos (Alonso et al., 2010a), and labeling data obtained in this study (Supplemental Tables S4 and S5).

Labeling information from Suc and starch reflect the labeling of the hexose-P pools from which they are synthesized, that is to say cytosol and plastidic hexose-Ps for Suc and starch, respectively. After labeling with 80% [1,2-13C2]-Glc and 20% [U-13C6]-Glc, the majority of the labeled carbon 1 in the Suc glucosyl has a labeled neighbor, carbon 2 (Supplemental Table S4), which refutes the occurrence of the OPPP in the cytosol. However, the substantial abundance of M + 1 in the starch glucosyl indicates that the OPPP is active in the plastid (Supplemental Table S5). When Alex maize embryos were labeled with 100% [U-13C5]Gln, the resulting mass isotopomer distribution for malate was not significantly different from that of its precursor from the tricarboxylic acid cycle, succinate (Supplemental Table S5; Student’s t test, P < 0.05), indicating that the malate pool is predominantly mitochondrial.

The exchange fluxes for metabolic steps known to be thermodynamically unfavorable (flux of the oxidative pentose-P pathway 1 [Vppp1], flux through cytosolic pyruvate kinase [Vpk], flux through plastidic pyruvate kinase [Vpkp], flux through cytosolic pyruvate dehydrogenase [Vpdh], flux through plastidic pyruvate dehydrogenase [Vpdhp], flux through citrate synthase [Vcs], flux through α-ketolactate dehydrogenase and succinyl CoA synthetase [Vkdh], flux through mitochondrial malic enzyme [Vme], and flux through plastidic malic enzyme [Vmep]; see Supplemental Table S6) were constrained to 0. After labeling Alex embryos with [U-13C5]Gln, there was no labeling detected in Suc and starch glucosyl units, Glc, and Fru (Supplemental Tables S4 and S5), indicating that there is no neoglucogenesis. Therefore, the following fluxes were considered irreversible: flux through cytosolic glycolysis (Vglyc), flux of pyruvate transported from cytosol to mitochondria (Vpyr), flux through cytosolic PEP carboxylase (PEPC; Vpepc), and flux through plastidic glycolysis (Vglycp). It has been shown that colorless plastids lack Fru 1,6-bisphosphatase (Entwistle and Rees, 1988, 1990; Borchert et al., 1993), and the exchange flux of this metabolic step (through plastidic Fru bisphosphate aldolase [Valdp]) was therefore constrained to 0. Finally, the following fluxes were set to be reversible: flux through hexokinase from Glc to G6P (Vhk1), flux through phosphoglucone isomerase (F6P to G6P; Vfg), flux through cytosolic Fru bisphosphate aldolase (Vald), flux through 2-oxoglutarate amidotransferase from Gln to Glu (Vgl), flux through Glu dehydrogenase from Glu to α-ketoglutarate (Vakg), flux of hexose-P exchanged between cytosol and plastid (Vhcp), flux through transketolase C5+C5 → C3+C7 (Vppp2), flux through transaldolase (Vppp3), flux through transketolase C4+C5 → C3+C6 (Vppp4), flux through aconitase (Vaco), flux through isocitrate dehydrogenase (Viso), flux through succinate dehydrogenase 1 (Vsdh1), flux through fumarase (Vfum), and flux through malate dehydrogenase (Vmdh). Using the settings described above, all the reversible fluxes were determinable by our mathematical model.

Supplemental Table S6 defines the reactions involving carbon metabolism extracted from the file used in the 13C-FLUX software (Wiechert and de Graaf, 1997; Wiechert et al., 1997, 1999; Möllney et al., 1999). In order to determine the fluxes, the following information was provided to the model: (1) the rates of substrate uptake for Glc (Vg), Fru (Vf), and Gln (Va); rates of biomass production for cell wall synthesis (Vwall), total starch synthesis (Vsta), FA synthase (Vfas1), glyceral incorporation into TAG (Vfas2), and proteinogenic amino acid synthesis; and free accumulation of Glu (Vgcd), Fru (Vfru), and Glu into Suc (Vgsuc); (2) the label input with a mixture of 20% [U-13C6]-Glc and 80% [1,2-13C6]-Glc, or 100% [U-13C5]Gln; and (3) the data from the two separate labeling experiments (Supplemental Tables S4 and S5). The resultant net and exchange fluxes calculated by the model are reported in Table 1, and the net fluxes are represented in Figure 4.

The optimization procedure was started with different values and repeated >1,000 times in order to avoid incorrect flux estimates. Over 70% of the optimizations led to the same ending point, which corresponded to the best fit. Finally, validation of the model was completed by comparing the measured labeling data with the model prediction (Supplemental Fig. S2), as previously described (Alonso et al., 2010a, 2011). Both the slope of the linear regression and the coefficient of correlation were close to 1, indicating that the model accurately describes the in vivo fluxes in developing Alex embryos.

**Flow of Carbon and Sources of Energy and Reductant for FAS**

According to the flux map presented in Figure 4, 94% of the carbon taken up by Alex embryos enters the plastid under the form of G6P, and then 55% is exported to the cytosol as triose-Ps (TPS), revealing a high exchange of carbon between these two compartments. Thirty-nine percent of the total carbon taken up from the embryos goes through the plastidic OPPP, an important pathway generating NADPH required for FAS. Glycolysis occurs in both cytosol and plastid, with the cytosolic pathway sustaining 62% of the total glycolytic flux. Seventy-eight percent of the carbon coming through the plastidic OPPP is used for starch synthesis (Vsta), FA synthase (Vfas1), glyceral incorporation into TAG (Vfas2), and proteinogenic amino acid synthesis; and free accumulation of Glu (Vgcd), Fru (Vfru), and Glu into Suc (Vgsuc); (2) the label input with a mixture of 20% [U-13C6]-Glc and 80% [1,2-13C6]-Glc, or 100% [U-13C5]Gln; and (3) the data from the two separate labeling experiments (Supplemental Tables S4 and S5). The resultant net and exchange fluxes calculated by the model are reported in Table 1, and the net fluxes are represented in Figure 4.

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Table 1. Net and exchange carbon fluxes in developing Alex embryos

Flux values are calculated by 13C-FLUX software (Wiechert et al., 2001) and expressed in nanomole molecules per hour per embryo. Vg, Vf, and Va were quantified by LC-MS/MS, as described in “Materials and Methods,” after embryo incubation for 7 d. Vfas2, Vsta, and Vwall were quantified according to the rate of DW and biomass accumulation (calculated from Supplemental Fig. S1). All of these rates and their standard deviations were set as free fluxes and fitted by the error minimization process. The fluxes toward amino acid for protein synthesis were determined by LC-MS/MS after protein hydrolysis at 22 DAP and set as constrained values in the model. All other fluxes were calculated from the model based upon 13C-labeling quantification after 7 d incubation with 100% [U-13C5]Gln and a mixture of 20% [U-13C6]Glc + 80% [1,2-13C2]Glc. The values are the best fit and also the most frequently determined optimized flux value means, with the confidence range shown in parentheses after each value.

<table>
<thead>
<tr>
<th>Flux Name</th>
<th>Flux or Rate Description</th>
<th>Net Values (nmol h⁻¹ embryo⁻¹)</th>
<th>Exchange Values (nmol h⁻¹ embryo⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vg rate of Glic uptake</td>
<td>338 (332–344)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vf rate of Fru uptake</td>
<td>385 (378–393)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Va rate of Gln uptake</td>
<td>27 (26–29)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vhk1 flux through hexokinase from Glic to G6P</td>
<td>337 (331–344)</td>
<td>246 (233–259)</td>
<td></td>
</tr>
<tr>
<td>Vhk2 flux through hexokinase from Fru to F6P</td>
<td>385 (378–392)</td>
<td>246 (233–259)</td>
<td></td>
</tr>
<tr>
<td>Vfg flux through phosphoglucose isomerase (F6P to G6P)</td>
<td>459 (385–533)</td>
<td>14,428 (5,643–∞)</td>
<td></td>
</tr>
<tr>
<td>Valsd flux through cytosolic Fru bisphosphate aldolase</td>
<td>–81 (~154 to ~8)</td>
<td>293 (199–407)</td>
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<tr>
<td>Vglyco flux through cytosolic glycolysis</td>
<td>632 (577–686)</td>
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<td>Vfas2 rate of glycerol incorporation into TAG</td>
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<td>Vpk flux through cytosolic pyruvate kinase</td>
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<td>Vpyr flux of pyruvate transported from cytosol to mitochondria</td>
<td>131 (118–143)</td>
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<td>Vgl flux through 2-oxoglutarate amidotransferase from Gln to Glu</td>
<td>27 (26–29)</td>
<td>2,686 (966–∞)</td>
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<td>Vakg flux through Glu dehydrogenase from Glu to α-ketoglutarate</td>
<td>13 (12–14)</td>
<td>14,428 (1,628–∞)</td>
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<tr>
<td>Vhscp flux of hexose-P exchanged between cytosol and plastid</td>
<td>704 (630–777)</td>
<td>955 (768–1,195)</td>
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<td>Vstsp flux of starch synthesized from hexose-P</td>
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<td>Valdp flux through plastidic Fru bisphosphate aldolase</td>
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<td>Vglycp flux through plastidic glycolysis</td>
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<td>Vpdp flux through plastidic pyruvate kinase</td>
<td>381 (330–432)</td>
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<td>Vpdpd flux through plastidic pyruvate dehydrogenase</td>
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<td>Vfas1 flux of FA synthase</td>
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<tr>
<td>Vmepl flux through plastidic malic enzyme</td>
<td>440 (389–491)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vtpc flux of TP exchanged between cytosol and plastid</td>
<td>823 (676–971)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vppp1 flux of the oxidative pentose-P pathway</td>
<td>294 (269–320)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vppp2 flux through transketolase C5+C5 → C3+C7</td>
<td>99 (90–107)</td>
<td>132 (92–174)</td>
<td></td>
</tr>
<tr>
<td>Vppp3 flux through transaldolase</td>
<td>99 (90–107)</td>
<td>691 (512–928)</td>
<td></td>
</tr>
<tr>
<td>Vppp4 flux through transketolase C4+C5 → C3+C6</td>
<td>95 (87–104)</td>
<td>192 (150–239)</td>
<td></td>
</tr>
<tr>
<td>Vpdhp flux through plastidic pyruvate dehydrogenase</td>
<td>180 (169–192)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vcs flux through citrate synthase</td>
<td>180 (169–192)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vaco flux through aconitase</td>
<td>180 (169–192)</td>
<td>1178 (340–7378)</td>
<td></td>
</tr>
<tr>
<td>Viso flux through isocitrate dehydrogenase</td>
<td>180 (169–192)</td>
<td>139 (108–172)</td>
<td></td>
</tr>
<tr>
<td>Vkdh flux through α-ketoglutarate dehydrogenase and succinyl CoA synthetase</td>
<td>193 (181–205)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vsdh flux through succinate dehydrogenase</td>
<td>194 (182–206)</td>
<td>5,554 (1,140–∞)</td>
<td></td>
</tr>
<tr>
<td>Vum flux through fumarase</td>
<td>193 (181–205)</td>
<td>3943 (1,140–∞)</td>
<td></td>
</tr>
<tr>
<td>Vmdh flux through malate dehydrogenase</td>
<td>296 (242–350)</td>
<td>3,431 (1,548–22,058)</td>
<td></td>
</tr>
<tr>
<td>Vme flux through mitochondrial malic enzyme</td>
<td>50 (39–60)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vpepc flux through plastidic PEPC</td>
<td>492 (436–549)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vcco2 flux of CO2 production</td>
<td>1,664 (1,626–1,702)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vwall rate of cell wall synthesis</td>
<td>86 (75–96)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vsta rate of total starch synthesis</td>
<td>39 (31–48)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vglic rate of Glic accumulation</td>
<td>1 (1–1)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vfru rate of Fru accumulation</td>
<td>0 (0–0)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vsuc rate of Suc accumulation</td>
<td>7 (7–7)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vp5pef flux of plastidic pentose-P to amino acids for protein synthesis</td>
<td>1 (1–1)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ve4pef flux of plastidic erythrose 4-P to amino acids for protein synthesis</td>
<td>4 (4–4)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vttepef flux of plastidic TP to amino acids for protein synthesis</td>
<td>12 (11–13)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vpepef flux of plastidic PPP to amino acids for protein synthesis</td>
<td>7 (7–7)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vpyrpef flux of cytosolic pyruvate to amino acids for protein synthesis</td>
<td>8 (8–8)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vpyrpep flux of plastidic pyruvate to amino acids for protein synthesis</td>
<td>25 (25–25)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vacoaeff flux of plastidic acetyl-CoA to amino acids for protein synthesis</td>
<td>5 (5–5)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Veoaaff flux of oxaloacetate to amino acids for protein synthesis</td>
<td>16 (15–17)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vglueff flux of Glic to amino acids for protein synthesis</td>
<td>14 (13–15)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
from cytosolic glycolysis enters the tricarboxylic acid cycle through the anaplerotic pathway, producing malate that is then exported to the plastid to feed into FAS. Indeed, in the plastid, pyruvate is produced at 440 nmol h\(^{-1}\) embryo\(^{-1}\) by the malic enzyme, while 380 nmol h\(^{-1}\) embryo\(^{-1}\) come from the pyruvate kinase (Fig. 5). Therefore, 54% of the plastidic pyruvate is synthesized from malate through the plastidic malic enzyme in developing Alex embryos. It is important to note that this enzyme is also key in producing NADPH for FAS.

FAS requires 790 nmol h\(^{-1}\) embryo\(^{-1}\) of ATP, NADH, and NADPH in developing Alex embryos. In the plastid, ATP is produced by phosphoglycerate
kinase and plastidic pyruvate kinase at 388 and 380 nmol ATP h$^{-1}$ embryo$^{-1}$, respectively (Fig. 5), which is just enough to sustain FAS. Glyceraldehyde-3-P dehydrogenase and pyruvate dehydrogenase synthesize 388 and 795 nmol NADH h$^{-1}$ embryo$^{-1}$, respectively, which exceeds the needs for FAS. Finally, a large portion of the NADPH is provided by the OPPP with 588 nmol h$^{-1}$ embryo$^{-1}$. The occurrence of the plastidic malic enzyme produces an additional 440 nmol NADPH h$^{-1}$ embryo$^{-1}$, covering the requirements for FAS (Fig. 5).

**Control of FA Content in Maize Embryos by the Plastidic Malic Enzyme**

A $^{13}$C-MFA study in developing LH59 maize embryos, low in oil content, previously reported that the plastidic malic enzyme was limiting the provision of NADPH for FAS (Alonso et al., 2010a). The in vitro activities of the enzymes producing NADPH (G6P dehydrogenase; 6-phosphogluconate dehydrogenase, and plastidic malic enzyme) were measured in Alex embryos accumulating more oil than LH59 (Table 2). In Alex embryos, the activity of these enzymes was found to be 2.1 to 2.5 times higher than in LH59 ($P < 0.05$). The in vivo fluxes through the plastidic OPPP and malic enzyme were 1.4 and 1.7 times higher in Alex embryos, respectively ($P < 0.05$), than in LH59. Interestingly, the flux through the OPPP is not limited by the quantity of the enzymes G6P dehydrogenase and 6-phosphogluconate dehydrogenase. However, the flux through the plastidic malic enzyme is not significantly different from the enzyme activity. Although Alex embryos accumulate more oil than LH59, the plastidic malic enzyme still limits the provision of carbon and NADPH for FAS.

**DISCUSSION**

**Challenges in Applying $^{13}$C-MFA to Various Cultivars**

Seed oil production depends on huge fluxes through central metabolism. In this study, we used the most well suited method for quantifying multiple central metabolic fluxes, $^{13}$C-MFA. $^{13}$C-labeling of developing embryos is required for this purpose. Therefore, establishing culture conditions that mimic the physiological development of maize embryos is essential to build carbon flux maps. It has been found for multiple species that developing plant embryos readily grow in liquid cultures (Schwender and Ohlrogge, 2002; Alonso et al., 2007a, 2010a; Lonien and Schwender, 2009; Chen and Shachar-Hill, 2012). Substrate composition, total osmotic pressure of the medium, and light intensity for photosynthetic embryos, are important factors influencing plant tissue development (Goffman et al., 2005; Allen et al., 2009; Alonso et al., 2010b) and hence have to be optimized for each species and each cultivar within a given species. Ideal culture conditions for plant embryos are validated when the DW gain and the biomass composition of embryos grown in culture are not significantly different from those of embryos grown in planta (Alonso et al., 2007a, 2010b, 2011).

Previous studies aiming at assessing the role of light (Allen et al., 2009; Carey et al., 2019) or nitrogen sources in FAS in developing oilseed embryos varied only one component of the culture conditions and performed comparative $^{13}$C-MFA on embryos from the same cultivar. However, for various cultivars/genotypes within the same species, several parameters of the culture conditions may have to be simultaneously altered because the mother plants are different. Therefore, culture conditions that mimic the development of in planta embryos has to be adapted and validated for each cultivar, which makes the application of $^{13}$C-MFA challenging. In the current study, we validated culture conditions that mimic the physiological development of Alex embryos in planta (Supplemental Fig. S1) and therefore can be compared to the physiological development of LH59 embryos (Alonso et al., 2010a).

**CCE Is Not Affected in Alex Embryos**

Heterotrophic maize embryos synthesize biomass components from carbons received from the mother plant. The conversion of these carbons into oil results in a loss under the form of CO$_2$, and is therefore costly. Indeed, acetyl-CoA is the precursor for de novo FAS. It is synthesized in the plastid through pyruvate dehydrogenase. This enzyme catalyzes the oxidative decarboxylation of pyruvate, generating acetyl-CoA and CO$_2$. Therefore for each two-carbon unit added to the nascent acyl-CoA chain, one carbon is lost as CO$_2$. 

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**Figure 5.** Sources of carbon, energy, and reducing power for FAS in Alex embryos. In this simplified overview of the pathway occurring in the maize plastid, the thickness of the blue arrows is proportional to the net flux values. The gray callout presents the requirements for FAS, while the white callouts present the reactions furnishing the carbon, energy, and reducing power. Units are nanomoles per hour per embryo.

p, plastidic; P5P, pentose-5-P; E4P, erythrose-4-P; PYR, pyruvate; MAL, malate; AcCoA, acetyl CoA.
which makes the process of oil biosynthesis less efficient in comparison to other macro-molecules (Goffman et al., 2005). Interestingly, the efficiency with which maize embryos convert carbon into biomass (i.e. the carbon conversion efficiency [CCE]) was estimated to be 61% to 64% for Alex, which is not substantially different from the one previously determined for LH59 embryos (Alonso et al., 2010a). Therefore, Alex embryos are capable of producing more oil without affecting the CCE.

The CCE in these heterotrophic embryos remains lower than in photosynthetic ones, such as soybean and rapeseed, for which it was determined to be 82% and 86%, respectively (Goffman et al., 2005; Allen et al., 2009). In rapeseed embryos, it was shown that the Rubisco bypass played a key role in refixing plastidic CO₂ (produced by the OPPP and by pyruvate dehydrogenase), resulting in a higher CCE (Schwender et al., 2004). Moreover, photosynthesis was the main supplier of energy and reductant rather than the tricarboxylic acid cycle, which releases CO₂ (Schwender et al., 2006). Additionally, after labeling rapeseed embryos with 50% [U-13C5]Gln, a considerable portion of the citrate was found to be labeled on five carbons. This phenomenon was explained by the in vivo reversibility of the isocitrate dehydrogenase that was able to catalyze the carboxylation of α-ketoglutarate, capturing CO₂ to produce isocitrate in developing rapeseed embryos. This reaction was thought to be key in providing citrate, and therefore carbon skeletons, for FA elongation (Schwender et al., 2006). In Alex maize embryos, labeling with 100% [U-13C5]Gln resulted in only 5% of citrate molecules containing five labeled carbons (Supplemental Table S5). This indicates that the reversibility of isocitrate dehydrogenase was minor in developing Alex embryos that mainly produce FA chains ≤18 carbons.

Comparative Metabolomics and Fluxomics Reveal Pathways That Are Enhanced in Alex Embryos

Comparative metabolomic and fluxomic studies were conducted in order to assess the redirection of the carbon flow into FAS in Alex embryos. Metabolomic data suggested a lower commitment of carbon toward plastid. In Alex, 94% of the carbon entering the embryos is transported into the plastids as G6P and 55% comes back into the cytosol in the form of TPs. This pattern of carbon flow between the plastid and the cytosol was first described in developing sunflower embryos (Alonso et al., 2007a) and then in maize endosperm and embryo of LH59 (Alonso et al., 2010a, Alonso et al., 2011). There is indeed less carbon from G6P committed to the plastid in Alex embryos, where 39% of the uptake carbon stays in the plastid, which is 1.5 times lower than the 57% for LH59 (Table 1; Supplemental Table S7).

Additionally, the comparative metabolomics did not indicate significant changes in the network that would suggest flux differences through the OPPP, which was corroborated by the in vivo flux map. In maize kernels and sunflower embryos, the net flux through the upper part of the glycolysis (Valdp) takes place in the plastid (Alonso et al., 2007a, 2010a, 2011), which was found to be the case for Alex embryos, too. In developing LH59 maize embryos, the lower part of glycolysis was found to be split between the two compartments as follows: 38% in the cytosol and 63% in the plastid. Interestingly, the overall glycolytic flux is 16% higher in Alex, due to a strong increase in the cytosolic compartment.

Then, it was inferred from our metabolomic study that the carbon flow through the anaplerotic pathway and the plastidic malic enzyme might be higher in developing Alex embryos than in LH59. Indeed, 13C-MFA determined that the anaplerotic pathway through the PEPC and the plastidic malic enzyme are 154% and 105% higher, respectively, in Alex embryos. The occurrence of these two enzymatic reactions results in malate contributing to 54% of the pool of plastidic pyruvate for FAS in Alex. This is significantly increased compared to maize LH59 and sunflower embryos, where the malic enzyme in the plastid produced 29% and 7%, respectively, of pyruvate (Alonso et al., 2007a, 2010a).

**Table 2. Comparison of in vitro enzyme activities and in vivo fluxes between Alex embryos and LH59 embryos**

<table>
<thead>
<tr>
<th>In vitro enzyme activities</th>
<th>Alex Embryos (nmol h⁻¹ embryo⁻¹)</th>
<th>LH59 Embryos (nmol h⁻¹ embryo⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P dehydrogenase</td>
<td>1,778 ± 348</td>
<td>856 ± 201</td>
</tr>
<tr>
<td>6-phosphogluconate dehydrogenase</td>
<td>4,807 ± 494</td>
<td>1,938 ± 377</td>
</tr>
<tr>
<td>Plasticid malic enzyme</td>
<td>425 ± 85</td>
<td>175 ± 21</td>
</tr>
<tr>
<td>In vivo fluxes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vppp1</td>
<td>294 ± 25</td>
<td>208 ± 18</td>
</tr>
<tr>
<td>Vmep</td>
<td>440 ± 51</td>
<td>163 ± 10</td>
</tr>
</tbody>
</table>

Comparison of in vitro enzyme activities and in vivo fluxes between Alex embryos and LH59 embryos at 22 DAP, as described in “Materials and Methods.” In vivo fluxes were determined by 13C-FLUX software (Wiechert et al., 2001) as described in Table 1. Data for Alex embryos are from this study; data for LH59 embryos are from Alonso et al. (2010a).
Therefore, to produce more oil, Alex embryos significantly increase the G6P entering into the plastid, the aldolase in the plastid, the export of TPs to the cytosol, the glycolytic flux in the cytosol, PEPC, and plastidic malic enzyme (Fig. 4).

Potential Factors Limiting FAS: The Plastidic Malic Enzyme Plays a Key Role in Maize Embryos

In Alex maize embryos, FAS requires 790 nmol h⁻¹ embryo⁻¹ of ATP, NADH, and NADPH. Our flux map shows that the needs in reductant power (NADH and NADPH) are covered by reactions localized in the plastid (Fig. 5). However, the ATP produced within the plastid is just enough to sustain FAS. It is important to note that numerous studies in isolated plastids from rapeseed and pea roots demonstrated that plastidic glycolytic metabolism is not sufficient to fully support FAS: exogenous addition of ATP is required to support maximal rates of carbon incorporation into FA (Qi et al., 1995; Kang and Rawsthorne, 1996). In heterotrophic organs, the major mechanism producing ATP is mitochondrial oxidative phosphorylation. The fluxes through reactions producing reductant in the mitochondria in the form of NADH (pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, malate dehydrogenase, and malic enzyme) and FADH₂ (succinate dehydrogenase), estimated by the flux modeling, synthesize 307 and 193 nmol h⁻¹ embryo⁻¹ of NADH and FADH₂, respectively. Oxidative phosphorylation produces 2.5 and 1.5 mol of ATP per mole of NADH and FADH₂, respectively (Hinkle, 2005), therefore reaching 1,058 nmol ATP h⁻¹ embryo⁻¹. It is therefore conservative to conclude that ATP synthesis is not limiting FAS in maize embryos.

Studies on isolated plastids from oilseed rape embryos (Kang and Rawsthorne, 1994), castor endosperm (Smith et al., 1992), and sunflower embryos (Pleite et al., 2005) have shown that malate can be transported into the plastid, providing carbon skeletons and NADPH for FAS through the plastidic malic enzyme. Although Alex embryos accumulate more oil than LH59, this work revealed that the plastidic malic enzyme still limits the provision of carbon and reductant necessary for FAS. Increasing the levels of this enzyme by selective breeding and/or genetic manipulation should enhance FA content in maize embryos. Finally, this study describes the use of state-of-the-art biochemical measurements combined with mathematical modeling to successfully identify novel biomarkers for breeding and targets for metabolic engineering, which will boost the process of crop improvement.

CONCLUSION

In the present work, we have compared the metabolism of embryos from two different maize lines, Alex and LH59, which accumulate 48% and 34% of oil, respectively. Comparative metabolomics and fluxomic studies demonstrated that Alex embryos have changed their metabolism without altering their CCE and highlighted the specific pathways that were enhanced. To achieve a higher oil content, Alex embryos significantly increased the carbon entering the plastid in the form of G6P and its cleavage through plastidic aldolase into TPs that are exported to the cytosol, where they feed into the glycolysis and phosphoenolpyruvate carboxylase. Malate reduced by the carboxylation of PEP enters the plastid, providing carbon skeletons and NADPH for FAS through the plastidic malic enzyme. Although Alex embryos accumulate more oil than LH59, this work revealed that the plastidic malic enzyme still limits the provision of carbon and NADPH for FAS. Increasing the levels of this enzyme by selective breeding and/or genetic manipulation should enhance FA content in maize embryos.

MATERIALS AND METHODS

Chemicals


Plant Materials and Growth Conditions

Maize (Zea mays) seeds for Alex (NSL 117227) and LH59 (PI 601466) were obtained from the U.S. National Plant Germplasm System. Maize lines Alex and

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Table 3. Transitions followed to determine the mass isotopomer distribution of soluble hexoses

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mass Isotopomer</th>
<th>Parent/ Daughter Transitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexoses</td>
<td>m₀</td>
<td>179/89</td>
</tr>
<tr>
<td></td>
<td>m₁</td>
<td>180/89; 180/90</td>
</tr>
<tr>
<td></td>
<td>m₂</td>
<td>181/89; 181/90; 181/91</td>
</tr>
<tr>
<td></td>
<td>m₃</td>
<td>182/89; 182/90; 182/91; 182/92</td>
</tr>
<tr>
<td></td>
<td>m₄</td>
<td>183/90; 183/91; 183/92</td>
</tr>
<tr>
<td></td>
<td>m₅</td>
<td>184/91; 184/92</td>
</tr>
<tr>
<td></td>
<td>m₆</td>
<td>185/92</td>
</tr>
</tbody>
</table>

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LHS9 were grown in a greenhouse kept at 28°C/21°C day/night temperature with a 16/8 h day/night photoperiod. To prevent cross-pollination between the lines, ears and tassels were covered with paper bags. Ears were hand pollinated and harvested at 15, 22, and 28 d after pollination (DAP). Embryos were dissected from the kernels, rinsed with water, freeze-dried, and stored at −20°C until further analysis.

For cultures, 15 DAP maize embryos were dissected under aseptic conditions as previously described (Koubaa et al., 2013). The incubation media was the same as for LHS9 (Alonso et al., 2010a), except for the concentration of Glu (5 mM) and polyethylene glycol (15%, w/v). For labeling experiments, substrates were replaced by (1) a mixture of 20% [U-13C5]Glc, 20% [U-13C5]Fru, and 20% [U-13C6]Glc; (2) a mixture of 20% [U-13C5]Glc and 80% [1,2-13C2]Glc; and (3) 100% [U-13C6]Glc. All plates were incubated for 7 d in the dark at 24°C and then harvested as previously described (Alonso et al., 2010a).

Biomass and Metabolite Extractions

Biomass extraction and quantification were performed in Alex embryos harvested at 15, 22, and 28 DAP, or after 7 d of culture in unlabeled media. The starting material consisted of one embryo per tube, except for 15 DAP, where two embryos were used. Fatty acids, proteins, and starch were sequentially extracted from ground freeze-dried maize embryos as previously described (Cocuron et al., 2014).

Intracellular metabolites were extracted from 10 mg of ground freeze-dried maize embryos using boiling water, and following the procedure described by Cocuron et al. (2014). It is important to note that 200, 50, and 200 nmol of [U-13C6]Glc, [U-13C2]Glmuramate, and [U-13C4]Gly, respectively, were added to each tube at the time of the extraction to serve as internal standards.

Biomass and Metabolite Quantifications

Biomass compounds (FAs, proteins, and starch) were sequentially extracted as previously described (Cocuron et al., 2014; Tsogbaatar et al., 2015). Briefly, the oil fraction containing an internal standard (C17:0; heptadecanoic triacyl-glycerol) was transmethylated into FA methyl esters before gas chromatography-mass spectrometry (GC-MS) analysis. FA quantification was referenced according to the internal standard. The kit DC Protein Assay from Bio-Rad was used as a colorimetric assay to measure total protein content. Starch extraction and quantification were performed using the α-amylase/ amyloglucosidase method in a Total Starch Assay Kit (Megazyme International Ireland).

Intracellular metabolite levels (sugars, sugar alcohols, amino acids, organic acids, and phosphorylated compounds) were measured using an Agilent 1290 ultra HPLC coupled with an AB Sciex 5500 QTRAP mass spectrometer, as described previously (Cocuron et al., 2014).

13C-Biomass and 13C-Metabolite Extractions

13C-Biomass and 13C-metabolites were extracted from Alex embryos after 7 d of culture, as described above. It is important to note that no internal standards were added at the time of extraction.

Isotopic Labeling Quantification

NMR Analyses for 13C-Suc

Lyophilized boiling water extract was resuspended in 1 mL deuterium oxide and freeze-dried overnight. The dried fraction was then resuspended in 550 μL deuterium oxide and analyzed by NMR using a Bruker 600 MHz spectrometer equipped with a 5 mm cryoprobe at the Campus Chemical Instrument Center (CCIC) NMR facility at The OH State University (Osu.). 1H and 13C NMR spectra were obtained and the absolute 13C enrichments were determined according to Alonso et al. (2010a).

GC-MS Analyses for 13C-EAs

FA butylamide (FABA) preparation was adapted from Allen et al. (2007). Oil extracted from one labeled embryo was resuspended in 1 mL hexane. Then, 0.2 mL were taken up and adjusted to 1.5 mL with hexane and 1 mL butylamine was added for the derivatization. The reaction occurred at 80°C for 24 h and quenched with 0.5 mL HCl (4 N). After derivatization, the tube was vortexed vigorously for 1 min and centrifuged for 2 min at 1,200 rpm. The upper phase, containing FABA derivatives, was transferred to a new glass tube. The lower phase was washed with 2 mL hexane, vortexed, and centrifuged as previously. The nonpolar fractions were then combined, dried under nitrogen flow at 60°C, and resuspended in 1 mL hexane for GC-MS analysis. FABA analysis was conducted on a GC-MS (ISQ Trace GC 1310) instrument with a TG-WaxMS capillary column (0.5 μm film thickness; 0.25 mm ID; 30 m length; Thermo Fisher). MS control and spectral processing were carried out using Xcalibur software (version 2.2; Thermo Fisher). The GC oven temperature was set to 50°C and held for 1 min, increased first to 100°C at 25°C min−1 and held for 2 min, then increased to 240°C at 10°C min−1 and held for 4 min, and finally increased to 250°C at 5°C min−1 and held for 26 min. The injection temperature was set at 225°C. The helium carrier gas flow was 1.4 mL min−1 and the split was set to 10. For mass spectrometry, the ion source and transfer line temperatures were set at 200°C and 250°C, respectively, with electron ionization mode at 70 eV. Detection was in full scan mode between m/z 20 and 400 with a dwell time of 0.071 s. 13C-labeling in FABA was quantified as described by Allen et al. (2007) following the fragment m/z 115 of the oleic acid butylamide derivative.

LC-MS/MS Analysis of 13C-Glucosyl from Starch

13C-starch was extracted as previously described (Cocuron et al., 2014). The glucosyl units were analyzed by LC-MS/MS using the method for the free 13C-sugars described below.

Purification of Water-Soluble 13C-Compounds

Water-soluble 13C-metabolites were extracted from one maize embryo labeled with 13C-substrate(s) for 7 d using boiling water as previously described (Cocuron and Alonso, 2014). Metabolite extract was separated and purified using stacked ion-exchange columns. Both columns were built using 5/6-inch Pasteur pipettes and adding sequentially a small amount of glass wool, 140 μL white quartz 70 mesh sand treated with HCl, 1 cm resin, then 140 μL more sand. The cation-exchange resin was Dowex 50×4-200. The prepared column was rinsed first with 5 × 1 mL double-distilled water (ddH2O), then 5 × 1 mL 1 M NH4OH, H2O again, 5 × 1 mL 1 M HCl, then ddH2O to keep the sample from drying out. The anion-exchange resin was Dowex 1×8-200 mesh. The prepared column was rinsed first with 5 × 1 mL ddH2O, then 5 × 1 mL 4 M formic acid, then ddH2O to keep the sample from drying out.

Lyophilized metabolite extraction samples were resuspended in 300 μL of ddH2O then transferred to NANOSEP 0.2-μm microfilter tubes to centrifuge and filter samples at 15,000g at 4°C for 45 min. Lastly, 1 mL 0.01 M HCl was added to each sample tube.

To purify the samples, the cation exchange column was stacked on top of the anion-exchange column, and the water was drained from the two columns. A glass tube was placed under the columns and the sample applied to the top column. The sample tube was rinsed with 1 mL 0.01 M HCl, and this was also applied to the column. Once the sample migrated through both columns, 5 × 1 mL ultrapure water was used to wash the columns. This first saved fraction, comprised of the run-through and washes, contained free sugars. Then, the two columns were separated to elute their compounds into separate glass tubes. The cation exchange column was washed with 1 mL NH4OH to elute amino acids and the anion-exchange column was washed with 4 M formic acid to elute organic acids and phosphorylated compounds. All fractions were dried under nitrogen gas at 50°C for 45 min. Tubes were frozen by pouring liquid nitrogen over them and then lyophilized for 24 h, and stored at −20 until LC-MS/MS analysis.

LC-MS/MS Analyses of 13C-Metabolites

The mass isotopomer distribution of the purified 13C-sugar fraction from the rinses was analyzed by LC-MS/MS in multiple-reaction monitoring mode, using the same column and gradient as described in Cocuron et al. (2014). In these chromatographic conditions, the retention times for Fru and Glc were 4.9 and 7.5 min, respectively. In order to determine the mass isotopomer distribution, 16 transitions were followed (Table 3).

The labeling of the 13C-amino acid fraction separated from the rinses was analyzed by LC-MS/MS, and data were published by Cocuron et al. (2017). 13C-organic acids purified through the rinses were injected into the LC-MS/MS, and labeling data were published by Koubaa et al. (2013). Finally, the labeling of 13C-phosphorylated compounds separated from the rinses was determined by LC-MS/MS as previously described by Cocuron and Alonso (2014).
Data Correction for Natural Isotope Abundances

13C-labeling data were corrected for natural isotope abundance using a home-written routine under Scilab software (http://www.scilab.org) based on the algorithms previously proposed (Lee et al., 1991; Nanchen et al., 2007).

Quantification of Substrate Consumption in the Medium

Glc, Fru, and Gln uptakes were determined from the unlabeled media incubated for 7 d with or without maize embryos. After incubation, all embryos were collected as described above. For each plate, the medium and the two glass filters were recovered in a 50 mL falcon tube, followed by three washes of the plate with 10 mL of ultrapure water; washes were pooled together with the recovered medium. All tubes were adjusted to 40 mL with ultrapure water and vortexed vigorously for 2 min. The obtained mixture was filtered through a 50 mL vacuum filtration system (SCGF00525, Millipore), and 1 mL of the filtrate was taken up and centrifuged for 10 min at 17,000 g at room temperature. All samples were then diluted first 20 times in ultrapure water, then 50 times in the presence of 40 μM Man and 1 μM [U-13C5]Gly, used as internal standards for sugar and Gln quantification, respectively. Sugar and Gln uptake were quantified using LC-MS/MS (Cocuron et al., 2014) after diluting 50 times in acetonitrile ultrapure water (60:40 v/v) and 10 times in 0.1% HCl, respectively. All peaks were integrated and area normalized according to Man and [U-13C5]Gly standards. Substrate uptakes were calculated in nanomoles per hour per embryo in three biological replicates.

Rates of Proteinogenic Amino Acid Synthesis

Amino acid synthesis rate was determined based on the amino acid composition and the total amount of proteins synthesized for 7 d from 15 to 22 DAP. The proteinogenic amino acid composition was determined by LC-MS/MS at 22 DAP, as previously described (McClure et al., 2017). Purified extract was resuspended in 1 mL HCl (0.01 N) and diluted 1,000 times in the same solvent; then, 2 μL was injected into the LC-MS/MS. All peaks for three biological replicates were integrated and the amino acid composition (%) was determined. The synthesis rate of each amino acid was calculated in nanomoles per hour per embryo.

Modeling Metabolic Pathways

The metabolic model was built using the software package 13C-FLUX, obtained from Dr. Wolfgang Wiechert (Department of Simulation, University of Siegen, Germany; Wiechert et al., 2001). The network (Supplemental Table S6) was adapted from the one previously published by Alonso et al. (2010a) describing the carbon metabolic fluxes in LH59 maize embryos, with slight modifications: (1) the tricarboxylic acid cycle was more detailed, including reactions for aconitate (Vaco), isocitrate dehydrogenase (Viso), α-ketoglutarate dehydrogenase (Vagk), succinate dehydrogenase (Vsdh1 and Vsdh2), and malate dehydrogenase (Vmdh); (2) storage reactions for Glc (Vg), Fru (Vfru), and Suc (Vsguc and Vfruc). In order to include data from the two separate labeling experiments, with (1) a mixture of 20% [U-13C6]Glc and 80% [1,2-13C6]Glc and (2) 100% [U-13C5]Gly, in a single flux estimation procedure, each flux and metabolite pool was duplicated (using "U" at the end of each label and metabolite name for [U-13C6]Glc labeling experiment, e.g. VgU, AlaU...). In the separate metabolic networks, the fluxes of duplicated reactions were forced to be equal. The following net fluxes were set to be free: Vg, Vf, Va, Vfas2, Valdp, Vfas1, Vppp1, Vmdh, Vme, Vpemc, Vwv, and Vsta (for more detail, see Supplemental Table S6). Flux values for biomass component accumulation (Vfas1, Vfas2, Vwv, and Vvsta) and the rates of substrate uptake (Vg, Vf, and Va) were included as measurements in the model. Fluxes toward proteinogenic amino acid synthesis and soluble sugar accumulation were constrained to their experimental values. The exchange fluxes of biomass accumulation ( proteinogenic amino acid synthesis, half the rate of glycerol incorporation into TAG [Vfas2a and Vfas2b], and flux of starch synthesized from hexose-P [Vstsp]), Vglyc, Vp, Vpyr, Valdp, Vglyc, Vpkp, Vpdp, Vnpe, exchange between plastidic and cytosolic triose-P (Vtpc), Vppp1, Vpdp, Vcs, Vkdh, Vme, and Vpemc were constrained to 0, and therefore considered as irreversible. The following exchange fluxes were set as free: Vkdh, Vg, Vald, Vg, Vagk, Vgcp, Vppp2, Vppp3, Vppp4, Vaco, Vso, Vsdh1, Vfum, and Vmdh. 13C-enrichment data obtained by 1H and 13C NMR (Supplemental Table S4), and 13C-labeling abundance obtained by GC-MS and LC-MS/MS (Supplemental Table S5), were included as labeling measurements in the model. The optimization program donlp2 was used to iteratively vary the values for the free fluxes in the metabolic model in order to minimize the sum of squared differences between the labeling data estimated by the model and those experimentally obtained. This procedure estimates the flux values that best fit our experimental data. Additionally, this optimization was repeated 1,000 times from different starting points in order to evaluate the reliability of the flux estimates.

In Vitro Enzyme Activities

In vitro activities of the 6-phosphogluconate dehydrogenase (EC 1.1.1.43), the G6P dehydrogenase (EC 1.1.1.49), and the malic enzyme (EC 1.1.1.40) were performed on five 22-DAP Alex embryos as previously described (Alonso et al., 2010a).

Statistical Analyses

For each biomass and intracellular metabolite quantification, the mean ± SD from three biological replicates was calculated. Significant differences between Alex and LH59 embryos at different stages of development were assessed using ANOVA at 95% confidence. Student’s t test was performed to determine statistical differences between metabolite levels, enzyme activities and flux values, and 13C-labeling, P < 0.05 was considered significant. PCA was performed using Meta/Analyst version 3.0 (Xia and Wishart, 2016) after the data for each variable were normalized using the log2 function, mean centered, and divided by the SD.

Supplemental Material

The following supplemental materials are available:

Supplemental Figure S1. Comparison of biomass composition between embryos in planta at 22 DAP and after incubation for 7 d with unlabeled substrates (in culture).

Supplemental Figure S2. Scatter plot of the comparison between the data predicted by the model and the measurements.

Supplemental Table S1. Targeted metabolomics analyses of Alex and LH59 embryos at different developmental stages expressed in picomoles per milligram DW.

Supplemental Table S2. Targeted metabolomics analyses of Alex and LH59 embryos at different developmental stages expressed in picomoles per embryo.


Supplemental Table S4. Steady-state 13C enrichments of Suc in Alex embryos.

Supplemental Table S5. Steady-state 13C isotope abundance of intracellular metabolites.

Supplemental Table S6. Metabolic network.

Supplemental Table S7. Metabolic fluxes in developing LH59 embryos.

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