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EXOGENOUS GLUTAMINE INCREASES LIPID ACCUMULATION IN DEVELOPING SEEDS OF CASTOR BEAN (*RICINUS COMMUNIS L.*) CULTURED IN VITRO

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Abstract: This report describes biomass production and compositional changes of developing castor seeds in response to change in the nitrogen resource (glutamine) of the medium. During the early developmental period (24-36 days after pollination), oil was found to initially accumulate in the developing seeds. Carbohydrates and oil were inversely related after glutamine provision (35 mM, in the culture medium). [U-^{14}C] sucrose labeling was used to investigate the effect of metabolic fluxes among different storage materials. Addition of glutamine led to a 7% increase of labeling in lipids and an inverse decrease of labeling in carbohydrates. It was postulated that changes in the glutamine concentration in the medium are likely to influence the partitioning of resources between the various storage products, especially carbohydrates and oil. These observations will contribute to a better understanding of assimilate partitioning in developing castor seeds and the development of molecular strategies to improve castor bean seed quality and plant breeding studies.

Key words: oil accumulation; glutamine; ^{14}C radioactive labeling; developing castor seed; RNA-Seq

Abbreviations: Gln, glutamine; **Suc**, Sucrose; **G-6-P,** Glucose-6-Phosphate; **F-6-P,** fructose 6-phosphate; **F-1,6-bP,** Fructose 1,6 phosphate; **GAP,** glyceraldehyde 3-P; **3-PGA,** 3 phosphoglyceric acid; **2-PGA,** 2 phosphoglyceric acid; **PEP,** phosphoenolpyruvate; **Pru,** pyruvate; **Lac,** lactate; **Ace,** acetic acid; **Ald,** aldehyde; **TAG,** triacylglycerol; **2-OG,** 2-oxoglutarate; **Succ,** succinic acid; **Mal,** malate; **OAA,** oxaloacetic acid; **Cit,** citrate; **Icit,** isocitrate; **DEGs**, differentially expressed genes; **GO**, Gene Ontology; **KEGG**, Kyoto Encyclopedia of Genes and Genomes; **RPKM**, Reads Per Kilo bases per Million reads; **FDR**, false discovery rate; **FA**, fatty acid; **DPM**, disintegrations per minute

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INTRODUCTION

The process of seed development is critical as it determines the accumulation of storage reservoirs (such as oil, protein and carbohydrates) in mature seeds. The synthesis of different storage products during seed development has been studied in detail, e.g. in soybean and Arabidopsis (Allen and Young, 2013; Sulpice et al., 2013). Carbon and nitrogen availability from the maternal plant plays an important role in protein, carbohydrates and oil synthesis in seeds and the relationship between C and N metabolism has been the target of numerous studies (Munier-Jolain et al., 2008; Peuke, 2010; Truong et al., 2013). Sucrose (Suc) and amino acids, mainly glutamine (Gln), are the primary sources of carbon and nitrogen available to the developing seeds (Hall and Baker, 1972; Hocking, 1982). As a primary form of translocated photosynthate to the seed, sucrose is the critical source of carbon and energy in the synthesis of protein, carbohydrates and oil (Hall and Baker, 1971). On the other hand, the principal N source supplying the seed for growth and protein synthesis is glutamine, accounting for over

85% of the amino-N in phloem sap destined for fruits (Hocking, 1982). The composition of individual seeds responds to variation in carbon and nitrogen availability from the maternal assimilate supply (Severino and Auld, 2013). Protein, carbohydrates and oil accumulation depend on both the capacity for assimilate uptake, metabolism and storage within the seed (zygotic control), and the assimilate supply to the seed (maternal control) (Rotundo et al., 2011). Although the accumulation of oil and other storage reservoirs is complexly controlled by carbon and nitrogen availability, the underlying metabolic control mechanisms are not fully understood (Hay and Schwender, 2011).

Castor bean (*Ricinus communis* L.) is considered as a potential bioenergy oilseed crop because of its oil quality and quantity for renewable non-food uses, including biodiesel and industrial chemical purposes (Brown et al., 2012). Castor seed is a highly specialized storage organ for the accumulation of lipids, proteins and carbohydrates in the endosperm. Because of the nearly uniform ricinoleic acid content of oil (approximately 90%) from castor bean seeds, castor oil is the only commercially available natural hydroxylated fatty acid extensively used in polymers, lubricants, polyurethane coatings, cosmetics, plastics, etc., and miscible with methanol and ethanol at normal temperatures (Qiu et al., 2010). With its high quality oil, various storage reserves and the recently sequenced genome (Chan et al., 2010), castor bean is considered to be a model plant for studying carbon assimilation in oilseeds (Hajduch et al., 2011). Furthermore, *in planta* evidences suggest that the growth and composition of castor bean seed are controlled largely by the availability of assimilates provided from the maternal plant, because this plant has a large capacity to adjust the sink (Grimmer and Komor, 1999). Hence, it provides an excellent test case for studying assimilate partitioning into various storage reserves in oilseeds.

Previous studies on assimilate partitioning were carried out on intact plants where many factors confound seed N supply and the accumulation of storage products (Reddy and Matcha, 2010). One approach to examine directly the seed response to N supply is to culture them *in vitro* (Saravitz and Raper, 1995; Allen and Young, 2013). In addition, due to the long life cycle of castor beans, using the *in vitro* culture of developing seed as an experimental system is an efficient and practical approach for evaluating the influence of carbon and nitrogen concentration of the culture solution on assimilate partitioning between storage products (Saravitz and Raper, 1995; Hayati et al., 1996; Allen and Young, 2013). Commonly, the biosynthesis of N-involving organic compounds (including amino acids and proteins), carbohydrates and lipid accumulation are closely interdependent (Huppe and Turpin, 1994; Lawlor, 2002). All these constituents require C-skeletons derived from imported sucrose during seed development, and hence, the observed negative relationship among seed storage proteins, carbohydrates and oil concentration is related to the regulation of carbon flux between these competing synthetic pathways (Weselake et al., 2009; Alonso et al., 2010). In particular, the aim of this study was to determine whether glutamine supply could influence the use of carbon derived from sucrose for proteins and carbohydrates versus oil synthesis. Hence, in this study, the first goal was to test a hypothesis that changes in the nitrogen source (glutamine) supply are likely to influence the partitioning of resources among the various storage products, such as proteins, carbohydrates and oils. The second goal was to identify the underlying metabolic control mechanisms that determine resource partitioning among proteins, carbohydrates and oils. Compared to soybean and sunflower, limited work has been done in castor bean, and a better understanding of the regulation of carbon flux among proteins, carbohydrates and oil synthesis may lead to the development of molecular strategies to improve castor been seed quality and in plant breeding studies.

MATERIALS AND METHODS

Plant material and culturing

Seeds of castor bean cv.ZB197 elite inbred line (kindly provided by the Zibo Academy of Agricultural Sciences, Shandong, China) were cultivated at the field station. Plants were watered daily and capsules with

three seeds were removed from the bottom half of plants at about 24-36 DAP (days after pollination, initial phase of lipid accumulation). The selected pods were fully expended and the seeds with a fresh weight greater than 250 mg were used for *in vitro* seed growth.

Capsules harvested from plants were surface sterilized by submerging them in constantly agitated water with 0.5% NaOCl (v/v) with 0.01% Tween 20 (v/v) for 5-10 min. The pods were rinsed three times with water to remove the detergent and then immersed in 70% ethanol for 10 min. Subsequently, capsules were rinsed three times with sterilized water; young seeds were carefully dissected and transferred aseptically to presterilized 150 mL Erlenmeyer flasks containing 30 mL of MS medium for seed culture *in vitro*. A modified MS medium was supplied with 20% (m/v) polyethylene glycol 4,000 and 50 mM MES (2-(N-Morpholino)ethanesulfonic acid) were added to adjust osmotic potential, and the pH was adjusted to 5.8 with 1 N NaOH. The concentration of agar in MS medium was initially screened from 0.4% to 0.8% to ensure that the seed surface was in good contact with the medium. Sucrose concentration was set as 85 mM to support growth and to establish an optimum rate under which cultured seeds grow well *in vitro*. Usually, the well-developed capsule is trilocular containing one large mottled seed per locule. One of the three seeds from the same capsule was considered an initial control (CK), and its dry weight and accumulated storage reserves (e.g., lipids, proteins and sugars) were measured; the other two seeds were used for *in vitro* culturing experiments, where one was cultured on the MS medium at 25°C and the other under the same conditions with glutamine supplementation. Using preliminary results and an extensive screening for sucrose concentration in the medium, an optimal concentration of solid medium with 85 mM sucrose and 0.45% agar was determined for culturing the young seeds at about 24-36 DAP. This medium allowed for stable growth of seed dry weight within 14 days. Castor seeds (DAP 24) were dissected from the capsules and cultured in medium with different glutamine concentrations (0, 5, 15, 25, 35 and 45 mM). Six days of culturing with or without glutamine resulted in growth rates between 0.55±0.12 and 0.60±0.07 mg dry weight per day per seed, consistent with *in planta* growth measured at 0.50 to 0.65 mg dry weight per day per seed in castor bean. All experiments were repeated at least six times, totaling more than 20 capsules containing 60 seeds with different treatments being tested. Seeds were maintained in culture for 6 days, and cultures visibly contaminated were discarded. At the end of the culturing period, seeds were washed and then quickly frozen in liquid nitrogen, lyophilized to dryness, and stored at -80°C until further processing. The initial fresh weight of each seed was measured and seeds with similar weights were matched and assigned randomly to one of the glutamine treatments and the control. The seeds were removed from the flasks after 6 days in culture, rinsed in water, blotted dry on filter paper and weighed. Seeds were placed in an oven at 65°C for 2 days for dry weight determination.

Biochemical quantification of storage reserves

For total lipid extraction, we used the method previously described by Xu et al. (2011) wherein ground seeds were weighed into pre-weighed test tubes and covered with 3 mL hexane:isopropanol solvent (3:2). The resulting supernatant (hexane phase) was collected via centrifugation at 5000 g for 5 min, and then total lipids dissolved in hexane phase were dried in a vacuum oven at a pressure of 60 KPa at 50°C and determined gravimetrically.

For total sugar extraction, we used the method previously described by Wen et al. (2009). In brief, 0.1 g fine powder (dry weight) of seeds was dissolved in 5 mL of deionized water with 200 μL ether, and the mixture was homogenized and heated in a water bath at 80°C for 30 min. After cooling, a saturated neutral lead acetate solution was added to remove proteins and then the total mixture was centrifuged at 5,000 g for 10 min, with 1 g sodium oxalate powder added to the supernatant to remove the lead acetate. Following centrifugation, 0.5 mL of transparent solution was mixed with 2.5 mL anthrone reagent (1 g anthrone dissolved in 1 L 72% (v/v) cool sulfuric acid) and

heated for 10 min in boiling water, and the OD value read at 620 nm in a DU-800 UV/visible light spectrophotometer (Beckman Coulter, USA).

For total protein extraction, we used the method previously described by Brandão et al. (2010), wherein 0.1 g (dry weight) of fine powder of seeds was dissolved in petroleum ether and gently agitated (ca. 15 min) to remove the oil. The powder was then mixed with a buffer containing 50 mM Tris-HCl, pH 8.8, 1.5 mM KCl, 10 mM dithiothreitol (DTT), and 1.0 mM phenylmethanesulfonyl fluoride (PMSF) in a 10:1 (v/m) ratio. The resulting mixture was centrifuged at 4°C and 5000 g for 5 min, and the supernatant was used for determining the protein content by the Coomassie Brilliant Blue method (Bradford, 1976).

RNA-Seq and RT-qPCR analyses

Young seeds cultured *in vitro* on medium with (G) and without glutamine (C) for three days were collected for RNA-Seq analysis. In total, three independent seeds from both control and glutamine treatments were respectively pooled to extract total RNA. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. cDNA libraries were constructed and then sequenced by Novogene (Beijing, China) using an Illumina HiSeq 2000 platform to generate single-end 50 bp reads. Sequencing raw data from each library was preprocessed using TopHat, allowing up to 2 mismatches (Trapnell et al., 2009). This also enabled the filtering out of clipped adapter sequences, low-quality reads with the Phred quality score <20 and also reads that mapped to more than one position with similar scores in the castor bean reference genome (http://castorbean.jcvi.org/index.php). For differential expression analyses, the RPKM (Reads Per Kilo bases per Million reads) method as described by Mortazavi et al. (2008) was performed to test the gene expression of clean reads mapped to exon regions by applying Fisher's exact test to the number of reads mapping uniquely to each locus. Resulting *P*-values (a threshold of 5% yields a false positive rate of 5% among all null features in the data set) for significance analysis were adjusted to Q-values (similar to the well known *P*-value, except it is a measure of significance in terms of the false discovery rate rather than the false positive rate) for multiple testing using the false discovery rate (FDR) (Storey and Tibshirani, 2003). A 5% significance level threshold FDR and normalized $\log_2$ transformed ratio >1 were applied for detecting differentially expressed genes between two libraries (Benjamini and Hochberg, 1995; Anders and Huber, 2010). Functional annotation of DEGs (differentially expressed genes) was performed separately to test for enrichments in GO (Gene Ontology) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, and significantly enriched GO and KEGG terms were identified. *P*-values ≤0.05 following Bonferroni correction were used for term identification in both GO and KEGG enrichment analyses (Kanehisa et al., 2004; Young et al., 2010).

Ten genes that were identified by RNA-Seq to be expressed differentially between C and G libraries were selected for validation by RT-qPCR (Table S5). Additionally, the primers for these genes were designed using Primer Express software (Applied Biosystems, ver. 3.0). For the analysis of gene expression by RT-qPCR, we used RNA isolated as described above, with three independent biological replicates. First-strand cDNA was synthesized according to the instructions of the Takara Bio for the Primer Script™ PCR Kit. The real-time PCR reactions were performed on an Applied Biosystems 7500 Real-Time PCR System using the intercalation dye SYBR as the fluorescent reporter. The optimized PCR reactions (25 μl) contained 200 ng of first-strand cDNA template, 2.5 μL 10×PCR buffer, 0.5 μM of each primer, 0.25 mM of dNTPs, and 1.2 μM of Trans-startTM HiFi DNA polymerase (Transgen, Beijing, China). The following conditions were used for PCR: initial denaturation at 95°C for 1 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55-58°C for 20 s and extension at 72°C for 5 min. The analysis and normalization of gene expression were performed in the 7500 system SDS software (Applied Biosystems), using the castor bean actin gene (30131.m007032) as internal reference to normalize the data for all samples.

Analysis of 14C labeling metabolic flux

According to the method described by Goffman et al. (2005), 10 uL [U-^{14}C]-sucrose was added to flasks containing 20 mL unlabeled culture medium and sealed with airtight lids. After 6 days in culture, the seeds were rinsed, dried and ground to a fine powder using a ball mill. Fine powder was extracted using 2 mL of mixture solution (hexane:isopropanol 3:2 v/v), centrifuged, and the hexane phase was removed, while the polar phase was re-extracted with an additional 1 mL of hexane. The pooled hexane extracts were used for radioactivity detection of oil. The remaining polar water/isopropanol phase was used for radioactivity detection of sugars. To recover proteins, the sediment was extracted with 1 mL of 0.01 M sodium phosphate saline buffer (pH 7.4) containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.02% sodium azide and 0.0125% (w/v) SDS; after centrifugation the supernatant was used for radioactivity detection of proteins. ^{14}C levels in each fraction were determined by mixing with a same volume of scintillation solution (1 or 2.5 mL) and the radioactivity was determined by a liquid scintillation counter.

Statistical analysis

Statistical analysis was carried out in SPSS statistical software (SPSS Institute, Version 16.0) using one-way ANOVA. Mean separations were conducted using Duncan's multiple range test (α=0.05) to determine significance. Unless otherwise indicated, all experiments were done with at least three replications per treatment.

RESULTS

Effect of glutamine supplements on biomass composition in developing castor seeds

Different concentrations of glutamine in the culture medium resulted in different levels of oil accumulation (Table 1). The oil in developing seeds was slightly enhanced (from 1.23±1.05 to 1.34±0.89 mg/seed) as the glutamine concentration in culture medium increased from 0 to 25 mM. However, when supplied with a higher concentration of glutamine (≥35 mM), there was a dramatic effect on the oil accumulation of developing seeds. Similarly, the oil content significantly increased (from 12.17±1.03% to 12.58±1.21%) as glutamine was increased from 35 to 45 mM (Table 1). In contrast, there was no effect on the dry weight of developing seeds after glutamine treatments. Thus, exogenous glutamine provision can increase lipid accumulation in developing seeds of castor bean under an *in vitro* culture environment.

To further assess the effect of glutamine supplement on lipid accumulation in developing seeds, *in vitro* culturing of developing seeds on medium with (35 mM) and without glutamine was done. Seeds cultured for six days were harvested and the seed dry weights were analyzed to determine the differences. As shown in Table 2, the dry weights did not

Table 1. Effect of glutamine on oil accumulation in developing castor seeds *in vitro*. Values are presented as averages of at least five independent replicates ± SD (standard deviation). DW, dry weight of developing castor seeds. "a" and "b" denote statistical significance, *i.e.,* values with the same letter indicate that no significant difference was detected (P< 0.05 level; Duncan's student range test).

Glutamine (mM in the medium)	DW (mg/ seed)	Oil (mg/ seed)	Oil content (% of DW)
0	[a] 13.60±1.42	[a] 1.23±1.05	[a] 8.23±1.05
5	[a] 13.04±2.02	[a] 1.24±0.92	[ab] 8.64±0.92
15	[a] 12.95±1.62	[ab] 1.26±1.16	[ab] 9.32±1.72
25	[a] 13.14±2.13	[ab] 1.34±0.89	[ab] 9.64±1.48
35	[a] 12.83 ±1.82	[b] 1.58±1.03	[b] 12.17±1.03
45	[a] 12.97±2.11	[b] 1.75±1.23	[b] 12.58±1.21

Table 2. Effect of glutamine on seed storage material accumulation *in vitro*. Values are averages of at least six independent replicates. CK, the blank control without culture; C, the developing castor seeds culture without glutamine; G, the developing castor seeds culture with glutamine (35mM); "a" and "b" denote statistical significance, *i.e.*, values with the same letter indicate no significant difference (P< 0.05 level; Duncan's studentized range test).

	DW (mg)	Oil (%)	Protein (%)	Sugar (%)	Sediment (%)
CK	[a] 8.15±0.42	[a] 8.16±1.10	[a] 18.50±1.46	[b] 50.83±1.13	[a] 20.51±0.23
C	[b] 11.36 ±2.82	[a] 8.60±1.44	[a] 18.69±1.17	[b] 50.29±1.03	[a] 20.92±0.38
G	[b] 11.60±3.01	[b] 12.42±1.35	[a] 18.67±1.07	[a] 46.81±1.41	[a] 21.60±0.46

significantly differ between glutamine treatment and control conditions (11.60±3.01 and 11.36 ±2.82 mg, respectively), nor did the proteins and sediments after six days in culture. However, the lipid concentration (12.42±1.35%) under glutamine treatment was significantly higher than under control conditions (8.60±1.44%). The carbohydrate concentration under glutamine treatment (46.81±1.41%) was significantly lower than under control conditions (50.29±1.03%). These observations indicated that the accumulation of carbohydrates and oil is inversely related.

Glutamine supplements affect partitioning of [U-^{14}C]-Sucrose between carbohydrates and oil

^{14}C-labeling experiments were performed to assess the fractional distribution of carbon used for oil, protein and carbohydrate production. In the culturing experiment, labeled [U-^{14}C]-sucrose was provided, while the other carbon substrates remained unlabeled. Therefore, any change in ^{14}C abundance in lipids, proteins and carbohydrates reflected the fractional contribution of the particular carbon distribution. The total ^{14}C enrichment for storage products of control conditions (4366±259 DPM, disintegrations per minute) is almost two times higher than in glutamine treatment (2206±249 DPM, Fig. 1). In addition, although the ^{14}C distribution pattern in Fig. 1 was similar for glutamine treatment and control, statistically significant differences between the subunits were observed. The increased abundance of ^{14}C labeling in lipids (from 22±2% to 29±5%) and decreased abundance in carbohydrates (from 43±4% to 37±3%) confirms that the accumulations of carbohydrates and oils have an inverse relationship under glutamine supplementation. Thus, the increased use of sucrose-derived carbon for lipid accumulation (relative to control conditions) under glutamine treatment reflected a concomitant increase in flux from carbohydrates to lipids. However, proteins are also derived from [U-^{14}C]-sucrose but do not display differences between glutamine treatment and control conditions (Fig. 1). This indicates that protein biosynthesis is independent of the metabolic control at the beginning stage of lipid accumulation in developing castor seeds. Labeling experiments for

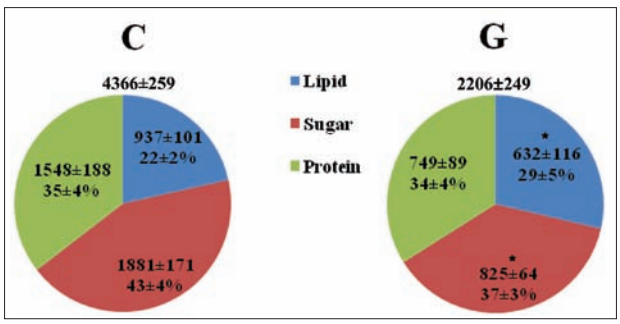

Fig. 1. Labeling changes in oil, protein and carbohydrates under glutamine treatment and the control condition. C, control; G, glutamine treatment. The number in the pie chart means radiolabeling of lipid, protein and carbohydrates (DPM, disintegrations per minute). The number above the pie chart means radiolabeling of developing castor seed under glutamine treatment and control condition, respectively (DPM); * denotes a significant difference of labeling fractional distribution compared to C and G (t-test $p<0.01$).

the glutamine treatment and control conditions revealed different patterns of carbon allocation into the three storage materials (Fig. 1, carbohydrates, lipids and proteins). Carbon partitioning varied between the glutamine treatment and control conditions, with sucrose providing more carbon for lipid accumulation under glutamine treatment.

Generation of flux maps for glutamine treatment from RNA-Seq analysis

To elucidate the potential molecular basis of the effects of glutamine on lipids, we performed RNA-Seq analysis on an Illumina platform. After filtering out the low-quality reads, a total of 7.08 and 6.18 million clean reads was obtained from control and glutamine treatment libraries (7.09 and 6.22 million raw reads, respectively as shown in Table 3). After mapping the clean reads against the reference castor bean genome (http://castorbean.jcvi.org/) and allowing two base mismatches, 68.58% and 49.64% reads were mapped to unique genes. To estimate our sequence quality and sequencing depth, the read coverage and saturation were analyzed for each library. Once the sequencing counts reached 2 million reads, the number of detected reads showed a trend towards saturation, indicating that the sequencing depth was sufficient to detect the global gene expression in each library (see Fig. S1).

Table 3. Summary of high throughput RNA-seq data. Total reads, the total number of raw sequence reads; Clean reads, high-quality reads after removing adaptor/acceptor sequences; Unique reads, reads that are mapped to more than one location; Uniquely mapped genes, reads that are mapped perfectly to a single location.

	Control	Glutamine treatment
Total reads (raw reads)	7,090,326	6,217,248
Clean reads	7,078,172	6,182,798
Nucleotides (nt)	353,908,600	309,139,900
Unique reads	5,395,783 (76.23%)	4,553,569 (73.65%)
Uniquely mapped genes	4,854,044 (68.58%)	3,069,433 (49.64%)

Global gene expression analyses

In total, 18552 unambiguous reads-mapped genes were identified with 2380 differentially expressed genes (DEGs), which comprised 827 upregulated and 1553 downregulated genes in the glutamine treatment library (Supplementary Table S1 and Fig. 2). For further analysis, 1955 highly up/downregulated DEGs were selected with a standard of reads ≥5, or $\log_2$ fold change ≥2 (Supplementary Table S2). To provide a functional overview of genes differentially expressed between the two libraries and gain a clearer picture of the differences, we employed gene ontology (GO) term enrichment analysis and found 921 GO-annotated DEGs categorized into 55 functional groups under biological process, cellular components, and molecular functions (Supplementary Fig. S2). Among the highly enriched groups (DEGs ≥50), most DEGs exhibited a downregulated expression in glutamine treatment, particularly the genes involved in ATP binding, catalytic activity and nucleotide binding in cell components, such as plasma membrane and chloroplast (Fig. 3). Further analysis of the KEGG annotations of the DEGs revealed the physiological functions of identified DEGs, yielding 1169 genes assigned to 111 pathways in the KEGG database (see Supplementary Table S3), with the most abundant being that of the metabolic pathway (243 genes), followed by biosynthesis of secondary metabolites (125 genes), ribosome (45 genes), oxidative phosphorylation (28 genes) and spliceosome (25 genes).

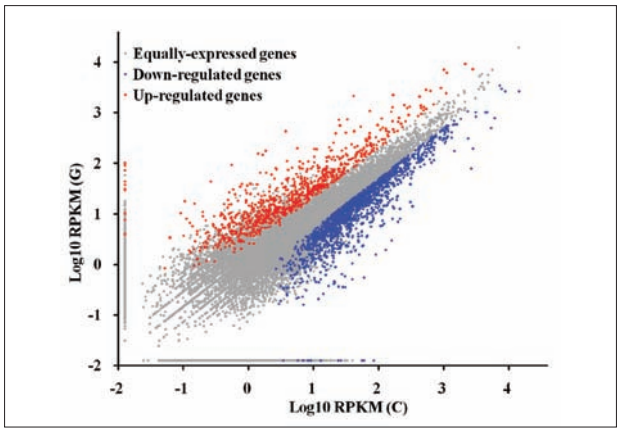

Fig. 2. Global analysis of differentially expressed genes *in vitro* cultured castor seeds with glutamine treatment. C, control condition; G, glutamine treatment; RPKM, reads per kilo bases per million reads.

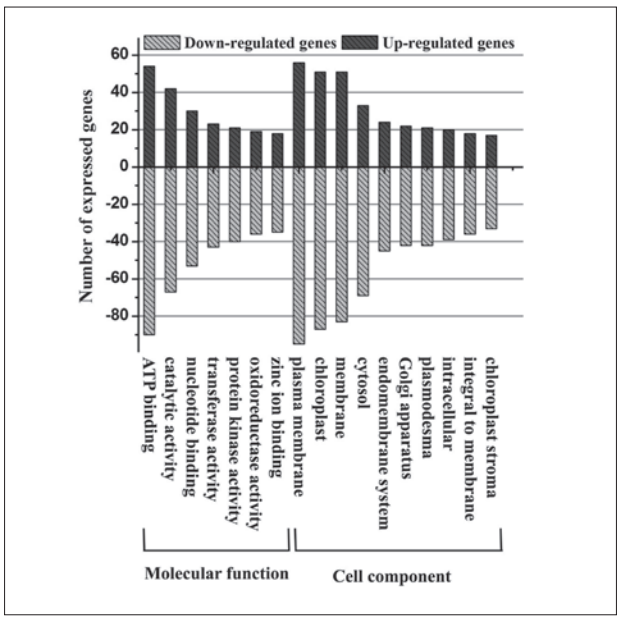

Fig. 3. Main DEGs (differently expressed genes) identified from GO enrichment analysis. Only the number of DEGs above 10 is shown.

To validate the initial RNA-Seq data, quantitative-real time qRT-PCR analysis was performed for 5 each of the upregulated and downregulated genes involved in the glutamine metabolism and lipid biosynthesis in the cultured seeds. Results of qRT-PCR showed that all 10 tested genes exhibited the same expression profile as the RNA-Seq results (Table 4).

Table 4. qRT-PCR validation of ten differentially expressed genes (DEGs) identified from RNA-Seq data. [1]The normalized number of reads identified in libraries prepared from control (C) and glutamine-treated (G) seeds; [2]Normalized $\log_2$ fold changes; [3]Normalized expression value as $2^{\Delta CT}$, with an average over three biological samples; [4]Average fold changes as $\log_2$ (normalized expression value G/ normalized expression value C); RPKM, Reads Per Kilo bases per Million reads.

		RNAseq (RPKM[1])			qPCR (Expression value[3])		
Gene-ID	**Annotation**	**C**	**G**	**Fold changes[2]**	**C**	**G**	**Fold changes[4]**
29594.m000267	asparagine synthetase	17.35	310.56	4.37	0.05	1.19	4.51
29950.m001132	glutamate dehydrogenase	15.14	94.98	2.86	1.31	9.20	2.81
30170.m013617	Phosphoenolpyruvate carboxykinase	79.51	214.34	1.64	0.51	1.66	1.71
29841.m002756	aspartate aminotransferase	43.82	109.50	1.53	0.44	1.41	1.68
29848.m004677	palmitoyl-acyl carrier protein thioesterase	92.54	188.51	1.23	1.00	2.77	1.48
47543.m000013	glyceraldehyde 3-phosphate dehydrogenase	489.67	218.60	-0.96	1.59	0.81	-0.98
29986.m001646	sucrose synthase	218.22	96.31	-0.97	2.86	1.37	-1.06
30131.m07299	pyruvate kinase	100.54	44.54	-0.97	2.59	1.23	-1.07
30128.m009036	pyruvate dehydrogenase	108.68	47.42	-0.99	2.21	1.09	-1.02
29092.m000447	enolase	209.48	70.54	-1.36	3.54	1.31	-1.42

Identification of genes encoding glutamine metabolism enzymes

Glutamine feeding resulted in an increment in lipid accumulation in developing castor seeds. To explore the molecular mechanism explaining how glutamine can promote storage lipid accumulation in castor bean seeds, we focused on identifying genes encoding glutamine metabolism enzymes, since these could potentially provide clues for understanding the carbon flux from glutamine feeding during seed development. Under glutamine treatment, a total of 48 and 84 DEGs involved in glutamine and amino acid metabolism were upregulated and downregulated, respectively, suggesting physiological involvement in responses to glutamine feeding treatment (Supplementary Table S2). Of these, several genes encoding enzymes involved in glutamate biosynthesis and transamination were dramatically upregulated in seeds exposed to glutamine treatment, such as glutamate dehydrogenase (29950.m001132 19) and asparagine synthetase (29594.m000267 21), while glutamate synthase (28076.m000425 18) and aspartate aminotransferase (29841.m002756 20) were slightly downregulated (see 18-21 in Fig. 4). In addition, two key enzymes for amino acid degradation and keto acid metabolism in the TCA cycle – isocitrate dehydrogenase (29933.m001413) and 2-oxoglutarate dehydrogenase (29585.m000604) – were substantially upregulated (see 23 and 24 in Fig. 4). The upregulation of these genes could likely be seen to reflect the active glutamine uptake and transformation that occurred in developing castor seeds during glutamine treatment.

Identification of sucrose metabolism and lipid biosynthesis genes

The aforementioned experiments demonstrated that exogenous glutamine supplementation has the capability of boosting lipid accumulation in developing castor seeds with a decrease in carbohydrate absorption and accumulation. There were 46 DEGs involved in sucrose metabolism, including 13 upregulated and 33 downregulated genes (Table S2), implying the downregulated sucrose metabolism and glycolytic pathway wholly. In particular, the two genes, sugar transporter (29634.m002113, 29737.m001257, 30169.m006599 and 29908.m006024) and sucrose synthase (29986.m001646), were significantly downregulated following glutamine treatment (see 1 and 2 in Fig. 4). In addition, most of the genes involved in glycolysis, which is the central flux of carbohydrates metabolism, were also dramatically downregulated (see 3-9 in Fig. 4).

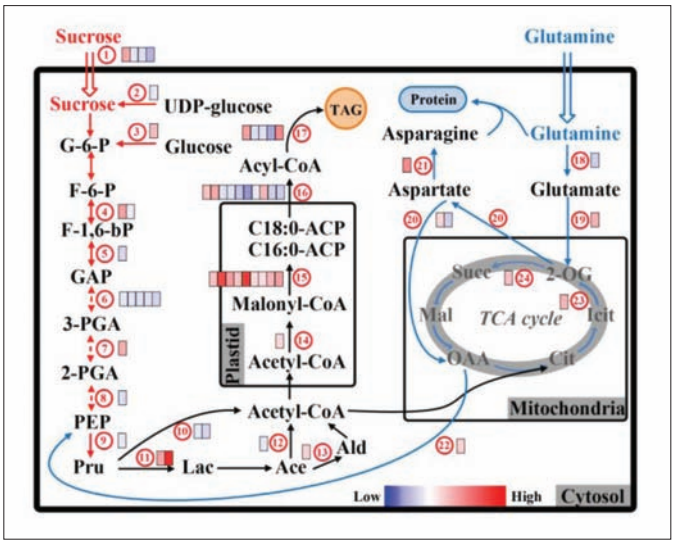

Fig. 4. Potential depiction of carbon conversion and lipid biosynthesis pathway in castor seeds after glutamine treatment. Key enzymes encoded by differentially expressed genes (DEGs) that may play critical roles in driving carbon conversion and lipid biosynthesis under glutamine treatment marked 1-26, representing the following: sugar transporter 1; sucrose synthase 2; hexokinase 3; phosphofructokinase 4; fructose-bisphosphate aldolase 5; glyceraldehyde 3-phosphate dehydrogenase 6; phosphoglycerate mutase 7; enolase 8; pyruvate kinase 9; pyruvate kinase 10; l-lactate dehydrogenase 11; acetyl-CoA synthetase 12; aldehyde dehydrogenase 13; acetyl-CoA carboxylase 14; enzymes involved in fatty acid biosynthesis and elongation (12-oxophytodienoate reductase, allene oxide cyclase, lipoxygenase, lipoxygenase, oxidoreductase, palmitoyl-acyl carrier protein thioesterase, palmitoyl-acyl carrier protein thioesterase, palmitoyl-protein thioesterase, palmitoyltransferase ZDHHC9) 15; enzymes involved in acyl-CoA synthetase and transport (acyl-CoA synthetase, acyl-CoA synthetase, acyl-[acyl-carrier-protein] desaturase, acyltransferase, acyltransferase, fatty acid desaturase, ketoacyl-ACP Reductase, omega-3 fatty acid desaturase, stearoyl-ACP desaturase, stearoyl-ACP desaturase) 16; enzymes involved in TAG assembly (choline-phosphate cytidylyltransferase, ER glycerol-phosphate acyltransferase, phospholipase C, phospholipase C, phospholipase D) 17; glutamate synthase 18; glutamate dehydrogenase 19; aspartate aminotransferase 20; asparagine synthetase 21; Phosphoenolpyruvate carboxykinase 22; NADP-specific isocitrate dehydrogenase 23 and 2-oxoglutarate dehydrogenase 24. The red and blue arrows indicate potential carbon flow from sucrose and glutamine to lipid biosynthesis, respectively. The number of rectangles represents a number of different isoforms of enzymes. The color bar represents expression level of DEGs from glutamine library. For the details of expression level (PRKM, reads per kilo bases per million reads) of the above-mentioned DEGs, see Table S4. Abbreviations: G-6-P, glucose-6-phosphate; F-6-P, fructose 6-phosphate; F-1,6-bP, fructose 1,6 phosphate; GAP, glyceraldehyde 3-P; 3-PGA, 3 phosphoglyceric acid; 2-PGA, 2 phosphoglyceric acid; PEP, phosphoenolpyruvate; Pru, pyruvate; Lac, lactate; Ace, acetic acid; Ald, aldehyde; TAG, triacylglycerol; 2-OG, 2-oxoglutarate; Succ, succinic acid; Mal, malate; OAA, oxaloacetic acid; Cit, citrate; Icit, isocitrate; acetyl-CoA, acetyl coenzyme A; malonyl-CoA, malonyl coenzyme A; acyl-CoA, acyl coenzyme A; C16:0-ACP, palmitic acid acyl carrier protein; C18:0-ACP, stearic acid acyl carrier protein; UDP-glucose, uridine diphosphate glucose.

Alongside the increased lipid accumulation in cultured seeds *in vitro* under glutamine treatment, 68 DEGs involved in lipid biosynthesis were identified, including 20 upregulated and 48 downregulated genes (Table S2). Likewise, most of the 16 key enzymes involving *de novo* FA synthesis, carbon chain elongation and modification and TAG assembly were greatly upregulated (Table S4). Moreover, in FA synthesis and carbon chain elongation, except for two genes *viz.*, pyruvate dehydrogenase (30128.m009036 and 29693.m001991 10) and acetyl-CoA synthetase (30131.m006926 12) catalyzing the acetyl-CoA formation that were downregulated, all the other 11 genes were upregulated (Table S4 and 11-15 in Fig. 4). For FA carbon chain modification, two genes, Acyl-CoA synthetase (30190.m010831 and 29844.m003365) and omega-3 fatty acid desaturase (29814.m000719) functionally associated with modified Acyl-CoA formation, were significantly upregulated after glutamine treatment, implicating their involvement in the biosynthesis of ricinoleic acid and triricinolein accumulation in developing castor seeds (see 16 in Fig. 4). As for TAG assembly, two potential genes, choline-phosphate cytidylyltransferase (CYT, 27704.

m000147) and phospholipase D (30190.m011102), were simultaneously upregulated following glutamine treatment (see 17 in Fig. 4). The upregulation of CYT catalyzing the cytidine triphosphate into cytidine diphosphate-choline and phospholipase D catalyzing the phospholipase into DAG in TAG assembly suggests an important role of these two genes in oil accumulation in castor seeds.

DISCUSSION

It is common to assume that storage product accumulation is a fraction of the total biomass (carbon and nitrogen resources) supplied by the mother plant to developing seeds (Allen and Young, 2013). In this study, glutamine-induced changes in the accumulation of storage products (protein, lipid and carbohydrates) in developing castor seeds were clearly observed. Increases in the amount of N source appeared to have a dose-dependent effect on the storage product accumulation. On one hand, contrary to the previous report of Masakapalli et al. (2013), the presence of Gln as an N source did not cause a dramatic increase in the protein content in developing castor seeds (Table 1). This probably could be due to the rapid consumption of absorbed Gln via transamination into other amino acids that contain organic acids as their carbon skeletons for protein biosynthesis. In tobacco and *Brassica napus,* oil and proteins are considered to be accumulating inversely in developing seeds (Zhang et al., 2005; Lock et al., 2009). However, our results indicated that the use of glutamine (≥35 mM) as an additional N source greatly reduced the content of carbohydrates and increased the lipid content, as reported previously in soybean (Saravitz and Raper, 1995). Interestingly, the effects of additionally supplied N sources on developing castor seeds are consistent with the observation that glutamine supply exerted different effects on storage reserve accumulation in soybean embryos (Saravitz and Raper, 1995). In the present study, the low labeling in total ^{14}C labeling from the glutamine provision indicated that the unlabeled carbons from glutamine metabolism probably take part in storage product accumulation, while glycolytic pathways are inhibited by exogenous glutamine supply in developing castor seeds. Recently, systems analysis of the growth and development of castor plant revealed that there is negligible change in the balance between those two components (oil and proteins) during castor seed growth (Severino and Auld, 2013). However, our results have clearly shown an inverse relationship in carbon partitioning between oil and carbohydrate accumulation. A similar inverse relationship was previously reported for developing oat seeds of two cultivars having different amounts of kernel oil (Ekman et al., 2008). Our studies clearly illustrate differences in the partitioning of carbon between carbohydrates and oil under experimental conditions with exogenous glutamine. Results of the present investigation on the characterization of metabolic states implied that both carbohydrate assimilation and lipid biosynthesis are differently regulated by Gln supply in developing castor seeds, warranting further analyses under a variety of growth conditions and development stages for a deeper understanding of the effects of Gln supply on carbon assimilation and partitioning in castor bean seeds.

The regulation of oil synthesis in developing seeds can occur at multiple levels in the biochemical conversion of photosynthetically fixed carbon into TAG. Sucrose represents the major form in which photosynthetically assimilated carbon is transported into sinks such as oil accumulating seeds (Baud and Lepiniec, 2010). Sucrose transport in conjunction with the regulatory mechanisms in the endosperm play a central role in determining the final amount of oil in the endosperm (Ekman et al., 2008). In this study, differences in storage reserve accumulation between glutamine treatment and control condition were found not only at the level of carbon partitioning to different storage reserve classes, but also at the level of carbon partitioning to total storage reserves. Gln supplementation failed to increase the biomass accumulation in developing castor seeds, but instead dramatically decreased the flux of sucrose uptake and utilization, indicating the use of additional carbon from Gln supplied by the media. The study of Allen and Young (2013) revealed that Gln is a major source of both carbon and nitrogen for soybean, with approximately 10-23% of all carbon (including 9-19%

of carbon in fatty acids) and 63-91% of all nitrogen coming from Gln. The amide-C group from glutamine that was probably in excess of that needed to maintain metabolism, did not go to protein synthesis but instead appeared to be directed to the lipid and carbohydrate pools through the TCA cycle (22, 23 and 24 in Fig. 4), which may provide oxaloacetic acid (OAA) used for the production of phosphoenolpyruvate (PEP) and subsequent *de novo* fatty acid synthesis in the plastid of the developing castor seed. As an alternative to the production of lipid precursors from glycolytic intermediates, OAA could be derived from Gln (as nutrient substrates). Gln uptake from the culture medium could have been diverted into OAA via deamination (glutamate dehydrogenase 19) and transamination (aspartate aminotransferase 20). Thus, the generation and cleavage of OAA along with the activities of aspartate aminotransferase and phosphoenolpyruvate carboxykinase (20 and 22) coordinate both glycolysis and sugar utilization with the uptake of the amino acid (Gln). Especially, phosphoenolpyruvate carboxykinase, which catalyzes the reversible decarboxylation of oxaloacetate to yield phosphoenolpyruvate and CO_2, is situated at important crossroads in plant metabolism, lying between keto acids and amino acids, lipids and sugars (Lea et al., 2001). Furthermore, the flux through OAA to PEP may be an important point of regulation that can partially account for the negative correlation between oil and carbohydrates, because carbon reallocated from pyruvate-derived downstream glycolic metabolite production to fatty acid biosynthesis could result in more dramatic changes in lipid levels. It is therefore likely that the difference in oil accumulation between glutamine treatments and control conditions lies in the competition for sugar utilization between glycolysis and lipid synthesis in the developing castor seeds, and the data with respect to a possible metabolic regulation involved in lipid biosynthesis are summarized in Fig. 4. In this study, the high accumulation of carbon in oil found in developing castor seeds under glutamine treatment during the early stages of development suggests that the regulation of genes involved in oil biosynthesis and/or glycolysis is important for carbon partitioning to oil. Furthermore, a combination with metabolic flux analyses and target gene modification (Allen et al., 2012; Bates et al., 2014) is likely to identify key enzymatic pathways regulating the carbon flux into oil in oilseeds and improve our understanding of those factors that contribute to the accumulation of lipids in oilseeds.

CONCLUSIONS

In this study, developing castor seeds were cultured on medium with varying levels of glutamine (0-45 mM). It was hypothesized that the synthesis of the storage products, proteins, oils and carbohydrates, and their proportional distribution would be influenced by the glutamine supplemented in the medium (Truong et al., 2013). We varied the nitrogen concentration in the medium (provided as glutamine) and found altered accumulation of storage products, with carbohydrates decreasing from 50.29% to 46.81% of total storage products and lipids increasing from 8.60% to 12.42%. In accordance with a previous report on developing soybean embryos (Allen and Young, 2013), the change in lipid and associated metabolic fluxes indicated that seeds are not merely a passive organ fully controlled by the supply from the mother plant but are units with specific internal characteristics with high influence on final composition. The information from this study should pave the way for further studies to investigate the metabolic control points regulating the partitioning of storage reserves in developing castor seeds and the role of seeds in the determination of seed storage products accumulation (Severino and Auld, 2013).

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Authors' contributions: YZ carried out the experiment and analyzed the transcriptome data. SM participated in the experiment and data analyses. AL conceived of the study, and participated in its design, data analyses and coordination. All authors read and approved the final manuscript.

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