

Targeted Metabolomics of *Physaria fendleri*, an Industrial Crop Producing Hydroxy Fatty Acids

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Physaria fendleri (syn. *Lesquerella*) is a Brassicaceae producing lesquerolic acid, a highly valued hydroxy fatty acid that could be used for several industrial applications, such as cosmetics, lubricating greases, paints, plastics and biofuels. Free of toxins, *Physaria* oil is an attractive alternative to imported castor (*Ricinus communis*) oil, and is hence on the verge of commercialization. Gas chromatography–mass spectrometry analysis of fatty acid methyl esters revealed that lesquerolic acid was synthesized and accumulated in the embryos, reaching 60% (w/w) of the total fatty acids. The sequential extraction and characterization of biomass compounds revealed that *Physaria* embryo metabolism switched from protein to fatty acid biosynthesis between 18 and 24 days post-anthesis (DPA). In order to unravel the metabolic pathways involved in fatty acid synthesis, a targeted metabolomics study was conducted on *Physaria* embryos at different stages of development. For this purpose, two novel high-throughput liquid chromatography–tandem mass spectrometry methods were developed and validated to quantify sugars, sugar alcohols and amino acids. Specificity was achieved using multiple reaction monitoring, and the limits of quantification were in the pmole–fmole range. The comparative metabolomic study underlined that: (i) the majority of the metabolites accumulate in *Physaria* embryos between 18 and 27 DPA; (ii) the oxidative pentose phosphate pathway, glycolysis, the tricarboxylic acid cycle and the anaplerotic pathway drain a substantial amount of carbon; and (iii) ribulose-1,5-bisphosphate is present, which specifically indicates that the Calvin cycle is occurring. The importance and the relevance of these findings regarding fatty acid synthesis were discussed.

Keywords: Hydroxy fatty acid • LC-MS/MS • *Lesquerella* • Lesquerolic acid • Metabolomics • *Physaria fendleri*.

Abbreviations: CE, collision energy; CXP, collision cell exit potential; DP, deconvolution potential; DPA, days post-anthesis; FAME, fatty acid methyl ester; GC-MS, gas chromatography–mass spectrometry; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring mode.

Introduction

Physaria fendleri (syn. *Lesquerella*; Fig. 1) is a promising industrial crop. Its seeds consist of about 25% oil (w/w) of which 60% is lesquerolic acid (Barclay 1962, Isbell et al. 2008), a monounsaturated 20-carbon fatty acid with a hydroxyl functional group on the 14th carbon. Lesquerolic acid is an unusual fatty acid whose structure and properties are similar to those of ricinoleic acid of castor oil, a major ingredient of industrial oils (Fig. 1). The hydroxy site on the fatty acid makes it suitable for esterification to yield the biodiesel additives estolides. The estolides from *Physaria* and castor have been shown to display far superior low temperature properties than any other estolides reported to date (Cermak et al. 2006). Due to the hazards of allergic reactions and presence of toxins (ricin), castor oil production has been halted in the USA. *Physaria* oil is toxin free and could therefore serve as an alternative source of hydroxy fatty acids to imported castor oil. In addition to its valuable hydroxy fatty acids, *Physaria* seeds produce a natural unique gum (Abbott et al. 1994, Wu and Abbott 1996). This gum is composed of the polysaccharide arabinogalactan with galacturonosyl residues, calcium cross-links and protein interactions. This composition confers unique chemical properties to *Physaria* gum which can be used as a food or industrial thickener. About 22% of *Physaria* seed weight—30% of the defatted meal—is made up of proteins. The composition of the proteinogenic amino acids was found to be very similar to that of soybean, with favorable accumulation of essential amino acids such as lysine, methionine and threonine (Carlson et al. 1990). This excellent distribution of amino acids gives *Physaria* the potential to be used as a protein supplement for livestock (Wu and Hojilla-Evangelista 2005).

Physaria will not compete with food crops. *Physaria* is a member of the Brassicaceae family and grows naturally in the southwestern USA. It is a perennial species which is grown commercially as a winter annual. Therefore, *Physaria* will not substitute current commodity crops but instead will be placed in rotation with existing crops. Several field trials have been conducted in order to determine the optimal planting and production practices for this alternative crop. *Physaria* seeds are

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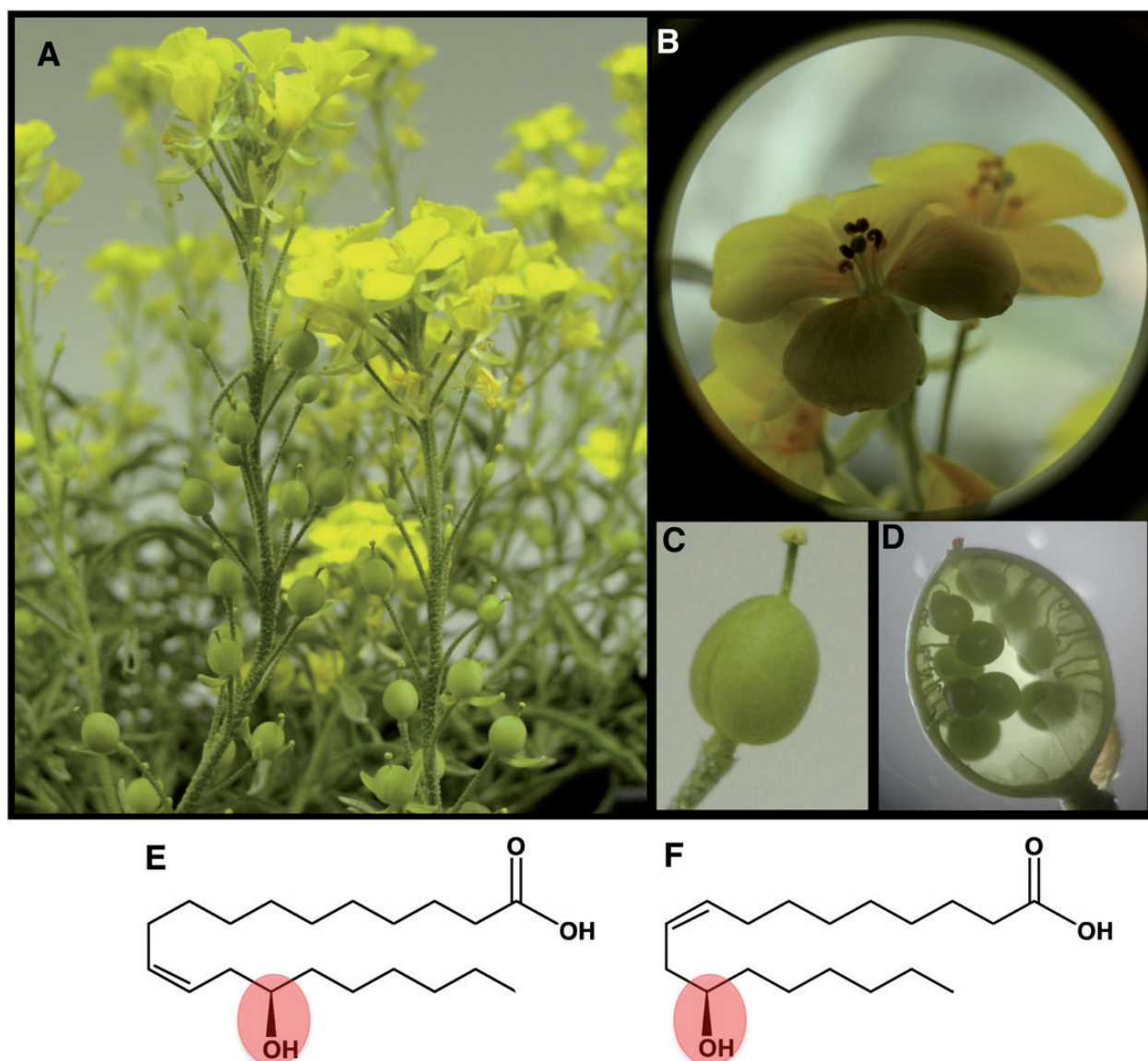


Fig. 1 *Physaria fendleri* plant anatomy and its unusual hydroxy fatty acid. (A) Mature plant; (B) flowers; (C) pod; (D) half pod with seeds. (E) (11Z, 14R)-14-Hydroxyicos-11-enoic acid (syn. lesquerolic acid) and (F) (9Z,12R)-12-hydroxyoctadec-9-enoic acid (syn. ricinoleic acid) present, respectively, in *Physaria* and castor plants. The hydroxyl group of each hydroxy fatty acid is highlighted in red.

sowed in October with an optimal planting rate of 8–12 kg ha⁻¹ (Brahim et al. 1996) via a broadcast planter, a technique commonly used for alfalfa. After about 10 weeks, emergence occurs and the plants remain small until the end of January. Then within 2 months, the vegetative part quickly develops into a full canopy. Flowering and seed development occurs from April until the middle of May. Finally, seeds are harvested in middle to late June using a conventional grain combine with the same sieves as used for alfalfa (Dierig et al. 2011). *Physaria* responds well to limited water and fertilizer (Nelson et al. 1996, Hunsaker et al. 1998, Dierig et al. 2011), making it more adaptable to land with marginal capacity. Irrigation has been shown to be important throughout the crop growth period going from late February to mid May (Hunsaker et al. 1998, Puppala et al. 2005). The current

seed yields are approximately 2,000 kg ha⁻¹ but the crop has the potential of yielding 2,500–3,000 kg ha⁻¹ (Dierig et al. 2011). Yields can be increased with the application of nitrogen fertilizer; however, too much nitrogen significantly reduces seed oil content (Nelson et al. 1996).

An important aspect when considering a crop for industrial use is the possibility of improving its phenotypic traits by genetic manipulation. As a member of the Brassicaceae, *Physaria* benefits from the fully sequenced genome of the closely related model plant *Arabidopsis thaliana*. It was reported that *P. fendleri* has a high capacity for plant regeneration from cultured cells (Skarzhinskaya et al. 1996), which is essential to recovering transgenic plantlets. Two effective protocols were developed to engineer *Physaria*: one was the biolistic transformation of

plastids from leaf samples (Skarjinskaia et al. 2003), and the other was *Agrobacterium tumefaciens*-mediated transformation of calli (Wang et al. 2008, Chen 2011). Transformation is no longer the main limitation to the successful genetic engineering of *Physaria* for increasing hydroxy fatty acid accumulation. The problem instead lies in identifying the targets. To advance toward this goal, a quantitative understanding of metabolic pathways underlying hydroxy fatty acid synthesis in *Physaria* embryos is needed.

In recent years, a new discipline known as metabolomics has emerged to be used as a complementary tool to genomics, epigenetics, transcriptomics, proteomics and fluxomics to better understand the metabolic changes in a given organism. Metabolomics studies the metabolome, which is defined as the complete set of small molecules or metabolites, and it also provides a signature of the cellular state. Metabolomics consists of two different approaches: a targeted one which quantifies a specific set of known molecules in a biological sample and an untargeted one which gives a global metabolic profile (Patti et al. 2012). Targeted metabolomics is the approach of choice to unravel the core metabolic pathways involved in hydroxy fatty acid synthesis in *Physaria*. It requires state-of-the-art equipment, such as liquid chromatography–tandem mass spectrometry (LC-MS/MS), to quantify with high accuracy and sensitivity known intermediaries of central metabolism (Huck et al. 2003, Bajad et al. 2006, Luo et al. 2007, Alonso et al. 2010b, Koubaa et al. 2013, Cocuron and Alonso 2014). In brief, metabolites from a biological mixture are separated by a column according to their chemical properties, and then a specific parent/daughter ion is detected by a triple-quadrupole mass spectrometer using multiple reaction monitoring (MRM) mode. The main intermediaries involved in central metabolism are: sugars, sugar alcohols, amino acids, organic acids and phosphorylated compounds. It is important to note that one unique method cannot separate all these compounds that are chemically and structurally diverse. Rather, several different methods were developed to investigate different categories of metabolites.

In this study, the metabolism of developing *Physaria* embryos was investigated quantitatively. First, the embryos were confirmed to be the organ synthesizing and accumulating lesquerolic acid. Secondly, the biomass composition in developing *Physaria* embryos was characterized thoroughly. Thirdly, two novel high-throughput LC-MS/MS methodologies were developed to target the quantification of sugars, sugars alcohols and amino acids in a small amount of biological sample (1–5 mg), expanding the set of tools for metabolite analysis. Finally, targeted metabolomics analyses of developing *Physaria* embryos highlighted the main pathways involved in hydroxy fatty acid production.

Results

Hydroxy fatty acid synthesis occurs in *Physaria* embryos

Even though fatty acid synthesis and accumulation usually occur in the embryo, only few studies on *Physaria* reported

to date were performed on isolated embryos (Reed et al. 1997, Moon et al. 2001). In order to unravel the metabolic pathways involved in hydroxy fatty acid synthesis, it is imperative to dissect and study the tissue that actually produces those compounds. Siliques from *Physaria* were harvested at different stages of development (every 3 days from 18 to 33 DPA) and embryos were dissected under a microscope. Fatty acids were extracted from *Physaria* embryos, and derivatized as previously described (Goffman et al. 2005, Alonso et al. 2007). In brief, the oil fraction containing an internal standard (triheptadecanoin) was transmethyated into fatty acid methyl esters (FAMES) before gas chromatography–mass spectrometry (GC-MS) analysis. **Supplementary Fig. S1** shows the GC-MS profiles of fatty acids at 18 and 27 DPA. For each stage, fatty acid quantification was referenced according to the internal standard (**Fig. 2**). Fatty acid composition evolved from long chain fatty acids at 18 DPA to very long chain hydroxy fatty acids, with predominance of lesquerolic acid (C20:1 OH) which represented about 60% of the total fatty acids at late stage (**Fig. 2**).

Biomass accumulation in developing *Physaria* embryos

Physaria embryos were dissected at different stages of development, frozen in liquid nitrogen and dried in a lyophilizer then the DW accumulation was measured (**Fig. 3A**). Fatty acids and proteins were sequentially extracted and quantified as previously described (Goffman et al. 2005, Alonso et al. 2007) (**Fig. 3A, B**). A *Physaria* embryo grew $13.7 \mu\text{g DW d}^{-1}$ ($R^2 = 0.95$) between 18 and 27 DPA with a rate of fatty acid synthesis of $6.9 \mu\text{g d}^{-1}$ ($R^2 = 0.97$). Then from 27 to 33 DPA, embryos grew $27.5 \mu\text{g DW d}^{-1}$ ($R^2 = 0.99$) and fatty acid accumulated at $17.1 \mu\text{g d}^{-1}$ ($R^2 = 0.99$). The protein biosynthesis rate stayed constant at $5.3 \mu\text{g d}^{-1}$ ($R^2 = 0.98$) among the different stages studied here (**Fig. 3A**). As shown in **Fig. 3B**, *Physaria* embryo metabolism changed during the development from protein biosynthesis (fatty acid:protein ratio = 0.7) at 18 DPA to fatty acid biosynthesis (fatty acid:protein ratio = 2.1) at 33 DPA. It is important to note that the time course did not cover the later stages of embryo development.

Targeted metabolomics analyses of developing *Physaria* embryos

LC-MS/MS quantification of sugars and sugar alcohols. Sugars and sugar alcohols were separated using a Shodex Asahipak NH2P-50 2D column (2.0×150 mm) with a Shodex Asahipak NH2P-50G 2A guard column. A triple-quadrupole QTRAP 5500 from AB Sciex was used in negative ion mode to monitor and quantify these compounds. Declustering potential (DP), collision energy (CE) and collision exit potential (CXP) were optimized for each metabolite using known standards and AB Sciex Analyst 1.6.1 software (**Supplementary Table S1**). In order to monitor the different sugars and sugar alcohols simultaneously, we used MRM mode. A gradient of acetonitrile was used to separate the metabolites. The gradient was started

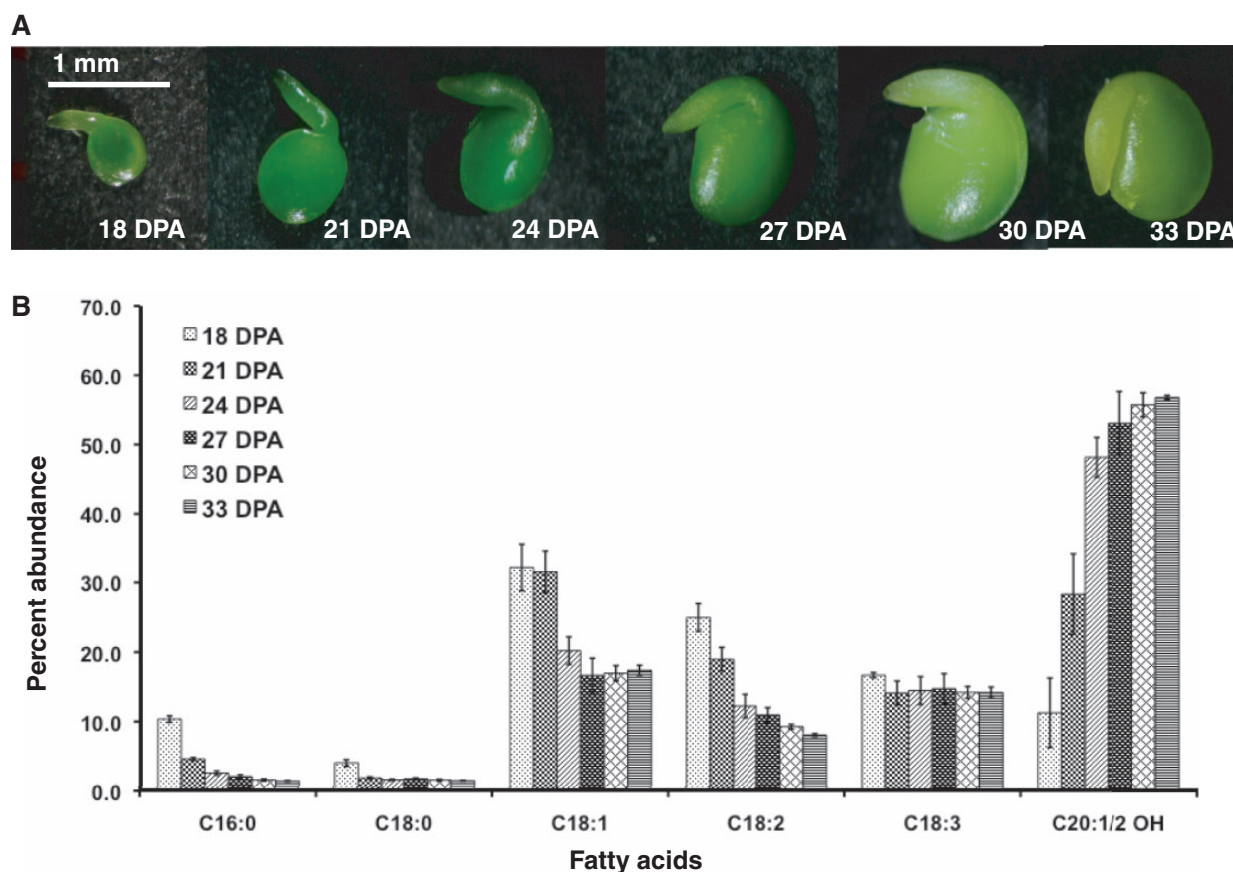


Fig. 2 Fatty acid composition in *Physaria* embryos across different stages of development. (A) Pictures of the embryos at different stages of development. Embryos were dissected under a binocular microscope. (B) Fatty acid composition from 18 DPA to 33 DPA. Error bars represent the SD of three biological replicates. C16:0, palmitic; C17:0, margaric as an internal standard; C18:0, stearic; C18:1, oleic; C18:2, linoleic; C18:3, linolenic; C20:1/2 OH, lesquerolic and auricolic acids.

with 85% acetonitrile for 8.5 min, which allowed the elution of glycerol, ribose, erythritol/threitol, arabinose, xylose, pentitols, fructose, sorbitol, mannose, mannitol, galactitol and galactose (**Supplementary Table S1**). Then the percentage of acetonitrile was lowered to 78% within 0.1 min and maintained at this level for 6.4 min to elute glucose, inositol, sucrose, maltitol, maltose and trehalose (**Supplementary Table S1**). Finally, the column was allowed to equilibrate under 85% acetonitrile for 5 min. It is important to note that this LC-MS/MS method is extremely sensitive, accomplishing the separation in 20 min and quantifying 18 sugars and sugar alcohols in the fmole range. The DP of sucrose for the biological sample was changed from -50 to -240 V to avoid saturation of the mass spectrometer detector. **Supplementary Table S1** reports the validation results for the established method. The calibration graphs for the sugars and sugar alcohols showed excellent linearity from low fmole to high pmole levels, with correlation coefficients >0.9912 . The limits of detection varied from 0.5 to 847 fmol and the limits of quantification were between 2.1 and 2823.3 fmol (**Supplementary Table S1**).

Sugars and sugar alcohols were extracted from *Physaria* embryos using boiling water, with a recovery determined as

previously described (Cruz et al. 2008, Alonso et al. 2010b) of $>87\%$ (**Supplementary Table S4**). When applying the LC-MS/MS method to extracts from *Physaria* embryos, three sugars and three sugar alcohols were detected and quantified according to: (i) standard curves generated for each metabolite; and (ii) the internal standard, $[U-^{13}C]$ glucose, added at the time of extraction (**Fig. 4**; **Supplementary Tables S5, S6**). The main sugars at 27 DPA were glucose, sucrose and fructose with 9421.4 ± 701.9 , 8600.6 ± 750.9 and 177.7 ± 19.8 pmol per embryo, respectively, while the main sugar alcohols were inositol, glycerol and erythritol/threitol with 5255.6 ± 737.2 , 1168.1 ± 101.8 and 8.7 ± 1.2 , respectively (**Supplementary Table S5**). Free sugars are common sources of carbon for developing plant embryos.

LC-MS/MS quantification of amino acids. Amino acids are not only involved in the synthesis of proteins and secondary compounds, they are also the source of nitrogen for plant embryos. We developed an LC-MS/MS method that allows the high-throughput separation and quantification of all the amino acids without derivatization. The separation and detection of amino acids were achieved using a Hypercarb column (100×2.1 mm, $5 \mu\text{m}$ pore) from Thermo Fisher Scientific and

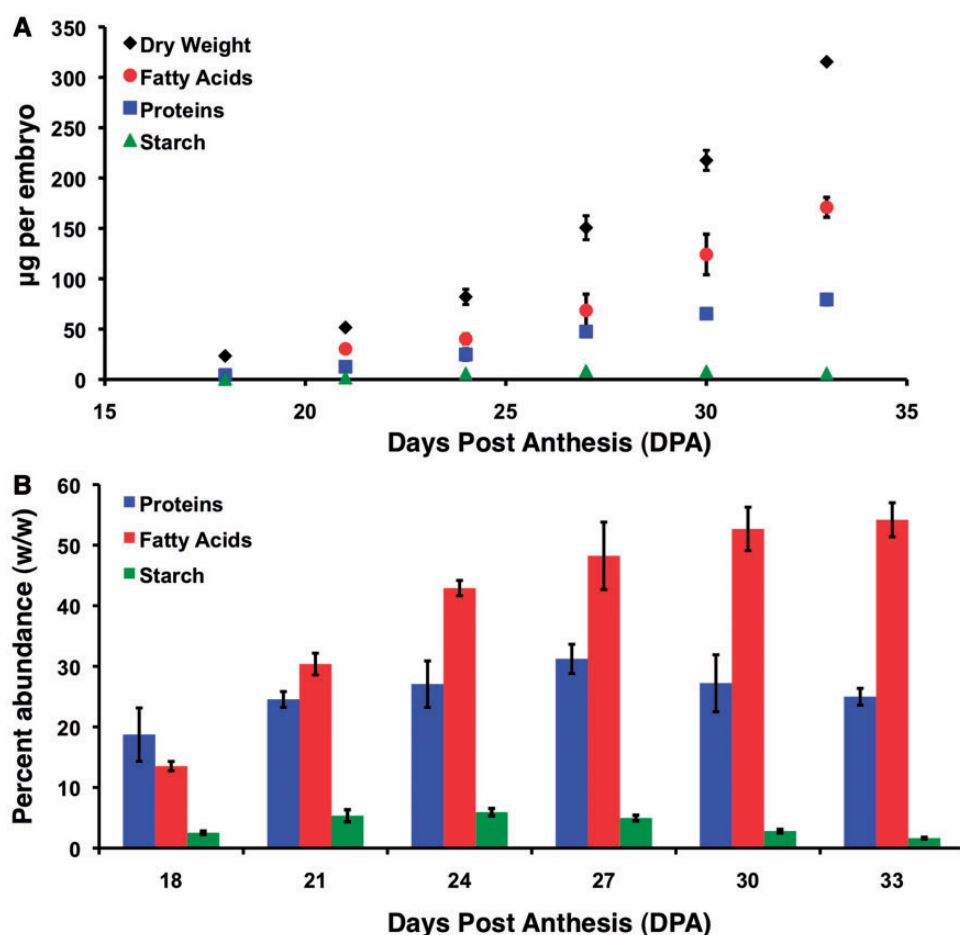


Fig. 3 Biomass characterization of *Physaria* embryos at different stages of development. (A) Biomass accumulation in *Physaria* embryos. The black diamonds, red circles, blue squares and green triangles are, respectively, the dry weight, the fatty acid, protein and starch contents ($n = 3$ biological replicates). (B) Biomass composition. The blue, red and green bars represent the percentage (w/w) of proteins, fatty acids and starch, respectively. Error bars represent the SD of three biological replicates.

a triple-quadrupole QTRAP 5500 from AB Sciex in positive ion mode. For each amino acid, commercial standards were individually injected to determine the optimal mass spectrometric parameters (DP, CE and CXP; [Supplementary Table S2](#)). MRM mode was used to follow all the compounds simultaneously. A gradient of acetonitrile was applied to separate the amino acids, while formic acid was maintained at 0.1% throughout the run. The gradient was started with 0% acetonitrile for 1 min to equilibrate the column, then was increased to 20% and linearly ramped to 45% in 1.6 min to elute lysine, ornithine, 4-aminobutyric acid (GABA), glycine, alanine, serine, threonine, hydroxyproline, asparagine, proline, valine, cysteine, aspartate, glutamine, leucine, isoleucine, glutamate and histidine ([Supplementary Table S2](#)). Next, acetonitrile was increased in a linear fashion from 45% to 60% in 2.3 min, which allowed the elution of methionine, citrulline, arginine, phenylalanine and tyrosine. Then the acetonitrile percentage was raised to 90% and maintained constant for 2 min to elute tryptophan ([Supplementary Table S2](#)). Finally, original conditions (0% acetonitrile) were restored and kept constant for 3 min.

Standard curves were generated for each metabolite using commercially available standards. The separation of all the amino acids, even isomers such as isoleucine and leucine or lysine and glutamine, was successfully achieved in 10 min with a sensitivity of quantification in the fmole range. The DPs of arginine, glutamate, histidine, proline and glutamine for the biological sample were modified from 60 to 170 V, 31 to 150 V, 50 to 160 V, 56 to 150 V and 40 to 130 V, respectively, to avoid saturation of the mass spectrometer detector. Validation results for the method described above are reported in [Supplementary Table S2](#). The calibration graphs for the amino acids showed excellent linearity from low fmole to high pmole levels, with correlation coefficients >0.9855 . The sensitivity of the method was demonstrated by extremely low limits of detection (from 0.1 to 24 fmol) and limits of quantification (between 0.3 and 80 fmol; [Supplementary Table S2](#)).

Free amino acids were extracted from *Physaria* embryos using boiling water with a recovery $>73\%$, except for cysteine (50%; [Supplementary Table S4](#)). The LC-MS/MS method described above was applied to extracts from *Physaria* embryos

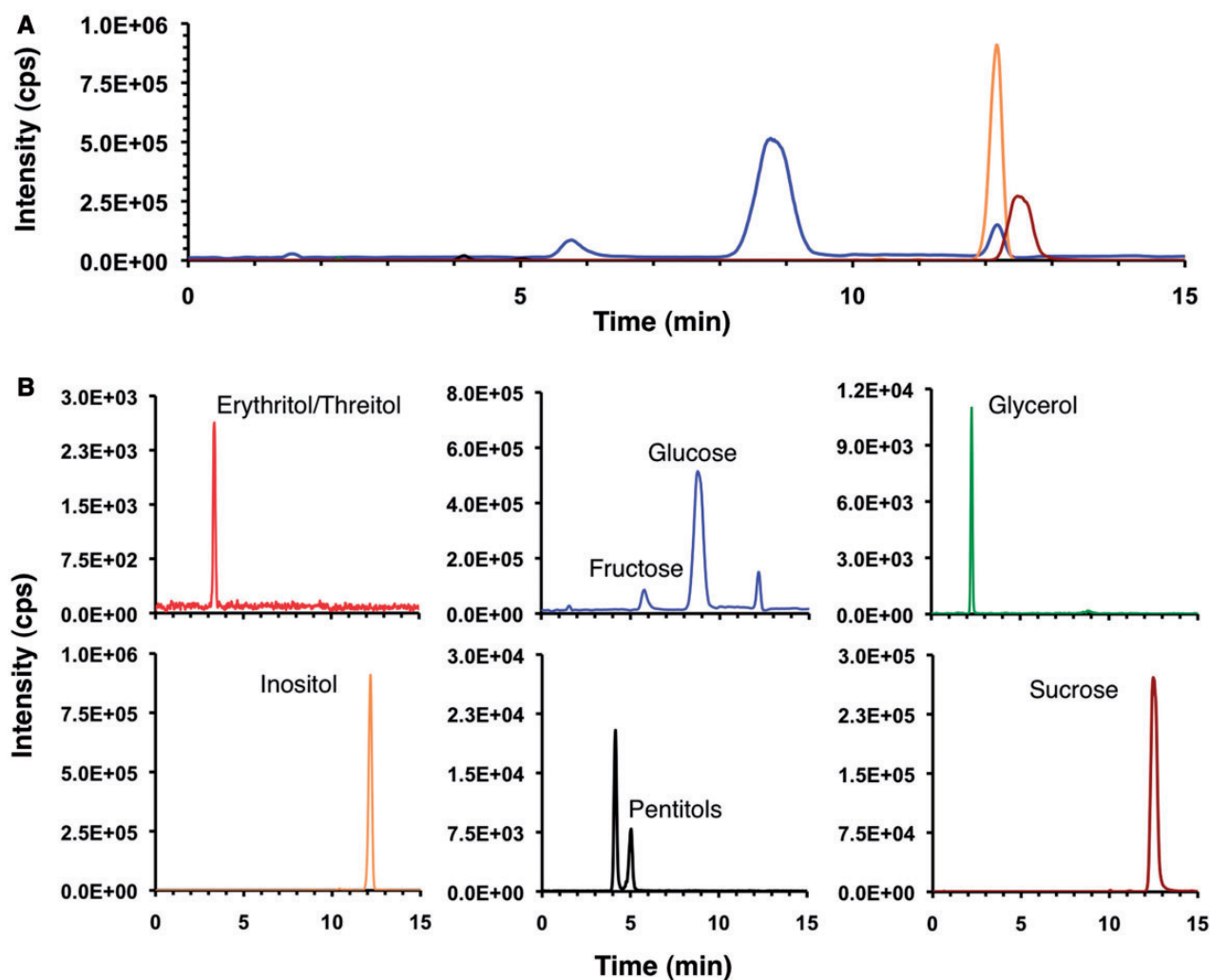


Fig. 4 LC-MS/MS analyses of sugars/sugar alcohols from 27 DPA embryos. (A) Total ion count (TIC) of sugars and sugar alcohols detected by multiple reaction monitoring (MRM). Each color represents a specific transition associated with a sugar or sugar alcohol metabolite. Separation and assignment of sugars and sugar alcohols were carried out using the LC-MS/MS conditions mentioned in the Materials and Methods and [Supplementary Table S1](#). (B) LC-MS/MS chromatogram for individual sugars/sugar alcohols. Each chromatogram represents a transition parent/daughter ion associated with sugar(s) or sugar alcohol(s). One transition could have several peaks corresponding to isomers (see fructose/glucose transition).

containing [$U\text{-}^{13}\text{C}$]glycine as an internal standard. **Fig. 5** shows the LC-MS/MS chromatograms of all the amino acids present in 27 DPA *Physaria* embryos. The main amino acid was found to be glutamine with 5715.4 ± 149.3 pmol per embryo at 27 DPA, which represents $>60\%$ of all the total free amino acid content ([Supplementary Tables S5, 6](#)).

LC-MS/MS quantification of organic acids and phosphorylated compounds. Organic acids and phosphorylated compounds are of particular interest because they are involved in core metabolic pathways, such as glycolysis, the pentose phosphate pathway, the Calvin cycle and the tricarboxylic acid cycle. These anionic metabolites were quantified in *Physaria* embryos using the LC-MS/MS method previously described (Alonso et al. 2010b, Koubaa et al. 2013, Cocuron and Alonso 2014) and

adding [$U\text{-}^{13}\text{C}$]fumarate as an internal standard. Optimized mass spectrometric parameters and retention times for each compound are listed in [Supplementary Table S3](#). The DPs for hexose phosphates, UMP, glycerol phosphates, AMP and malate for the biological sample were changed from -40 to -160 V, -45 to -180 V, -60 to -140 V, -60 to -180 V and -50 to -150 V, respectively, to avoid saturation of the mass spectrometer detector. A previous study (Alonso et al. 2010b) and [Supplementary Table S3](#) report the validation results for the established method. The calibration graphs for the organic acids and phosphorylated compounds showed excellent linearity from low fmole to high pmole levels, with correlation coefficients >0.9749 . The limits of detection varied from 0.1 to 122.5 fmol and the limits of quantification were between 0.5 and 408.5 fmol ([Supplementary Table S3](#)). The erythrose

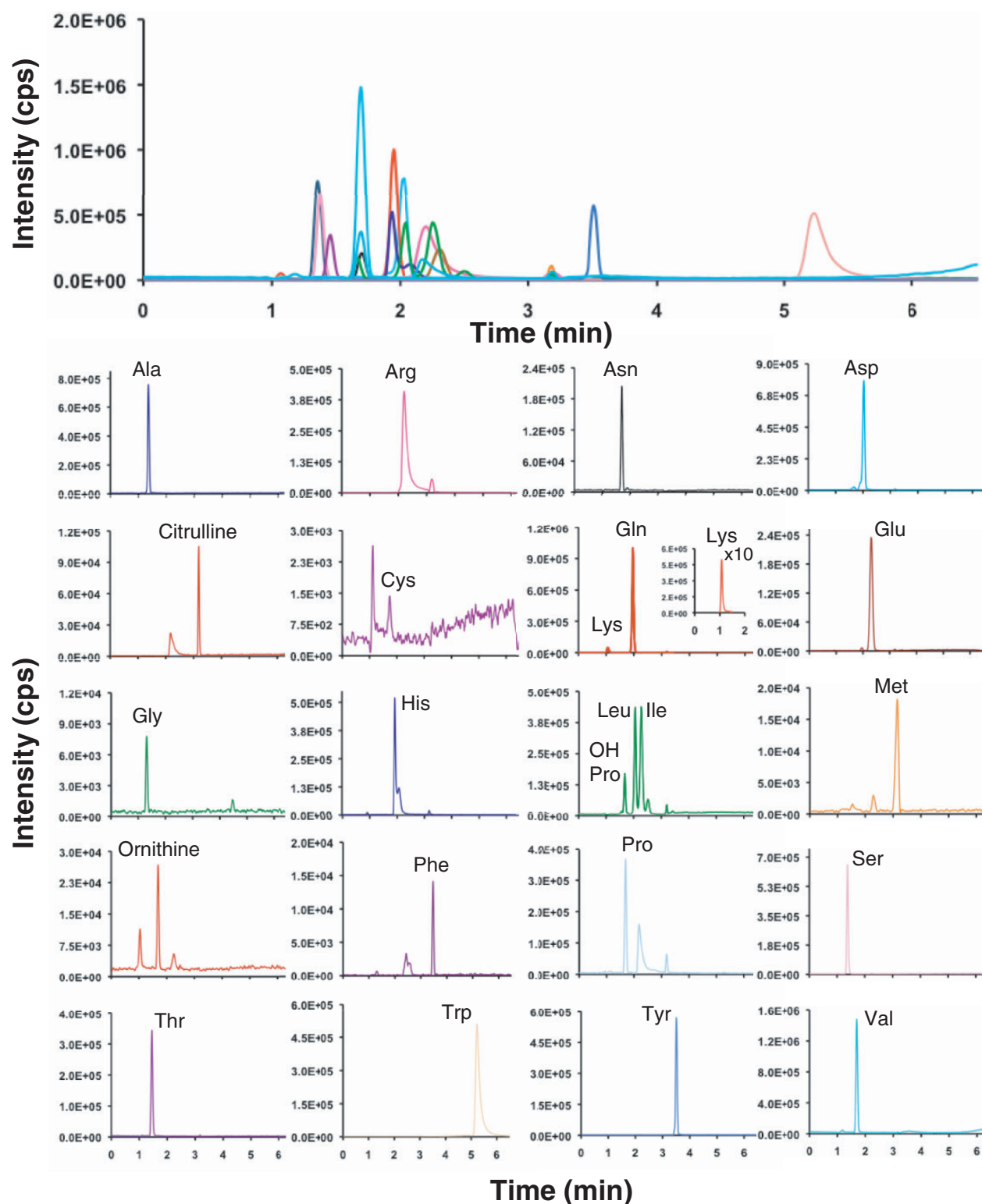


Fig. 5 LC-MS/MS analyses of amino acids from 27 DPA embryos. (A) Total ion count (TIC) of amino acids detected by multiple reaction monitoring (MRM). Each color represents a specific transition associated with an amino acid. Separation and assignment of amino acids were carried out using the LC-MS/MS conditions mentioned in the Materials and Methods and [Supplementary Table S2](#). (B) LC-MS/MS chromatogram for each individual amino acid. Each chromatogram represents a transition parent/daughter ion associated with amino acid(s). One transition could have several peaks corresponding to isomers (see lysine/glutamine). Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; Cys, cysteine; Lys, lysine; Gln, glutamine; Glu, glutamate; Gly, glycine; His, histidine; OHPro, hydroxyproline; Leu, leucine; Ile, isoleucine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

4-phosphate was the exception, with higher limits of detection and quantification (3157.9 and 10526.3 fmol, respectively).

Phosphorylated metabolites and organic acids were extracted from *Physaria* embryos using boiling water, with a recovery of >50% and 72%, respectively (**Supplementary Table S4**). It is important to note that this extraction process was not suitable for the oxaloacetate or the ribose 1-phosphate. The main organic acids at 27 DPA were found to be malate and citrate, with 275.6 ± 39.1 and 261.4 ± 29.4 pmol per embryo, respectively, whereas the major phosphorylated compounds were UDP-glucose and glucose 6-phosphate with 297.7 ± 41.7 and 238.8 ± 3.1 pmol per embryo, respectively (**Supplementary Table S5**). Malate and citrate are involved in the tricarboxylic acid cycle as well as in fatty acid synthesis, while UDP-glucose is a precursor of the cell wall. Glucose 6-phosphate is a key phosphorylated compound that is located at the crossroads of several metabolic pathways: glycolysis, the oxidative pentose phosphate pathway, the Calvin cycle, and starch and cell wall synthesis.

Comparative metabolic analyses of Physaria embryos. Using the techniques described above, metabolite levels involved in core pathways of *Physaria* embryos at six developmental stages were measured and reported (**Fig. 6; Supplementary Tables S5, S6**). Intermediates of glycolysis, the oxidative pentose phosphate pathway, the Calvin cycle and the tricarboxylic acid cycle were quantified, indicating the occurrence of these pathways in *Physaria* embryos. Furthermore, the level of the majority of metabolites increased from 18 to 27 DPA, which underlines a net synthesis during these developmental stages. The order of abundance for intermediates, from highest to lowest, was found to be sugars (glucose and sucrose), then amino acids (glutamine), then finally phosphorylated compounds (glucose 6-phosphate and UDP-glucose) and organic acids (malate and citrate). The levels of glucose and sucrose increased across the developmental stages, indicating that there is a substantial source of carbon available to *Physaria* embryos. Most of the phosphorylated compounds are commonly shared between glycolysis, the oxidative pentose phosphate pathway and the Calvin cycle. However, the level of ribulose 1,5-bisphosphate, a metabolite specific to the Calvin cycle, increased until 30 DPA, which reveals that *Physaria* embryos are capable of photosynthetic activity. Most free amino acids accumulated over time and their levels remained high, indicating the following: (i) histidine is produced from pentose phosphate and the increase of its level underlines the occurrence of the oxidative part of the pentose phosphate pathway; (ii) the accumulation of amino acids produced from intermediaries of the tricarboxylic acid cycle shows that the anaplerotic pathway (phosphoenolpyruvate) is active in developing *Physaria* embryos; and (iii) glycine, serine, phenylalanine, tyrosine, tryptophan, alanine, lysine, valine and leucine accumulated to much higher levels than their respective glycolytic precursor, which indicates that a substantial amount of carbon is drained from glycolysis to sustain the production of these amino acids.

Discussion

High-throughput LC-MS/MS methods for sugars, sugar alcohols and amino acids

We have confirmed that in *Physaria*, lesquerolic acid synthesis occurred in the embryos (**Supplementary Fig. S1**), which is fundamentally and metabolically different from castor that produces and accumulates ricinoleic acid in its endosperm (Donaldson 1977, Bafor et al. 1991). In order to unravel the metabolic pathways involved in hydroxy fatty acid synthesis, it was imperative to dissect and study the organ which actually produces those compounds: the embryo. Furthermore, it was necessary to develop methods that were simple in terms of sample preparation yet highly sensitive to quantifying intracellular metabolites at the early stages of development (when lesquerolic acid synthesis occurs). Although numerous studies reported methods to analyze sugars and sugar alcohols, these methods had significant drawbacks: (i) GC-MS analysis involving a derivatization step of the sugars to increase their volatility (Ruiz-Matute et al. 2011, Koubaa et al. 2012); (ii) HPLC separation coupled to a differential refraction detector (low sensitivity) or to a UV detector after derivatization of the sugars (Grimble et al. 1983, Kelebek and Selli 2011); and (iii) LC-MS methods that also required a step of derivatization or formation of adducts (Kato and Numajiri 1991, Rogatsky et al. 2005). In this study, we used LC-MS/MS in MRM mode to achieve a highly sensitive quantification of sugars and sugar alcohols without additives or derivatization. In comparison with existing LC-MS/MS methods (Bajad et al. 2006), the one described here has the advantage of separating isomers particularly important in plant metabolism, such as but not limited to fructose and glucose. The most commonly used method to quantify amino acids in a biological sample is HPLC coupled to UV detection of the AccQTag derivatives (Narayan et al. 2011). Recently, new techniques have been developed to analyze underivatized amino acids by LC-MS/MS (Bajad et al. 2006, Gu et al. 2007, Thiele et al. 2012). One of these new techniques makes use of a strong cation exchange column which achieved a good separation of the 20 amino acids but expanded the runs to >1 h (Thiele et al. 2012). Another new technique employs a reverse phase column that did considerably shorten the runs but required the addition of an ion-pairing agent. Furthermore, several isomers, such as isoleucine and leucine, co-eluted and were separated by their isomer selective daughter ion (Gu et al. 2007). However, this technique is actually dependent upon the mass spectrometer instrument. In the present study, the development of a high-throughput method that separated all underivatized amino acids without ion-pairing agent is reported.

Potential important pathways in developing Physaria embryos

The mother plant supplies substrates (sugars and amino acids) to the developing seeds. The main sugars and amino acids in developing *Physaria* embryos were found to be glucose, sucrose

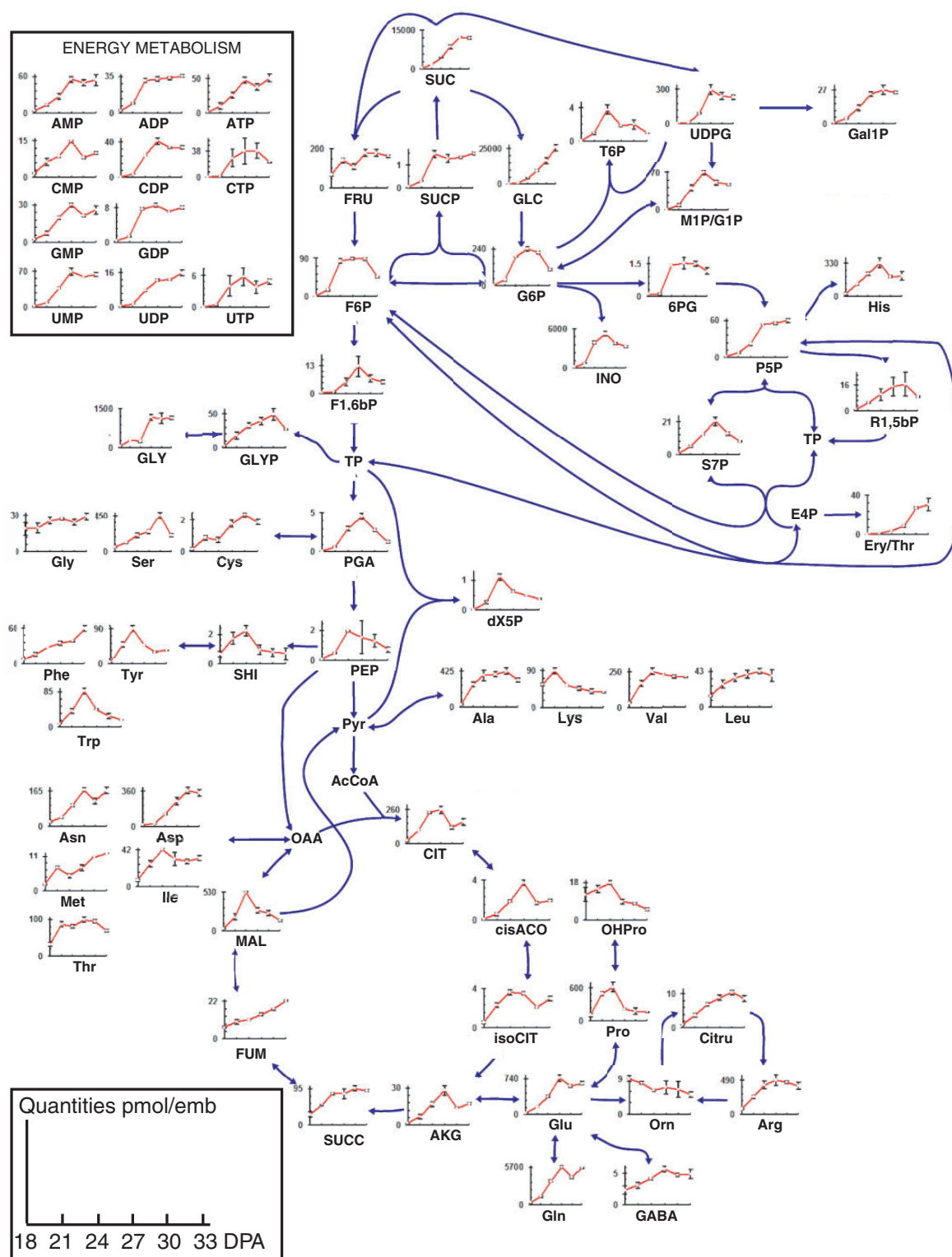


Fig. 6 Metabolic map of *Physaria* embryos at different stages of development. This is a representation of the data from [Supplementary Table S5](#). Values are expressed in pmol per embryo and are the average $\pm$ SD of three biological replicates from embryos harvested at 18, 21, 24, 27, 30 and 33 DPA. SUC, sucrose; FRU, fructose; GLC, glucose; INO, inositol; GLY, glyceral; Ery/Thr, erythritol/threitol; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; Cys, cysteine; Lys, lysine; Gln, glutamine; Glu, glutamate; Gly, glycine; His, histidine; OHPro, hydroxyproline; Leu, leucine; Ile, isoleucine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; GABA, 4-aminobutyric acid; Orn, ornithine; Citru, citrulline; T6P, trehalose 6-phosphate; UDPG, UDP-glucose; SUCP, sucrose 6-phosphate; G1P, glucose 1-phosphate; M1P/G1P, mannose 1-phosphate/glucose 1-phosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; 6PG, glucose 1-phosphate; P5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; TP, triose phosphate; E4P, erythrose 4-phosphate; Ery/Thr, erythritol/threitol; dX5P, deoxyxylulose 5-phosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; AcCoA, acetyl-coenzyme A; OAA, oxaloacetate; CIT, citrate; cisACO, cis-aconitate; OHPro, hydroxyproline; isoCIT, isocitrate; Pro, proline; Citru, citrulline; Glu, glutamate; Orn, ornithine; Arg, arginine; Gln, glutamine; GABA, 4-aminobutyric acid; SUCC, succinate; AKG, α -ketoglutarate; FUM, fumarate; MAL, malate; Ile, isoleucine; Met, methionine; Thr, threonine; Asn, asparagine; Asp, aspartate; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; Gly, glycine; Ser, serine; Cys, cysteine; and PGA, 3-phosphoglycerate.

(continued)

and glutamine, suggesting that those might be the main sources of carbon and nitrogen taken up by the embryos. Sugars are metabolized in the cytosol to provide the carbon precursors for biomass synthesis and accumulation in the embryos (Hills 2004, Baud and Lepiniec 2010). The comparative metabolomics analyses performed in this study have shown that (i) the majority of metabolites accumulate in *Physaria* embryos between 18 and 27 DPA; and (ii) several core metabolic pathways substantially drain carbon: the oxidative pentose phosphate pathway, the Calvin cycle, glycolysis, the tricarboxylic acid cycle and the anaplerotic pathway (**Fig. 6; Supplementary Table S5**).

In plants, fatty acid synthesis occurs predominantly in plastids and requires carbon, ATP (energy) and NAD(P)H (reducing power). The carbon source for fatty acid synthesis is in the form of acetyl-coenzyme A (acetyl-CoA). Malate and citrate were the main organic acids in *Physaria* embryos (**Fig. 6; Supplementary Table S5**). As in developing maize embryos, malate may play a key role in supplying carbon and NADPH necessary for fatty acid synthesis through plastidic malate dehydrogenase (Alonso et al. 2010a). Citrate may contribute to fatty acid elongation, providing cytosolic acetyl-CoA via ATP:citrate lyase (Fatland et al. 2000, Nikolau et al. 2000, Baud and Lepiniec 2010). Fatty acid synthesis also depends upon supplies of ATP and reducing power. In green seeds, light energy can be used by chloroplasts to generate ATP and NADPH (Browse and Slack 1985, Ohlrogge et al. 2004, Schwender et al. 2004, Goffman et al. 2005, Schwender et al. 2006, Allen et al. 2009), whereas plastids isolated from heterotrophic tissues must either generate these compounds internally or import them from the cytosol (Browse and Slack 1985, Hill 1991, Kleppinger-Sparace 1992, Smith et al. 1992, Neuhaus et al. 1993, Kang 1996, Alonso et al. 2007, Alonso et al. 2010a, Alonso et al. 2011). As shown in **Fig. 1**, developing *Physaria* are green and hence might be capable of photosynthesis. Indeed, the presence of increasing amounts of ribulose 1,5-bisphosphate across the developmental stages (**Fig. 6; Supplementary Table S5**) provides evidence for Calvin cycle activity. Taken together, these results indicate that part of the ATP and NADPH required for fatty acid synthesis is provided by photosynthetic conversion of light energy. Besides photosynthesis, the main metabolic pathways generating ATP and NADPH usually are mitochondrial respiration and the oxidative pentose phosphate pathway, respectively. According to the metabolomic data in developing *Physaria* embryos (**Fig. 6; Supplementary Table S5**), there is indeed a substantial amount of carbon going through: (i) the oxidative part of the pentose phosphate pathway generating NADPH; and (ii) the tricarboxylic acid cycle, producing not only NADH but also ATP via oxidative phosphorylation. In conclusion, the metabolomic analyses conducted here highlighted the

pathways that are active in developing *Physaria* embryos. This study shows the importance and power of such metabolomic data to map metabolic pathways (**Fig. 6**), and to reveal insights into fatty acid synthesis in *P. fendleri*. However, the contribution of each pathway to fatty acid synthesis in terms of carbon, energy and reductant provision can only be accessed by measuring the carbon flow through the metabolic network. For this purpose, a ^{13}C -metabolic flux analysis (^{13}C -MFA) of developing *Physaria* embryos would be required.

Materials and Methods

Chemicals

All of the standards, as well as 3 N methanolic/HCl and toluene, were obtained from Sigma. $[\text{U-}^{13}\text{C}]$ Glucose, $[\text{U-}^{13}\text{C}]$ glycine and $[\text{U-}^{13}\text{C}]$ fumarate were purchased from Isotec. Solvents for GC-MS and LC-MSMS experiments were ordered from Fisher Scientific.

Plant growth

Physaria fendleri plants were grown in 18 cm pots in a growth chamber maintained at a temperature of 22°C with a light intensity of approximately $320\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ and a 16 h/8 h day/night photoperiod. At 2 weeks, seedlings were thinned to one per pot. Flowers were hand-pollinated, tagged at pollination and embryo development was recorded as DPA.

Biomass extraction

Oil, protein and starch were extracted from dissected embryos as described previously (Goffman et al. 2005, Alonso et al. 2007) with minor modifications.

Oil. Dried embryos (between 2 and 10 mg DW) were ground in a 2 ml Startedt tube containing one 5 mm tungsten bead, 590 nmol of triheptadecanoin as internal standard and 1 ml of hexane/isopropanol (2 : 1). Tubes were then placed into a mixer mill and were shaken for 5 min at a frequency of 30 Hz. Samples were centrifuged at $15,000\times g$ for 15 min at room temperature. The supernatant was carefully removed and pipetted into a 13×100 mm glass tube. To ensure a total extraction of oil, the steps above were repeated twice more. The pooled oil fractions were dried at 60°C under nitrogen and stored at -20°C or were subject to methylation. Fatty acids were methylated using methanolic/HCl. In brief, 150 μl of toluene and 0.5 ml of 3 N methanolic/HCl were added to the dried fatty acid extracts. The samples were transmethylated for 120 min at 80°C. The reaction was quenched using 250 μl of 5% (w/v) sodium bisulfate,

Fig. 6 Continued

6-phosphogluconic acid; P5P, pentose 5-phosphates; R1,5-bP, ribulose 1,5-bisphosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; F1,6bP, fructose 1,6-bisphosphate; GLYP, glycerol-phosphates; TP, triose phosphates; PGA, 2–3 phosphoglycerates; dX5P, deoxyxylulose 5-phosphate; PEP, phosphoenolpyruvate; SHI, shikimate; PYR, pyruvate; AcCoA, acetyl-CoA; CIT, citrate; cisACO, cis-aconitate; isoCIT, isocitrate; AKG, α -ketoglutarate; SUCC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate.

and 1 ml of hexane was added to extract FAMES. Samples were mixed and spun at $800\times g$ for 10 min at room temperature. A 200 μ l aliquot of supernatant was transferred into a 2 ml glass vial containing 800 μ l of hexanes, and FAMES were ready for GC-MS analysis.

Proteins. Proteins were extracted from the residual pellet after fatty acid extraction. Each tube received 0.5 ml of extraction buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1% (w/v) SDS] previously warmed to 42°C. Tubes were then vortexed for 15 min at 42°C. Samples were centrifuged at $14,000\times g$ for 10 min and the supernatant was transferred into a 2 ml tube. The process was repeated once more. The supernatants were combined and ready for quantification.

Starch. Starch extraction was performed on the pellets after protein extraction. Pellets were resuspended in 1 ml of water, spun at $14,000\times g$ for 10 min at room temperature then the supernatant was discarded. This step was repeated once more. A 250 μ l aliquot of 0.1 M acetate buffer pH 4.8 was added to the pellet and samples were autoclaved for 1 h at 120°C and 21 psi. After autoclaving, 490 μ l of 0.1 M acetate buffer pH 4.8 and 33 U of amyloglucosidase from the Megazyme International Ireland Ltd Total Starch Assay Kit were incorporated. Starch samples were incubated for 1 h at 55°C and centrifuged at $14,000\times g$ for 15 min. A 500 μ l aliquot of supernatant was pipetted into a 1.5 ml tube to be used for quantification by LC-MS/MS.

Biomass quantification

Oil. Oil content was determined by GC-MS of FAMES. FAMES were analyzed using a Thermo Trace gas chromatograph ultra coupled to a single-quadrupole DSQ II mass spectrometer. FAME derivatives were separated using a VF-23MS capillary (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) column from Agilent at a constant flow rate of 1.5 ml min⁻¹. Helium was used as the carrier gas. The GC conditions were as follows: initial temperature was set to 150°C and held for 3 min. The oven temperature was then raised to 210°C at 5°C min⁻¹. A second ramp was applied at a rate of 25°C min⁻¹ to reach a final temperature of 250°C which was held for 1 min. The injection temperature was fixed at 240°C and the injection mode was set to split with a split ratio of 20. For the MS analysis, the mass spectra were acquired using electron impact (EI) ionization in positive ion mode. The ion source and the interface temperatures were set to 280 and 240°C, respectively. GC-MS data were acquired and processed using Xcalibur software. FAME derivatives were identified using the NIST library and neat FAME standards purchased from Sigma.

Proteins. Proteins were quantified using the DC Protein Assay kit from Bio-Rad. Bovine serum albumin (BSA) was chosen as the standard to determine the amount of proteins in *Physaria* embryos.

Starch. Starch glucosyl units were quantified using LC-MS/MS (see section 'LC-MS/MS quantification of intracellular

metabolites'). In brief, 5 μ l of extract was diluted in 995 μ l of acetonitrile/H₂O (60:40) solution and 10 μ l of sample was injected onto the column. The LC and MS conditions were exactly the same as in the sugars/sugar alcohol method (see below).

Metabolite extraction

Embryos were collected, in a 2 ml screw cap tube sitting on ice, at a consistent hour of the morning to avoid biased data due to metabolism changes over the day. Embryo samples were lyophilized and weighed before the extraction of metabolites. Dried embryos (between 1 and 5 mg DW) were ground in a 2 ml screw cap tube containing a 5 mm tungsten bead for 3 min at 30 Hz in a mixer mill. Then 500, 500 and 1,000 nmol of U-¹³C-labeled glucose, glycine and fumarate were added, respectively, to each tube as internal standards. Water-soluble metabolites (soluble sugars, sugar alcohols, amino acids, organic acids and phosphorylated compounds) were extracted using 1 ml of boiling water and then placed for 10 min in a water bath set at 100°C as previously described (Alonso *et al.* 2010b, Cocuron and Alonso 2014). The extracts were then transferred onto ice and centrifuged at $14,000\times g$ for 5 min at 4°C. The supernatants were recovered and filtered using 5 ml syringes and 0.22 μ m filters. The remaining pellets were rinsed once with 1 ml of cold water (4°C), vortexed, centrifuged and then filtered as described above. The syringes and filters were rinsed once with 1.5 ml of cold water. Finally, the water-soluble metabolite fractions were freeze-dried overnight.

LC-MS/MS quantification of intracellular metabolites

The LC was performed with an UHPLC (ultra high pressure liquid chromatography) 1290 column from Agilent Technologies, Inc. The MS/MS analyses were performed with a hybrid triple-quadrupole/ion trap mass spectrometer QTRAP 5500 from AB Sciex. LC-MS/MS data were acquired and processed using Analyst 1.6.1 software.

After lyophilization, extracts were resuspended in 300 μ l of water, vortexed and loaded on a 0.2 μ m nanosep MF centrifugal device. The extracts were centrifuged at $14,000\times g$ for 10 min at 4°C.

Sugars and sugar alcohols. Extracts were transferred to LC-MS/MS glass vials and then placed in the autosampler kept at 20°C. A 10 μ l aliquot of extract was diluted in 90 μ l of acetonitrile/water (60:40) solution and 5 μ l of sample was injected onto the column. The LC analysis was carried out at 30°C. The sugars and sugar alcohols were separated using a Shodex Asahipak NH2P-50 2D column (2.0 $\times$ 150 mm) with a Shodex Asahipak NH2P-50G 2A guard column from Showa Denko America. A gradient used to separate the compounds consisted of acetonitrile (solvent A) and water (solvent B). The total LC-MS/MS run was 20 min with a flow rate of 400 μ l min⁻¹. The gradient was as follows: A = 0–8.5 min 85%, 8.5–8.6 min 78%, 8.6–15 min 78%, 15–15.1 min 85%, 15.1–20 min

85%. The mass spectra were acquired using turbo spray ionization of 4,500 V in negative ion mode and MRM. The curtain gas (nitrogen) and CAD (collision activated dissociation) were set to 30 psi and medium, respectively. The MS was set to have a dwell time of 100 ms. The parameters for each sugar were set as shown in [Supplementary Table S1](#).

Amino acids. A 10 μ l aliquot of embryo extract was added in a vial containing 90 μ l of water with 1 mM hydrochloric acid, and 5 μ l of sample was injected onto the column. Samples in the autosampler were kept at 15°C while the LC analysis was carried out at 30°C. The amino acids were separated using a Hypercarb column (100 $\times$ 2.1 mm, 5 μ m pore) from Thermo Fisher Scientific. A gradient used to separate the compounds consisted of acetonitrile with 0.1% formic acid (solvent A) and water with 0.1% formic acid (solvent B). The total LC-MS/MS run was 10 min with a flow rate of 200 μ l min⁻¹. The gradient was as follows: A = 0–1 min 0%, 1–1.1 min 20%, 1.1–2.7 min 45%, 2.7–5 min 60%, 5–5.1 min 90%, 5.1–7 min 90%, 7–7.1 min 0%, 7.1–10 min 0%. The mass spectra were acquired using turbo spray ionization of 2,500 V in positive ion mode and MRM. The curtain gas (nitrogen) and the CAD were set to 30 psi and medium, respectively. The MS was set to have a dwell time of 35 ms. The parameters for each amino acid were defined as shown in [Supplementary Table S2](#).

Phosphorylated compounds and organic acids. A 40 μ l aliquot of extract was resuspended in 200 μ l of water, and 15 μ l was injected onto the column.

The separation and quantification of phosphorylated compounds and organic acids were performed as previously described (Alonso et al. 2010b, Koubaa et al. 2013, Cocuron and Alonso 2014). The parameters for each metabolite were set as shown in [Supplementary Table S3](#).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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