Chapter 9

Amino Acid Changes during Energy Storage Compounds Accumulation of Microalgae under the Nitrogen Depletion

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Additional information is available at the end of the chapter

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Abstract

The nitrogen depletion stress is widely used to promote energy storage compound (ESC) production of microalgae, such as starch and lipids. Previous reports show that the fast ESC's accumulation happens around the overall nitrogen content lowered to the half of normal cells. It indicates that the cells take an active nitrogen reassembly to rebalance the requirement of nitrogen, in which the amino acid conversion should play an important role. So here, using a marine strain, Isochrysis zhanjiangensis, as the model to give a detail view on metabolic, transcriptomic and proteomic levels during ESC's fast accumulation. The intracellular metabolite fluctuation within 32 h was profiled by GC-MS and LC-MS. These techniques identified and quantified the levels of 14 SMAs, 2 carbohydrates involved in the TCA cycle and glycolysis, and 28 free amino acids (AAs). The pulsed increase of pyruvate indicated a potential to produce more FAs. Although overall AAs showed a decreasing trend, Ala and Phe increased initially. Meanwhile, the transcriptomic and proteomic studies were utilized to show the nitrogen metabolic pathways changes. It is found that gamma-aminobutyric acid (GABA) and other nonprotein AAs play important roles in the regulation of energy metabolism.

Keywords: nitrogen reassembly, microalgae, energy storage compound, *Isochrysis*

1. Introduction

As the potential producer of the third-generation biofuels [1], and the origin of plenty of high-value products [2], microalgae have attracted more and more attentions from the last decade. An ideal algal biofuel production process should have high biofuel yield, with economic raw material utilization and proper biomass formulation. To meet these requirements, a two-stage culture method has been widely utilized, with nitrogen deficiency (N-deficiency) stress applied after the growth stage of the culture [3–7]. Nitrogen is the key element among the creatures, and the response to nitrogen deficiency varies among different plants [8]. For example, *Isochrysis zhangjiangensis* (synonym *Isochrysis zhanjiangensis*), a marine microalga with high carbon fixation capacity, will accumulate both polysaccharides and lipids as ESCs under N-deficiency conditions [3, 6, 9], while wild *Chlamydomonas reinharditii* stores starch and *Nannochloropsis oceanica* stores lipid preferentially.

However, the regulatory mechanism beneath the coordination of carbon and nitrogen metabolism is unclear, which is important for the design of high efficient process. For photoautotroph microalgae, carbohydrates are from the complex photosynthesis system. The synthesis of lipids is a more complex process than that of carbohydrates, which needs more ATP together with the reduction power from NADPH [10]. During the accumulation of ESCs, it can be reasonably assumed that plenty of enzymes are involved and a considerable portion of amino acids (AAs) are consumed or turned over to produce proteins (enzymes) for metabolism adjustment. Otherwise, some AAs involve the ESCs production directly. For instance, branched AAs (leu, Ile and Val) take part in the Ac-CoA production [11, 12], which is the raw material for the fatty acid synthesis; Glu is the precursor of chlorophyll synthesis. The clear understanding of carbohydrate and lipid accumulation coordinating process will contribute to oriental enrichment of bio-products according to the interest of industry.

The fast development of various "-omics" analyses provides versatile tools for probing complex biological problems. Different levels of "-omics" are combined to show a multidimensional information and widen our views on the essence of biological processes. Therefore, it gives us the opportunity to investigate the facts involving in this "golden period" of ESC's production, especially here, focused on the AA-related processes.

2. The methods used for "-omics" studies of *I. zhangjiangensis*

2.1. Strain and cultivation

I. zhangjiangensis FACHB-1750 was maintained by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences. The microalga had been previously cultivated in F/2 medium without silica under 14:10 light/dark cycle [3]. Algal cells were harvested in the exponential phase and re-suspended in nitrate-free medium to a final concentration of 3.0–4.0 × 10^6 cells/mL. For flask cultures, 200 mL seeds were inoculated in 500-mL glass shaking flasks with hand shake after inoculation and sampling. For bioreactor

cultures, 500 mL seeds were inoculated in 600-mL glass bubbling reactors and aired by CO_2 enriched air (2%, v/v) at the speed of 100 mL/min. The cultivation was under the control of self-made Algal Station Platform for reproducible growth (http://www.mbpe.dicp.ac.cn/yjcg/kyjz/kyjzpage.html).

2.2. "-omics" analysis procedures

I. zhangjiangensis cells from standard 7 day's cultivation were used as sample pools for the transcriptomic database construction. Sequencing was performed on Illumina HiSeq™ 2000 by BGI Tech (Shenzhen, China). Reads were assembled to unigenes (7511 clusters and 16,712 singleton transcripts) by Trinity [13] and further annotated against NCBI NR database (non-redundant protein database) and using Nt (non-redundant nucleotide database), SwissProt, KEGG and COG database by blastx (e-value $<10^{-5}$). The calculation of unigene expression uses FPKM method (Fragments Per kb per Million fragments) [14]. The COG cluster enrichment analysis was performed basing on the expression. The RNA-seq data are available with accession No. PRJNA217946 on NCBI.

The proteomics analysis was performed according to previous report [15] against above RNA-seq database. Totally 1862 proteins were identified and quantified.

The AAs and other small molecular acids (SMAs) were inspected by SIM LC-MS and GC-MS, respectively. The experimental details can be found from Zhang et al. [16]. Furthermore, by the pulse-isotope label of ^{15}N on nitrogen containing compounds (NCCs) during the "golden period," the turnover of nitrogen between AAs was investigated.

2.3. Growth, photosystem II (PS II) activity and other biochemical composition analyses

Other biochemical and physiological parameters were detected as previous description [3, 6, 9]. In brief, dry weight was determined gravimetrically after filtration by Whatman GF/C filters (47 mm diameter) and air dried in the air until constant weight achieved. Nitrate analysis was conducted with SEAL Analytical AutoAnalyzer 3 following the manufacturer's instructions. Lipid analysis was performed using Nile Red or GC according to Wang's method [6, 9]. The chlorophyll fluorescence measurements were performed using a chlorophyll fluorometer (Water-PAM, Heinz Walz GmbH).

3. Overall of cell growth and turnover of AAs

3.1. The newly synthesized AAs during the fast accumulation of ESCs by pulsing label

The assimilation of nitrogen starts from the reduction of nitrate and the formation of glutamate. Without nitrate supply from the medium, free AAs were turn over between different NCCs, including proteins, nucleic acids and AAs themselves. By pulsing adding 12.3 mg/L ^{15}N (in form of $Na^{15}NO_3$) at day 2, when initial 24.6 mg/L N was nearly used out, the labeled ratio of free AAs was detected within 32 h.

It has been reported, while the external nitrogen supply depleted, the protein, especially ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), was partially degrade as a nitrogen pool to reassemble AAs and other NCCs [17]. So, the final labeled ratio of AAs was expected near 33% (according to total nitrogen supplied). Most of AAs (15/23) consisted with the theoretical value with about 33% labeled ratio. However, some AAs showed interesting changing patterns (**Figure 1**).

The labeled AAs were newly synthesized, and therefore, most of free AAs show a high labeled ratio at the beginning (day 3-1 of **Figure 1**), except Arg, which increased stepwise. The Arg is mostly from the conversion of ornithine. The low level of labeled Arg indicates a slower than average AAs' synthesis rate of it in the early of exponential growth phase.

Among 23 AAs detected, 6 of them (His, taurine, Asn, Gln, Arg, and Trp) show a the final label ratio higher than 35%, indicating more than average newly synthesized of them were left inform of free AAs. Together with the low label level of Arg, the nitrogen assimilation may mostly form Gln branch.

The first undetectable labeled AA was methionine sulfoxide, which disappeared within 24 h. It is the oxidation form of methionine and formed post-translationally. The content of methionine sulfoxide is about one-fifth of that of Met in *I. zhangjiangensis* [16].

The second disappeared labeled AA was ornithine. It may be the result of the increasing labeled Arg and function involving in the urea cycle [11].

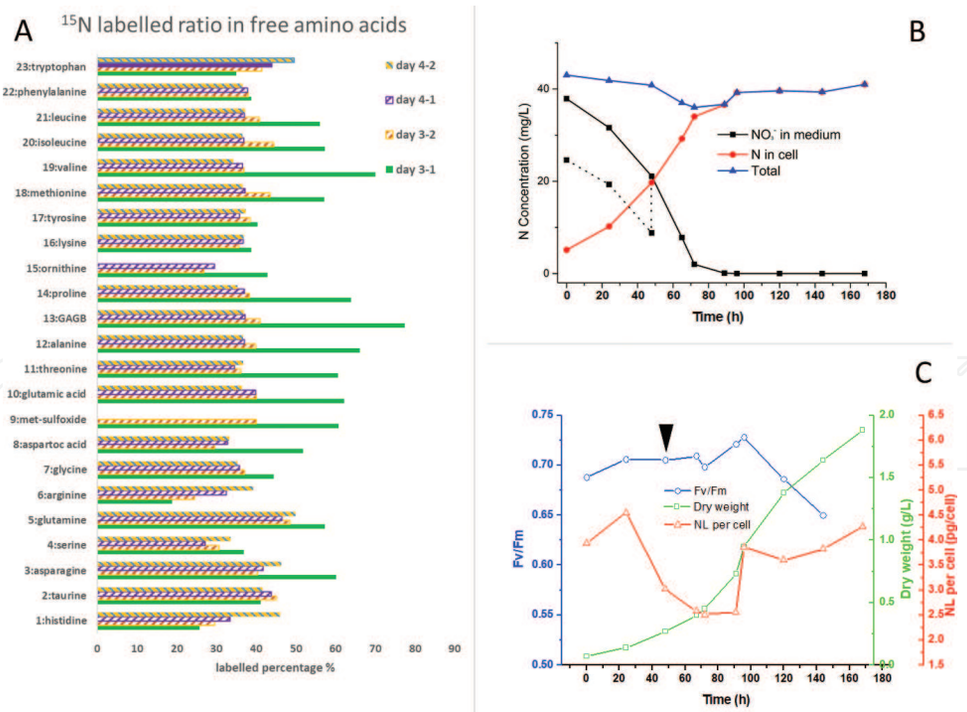

Figure 1. ^{15}N labeling percentage of AAs during the ESC fast accumulation period. (A) The labeling ratio of AAs changed within 32 h. The theoretical labeled ratio is about 33%. (B) The nitrogen changes during a standard bioreactor cultivation. The dotted line is nitrate concentration in medium of pulsing label experiment. (C) The dry weight, F_v/F_m and neutral lipids content changes in above cultivation. The black arrow indicates the start of pulsing label of ^{15}N.

3.2. The change of free AAs together with SMAs

The AAs metabolism relates to other SMAs closely. Zhang et al. have reported a detailed metabolic network change, with 18 protein AAs, 11 non-protein AAs, 14 SMAs and 2 carbohydrates [16]. As shown in **Figure 1B**, the total nitrogen was steady during the fast ESC accumulation stage, so the per cell-based qualification was used to tracing the changes. The cell number doubled after nitrogen depletion, and it is reasonable that the AA content level decreased to about half level. However, Phe had an evident 3-time increase during the early stage of nitrogen depletion. Other AAs maintained a steady state or increased in quantity slightly at the beginning and then decreased below their initial amounts.

The relative constant of protein AAs may contribute to the release of AAs from Rubisco. **Table 1** is the comparison of AA content in Rubisco large subunit and whole cell of *I. zhangjiangensis*. The data for Rubisco are from the putative AA sequence translated based on RNA-seq and that for whole cell is measured by MS from nitrogen-rich cultured cells. They share a similar composition. Only Glu shows a great difference.

In non-protein AAs, only Gamma-aminobutyric acid (GABA) shows a profoundly increase after nitrogen depleted, indicating an important role in the response of nitrogen stress.

Together with the changes of AAs, including the metabolites of glycolysis (D-glucose-1p,dihydroxyacetone-p, glyceraldehyde-3p, D-fructose-6p) and the glyoxylate or TCA cycles (citrate, cis-aconitate, isocitrate, α-ketoglutarate) showed a pattern of initial increase in quantity followed by subsequent decrease. Malate, fumarate and succinate, all from the TCA cycle, shared the same tendency of steady decline. Glucose and lactate showed steady increase in quantity [16].

In general, nitrogen has positive effects on the growth of microalgae, while having a negative impact on the accumulation of lipids, for aims of biofuel production. When carbon is not a limiting factor, intracellular energy substrates will accumulate under nitrogen starvation. Evidence shows that glutamate dehydrogenase as a metabolism shunt plays an important role in ensuring that the nitrogen metabolism does not affect the function of mitochondria and nitrogen recycling [18]. As a consequence, Gln, Glu, Asp, and Asn form the basis of several other organic nitrogen compounds, especially AAs [18].

3.3. AA metabolism–related transcriptomics and proteomics change

The overall transcriptomics and proteomics annotation results by KEGG pathways show opposite changes, up-regulated in transcriptomics while down-regulated in proteomics (**Figure 2**). The transcription and translation are controlled complex mechanisms. Their trends difference is a result of different level responses to the nitrogen deficiency.

To have a more global view of cell response, basing on the Clusters of Orthologous Groups (COGs) enrichment analysis of differentially expressed genes, amino acid transport and metabolism cluster (E) is the most significantly down-regulated cluster (**Figure 3**).

AA	Mole % in		Mass % in	
	Rubisco large subunit	Whole cell	Rubisco large subunit	Whole cell
His	1.5%	0.3%	1.8%	0.4%
Cys	1.8%	0.4%	1.7%	0.4%
Trp	1.8%	0.0%	2.9%	0.0%
Glu	3.4%	***9.0%***	3.8%	***10.9%***
Met	3.7%	2.1%	4.2%	2.6%
Phe	3.8%	4.8%	4.9%	6.5%
Asn	4.0%	/	4.1%	/
Ile	4.1%	5.3%	4.2%	5.7%
Tyr	4.3%	2.1%	***6.0%***	3.1%
Gln	4.4%	0.7%	5.0%	0.8%
Pro	4.7%	4.5%	4.2%	4.2%
Lys	4.9%	***6.2%***	5.5%	***7.4%***
Ser	5.0%	***7.9%***	4.1%	***6.9%***
Arg	***6.0%***	4.8%	***8.1%***	***6.9%***
Thr	***6.4%***	4.8%	***5.9%***	4.7%
Asp	***6.6%***	4.5%	***6.8%***	4.9%
Gly	***7.3%***	13.0%	4.3%	***8.0%***
Val	***7.6%***	***7.4%***	***6.9%***	***7.2%***
Leu	***8.0%***	***9.0%***	***8.1%***	***9.7%***
Ala	***10.6%***	***13.2%***	***7.3%***	***9.7%***

*The bold italic underlined AAs are first 7 abundant AA in each group.

Table 1. The AA profiling in Rubisco large subunit and whole cell*.

3.4. Glu vs. α-ketoglutarate

Glu is the key AA for photosynthesis cell as the precursor of chlorophyll. Also, it is the second AA products after the series reduction of nitrate to ammonia. It is the most abundant AA (in weight ratio) in *I. zhangjiangensis* (**Table 1**). α-Ketoglutarate plays two vital roles in the biochemical pathways of microalgae. One role is its participation in the TCA cycle, and the other is its involvement in the synthesis of glutamate. α-Ketoglutarate accumulates in the early stage of nitrogen deficiency in *Phaeodactylum tricornutum* and *I. zhangjiangensis* [10, 16]. α-Ketoglutarate accumulated incipiently instead of participating in the synthesis of glutamate, as indicated by the smooth decline in glutamine levels during the same time period. Then, both the level of glutamate and glutamine decreased significantly and the high level of α-ketoglutarate then resulted in feedback inhibition for its precursors, leading to their accumulation and apparent

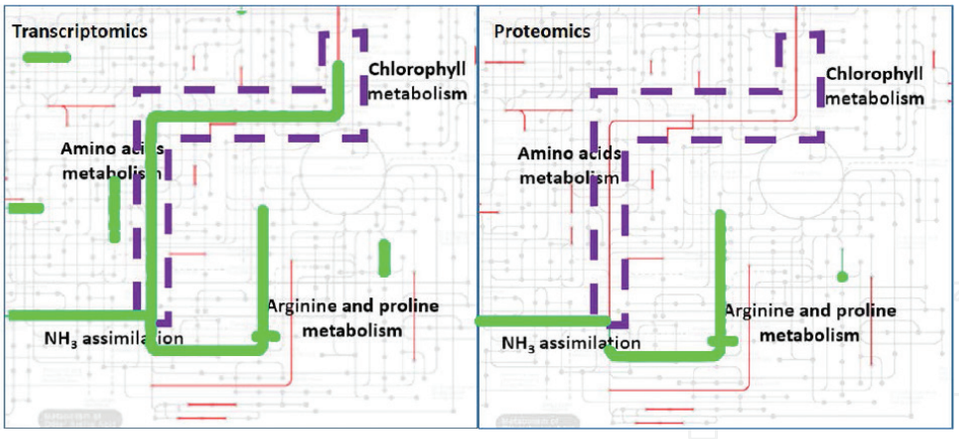

Figure 2. The mapping of significant changed AA metabolism related genes and proteins on KEGG metabolic pathways (map01100). The thin line indicates the significantly down-regulated elements, while the thick line indicates the significantly up-regulated elements.

increase (isocitrate, cis-aconitate, citrate) consequently [10, 16]. Nitrogen supply status showed directly influence on nitrogen assimilation, and by way of α-ketoglutarate intrigued the rebalance the carbon metabolism on a certain degree. AKG-Gln-Glu is the linker between nitrogen assimilation and central carbon metabolism in *I. zhangjiangensis*' response to nitrogen starvation.

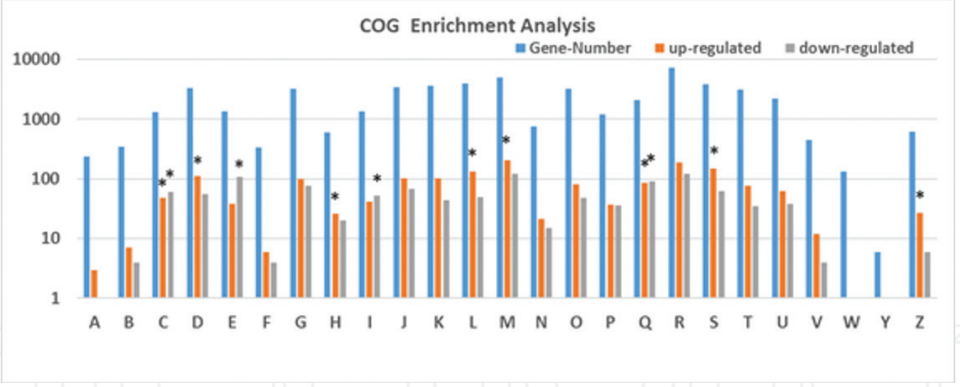

Figure 3. Enrichment of COG clusters analysis. Bars indicated the number of identified genes in different COG clusters and up or down regulated gene number in each cluster. The significant enrichment classes were mark with asterisk (*) on the top of the bar. The meaning of letters (A–Z) is listed below and significantly up or down regulated clusters were mark with up* or down* after their names correspondingly and the number after * was the *Q*-value. RNA processing and modification (A); chromatin structure and dynamics (B); energy production and conversion (up*, 2.95E−02) (down*, 1.00E−05) (C); cell cycle control, cell division, chromosome partitioning (up*, 1.05E−02) (D); amino acid transport and metabolism (down*, 0.0) (E); nucleotide transport and metabolism (F); carbohydrate transport and metabolism (G); coenzyme transport and metabolism (up*, 2.95E−02) (H); lipid transport and metabolism (down*, 1.88E−03) (I); translation, ribosomal structure and biogenesis (J); transcription (K); replication, recombination and repair (up*, 1.93E−03) (L); cell wall/membrane/envelope biogenesis (up*, 2.42E−11) (M); cell motility (N); posttranslational modification, protein turnover, chaperones (O); inorganic ion transport and metabolism (P); secondary metabolites biosynthesis, transport and catabolism (up*, 6.57E−05) (down*, 1.54E−07) (Q); general function prediction only (R); function unknown (up*, 3.60E−07) (S); signal transduction mechanisms (T); intracellular trafficking, secretion, and vesicular transport (U); defense mechanisms (V); extracellular structures (W); nuclear structure (Y); Cytoskeleton (up*, 2.54E−02) (Z).

It's reported that Gln and Glu subject influence on the intermediates of the TCA cycle. The transcription repressor of aconitase, CcpC, can be suppressed by glutamate, when nitrate is assimilated in phototrophic eukaryotes, glutamate or its precursors can arrest the glutamate synthase operon, which is closely affected by both carbon and nitrogen sources, and induced in the presence of ammonium and glycolytic carbon sources to form a feedback inhibition cycle [19]. The nitrogen source for the increased glutamine was considered as the recycling of internal nitrogen, indicating by the higher level of a key intermediate in the urea cycle, citrulline. Proteolysis and AA metabolism consume plenty of energy and release CO_2, resulting in the low level of intermediates in central carbon metabolism in the early stage of nitrogen deficiency [16].

Furthermore, in *I. zhangjiangensis*, the photosynthesis brought enough glucose to cell after the growth was inhibited by nitrogen depletion, and further stored in form of β-1,3 glucan [9, 16]. The wholly increased intermediates in glycolysis process inhibit glyoxylate cycle from the very beginning. By sharing the intermediates with TCA, the influence also causes the transitory increase of α-ketoglutarate, and further influence Gln and Glu metabolism.

3.5. Succinate and Asp, pyruvate and Ala: two secondary intersections of carbon and nitrogen metabolism

In most microalgae species, aspartate and glutamate usually constitute a large proportion of the total amino acid content, while GABA, Trp, Met, His, ornithine and others only represent a small proportion [20]. Asp makes up 6.9% or more of the total AA content and approximately 1.5–1.8% of total detected free AAs in *I. zhangjiangensis*. Asp serves as the source of nitrogen for transaminations and as the early product of the carbon fixation pathway for carbon storage in blue-green algae [21]. Succinate and Asp are intermediates between the glyoxylate cycle and the TCA cycle in cells. The glyoxylate cycle is involved in the formation of glucose and further AAs and nucleotides, whereas the TCA cycle targets the generation of ATP as an energy carrier. For *I. zhangjiangensis*, when nitrogen was limited, some cell physiological activities related to growth receded or stopped, and the product of carbon fixation, glucose, accumulated, while the surplus energy was stored in the form of polysaccharose or lipids [3, 6, 9]. The increase of intermediates of glycolysis and the TCA cycle, as well as AMP and ADP [10], blocked the glyoxylate and TCA cycles. Most of metabolites of the TCA cycle in the inhibitor-treated sample showed a stable concentration at the beginning, except succinate and fumarate. Subsequently, with a further decrease in photosynthetic activity, the TCA cycle activity increased to produce more energy, and the levels of all TCA cycle metabolites decreased. While lipid accumulation increased lately, malate, α-ketoglutarate and other metabolites in TCA decreased. More energy consumed in lipid synthesis than that of polysaccharose may be the reason for this.

Due to the share of intermediates of above cycles, citrate, cis-aconitate and isocitrate accumulated rapidly. In one glyoxylate cycle, two molecules of Ac-CoA are consumed, while one molecular Ac-CoA is consumed during one TCA cycle. Otherwise, from the stable level of pyruvate, it is postulated the initiative synthesis of Ac-CoA was intact. Therefore, the block of above two cycles induced a transitory net accumulation of Ac-CoA, which redirected to FAs, which also consists with our previous work [6, 9].

Pyruvate and Ala are another pair of metabolites at the carbon-nitrogen metabolism intersection. Pyruvate is the key linker between different sub cell component (**Figure 4**), and the precursor of acetyl-CoA, the precursor for FAs synthesis and TCA cycle. As the most abundant AA in Rubisco, which contributes about 3% dry weight of normal *I. zhangjiangensis* cells, Ala was considered to release ammonium after nitrogen depleted by consuming α-ketoglutarate and producing Glu and pyruvate. The free Ala kept decrease in this period.

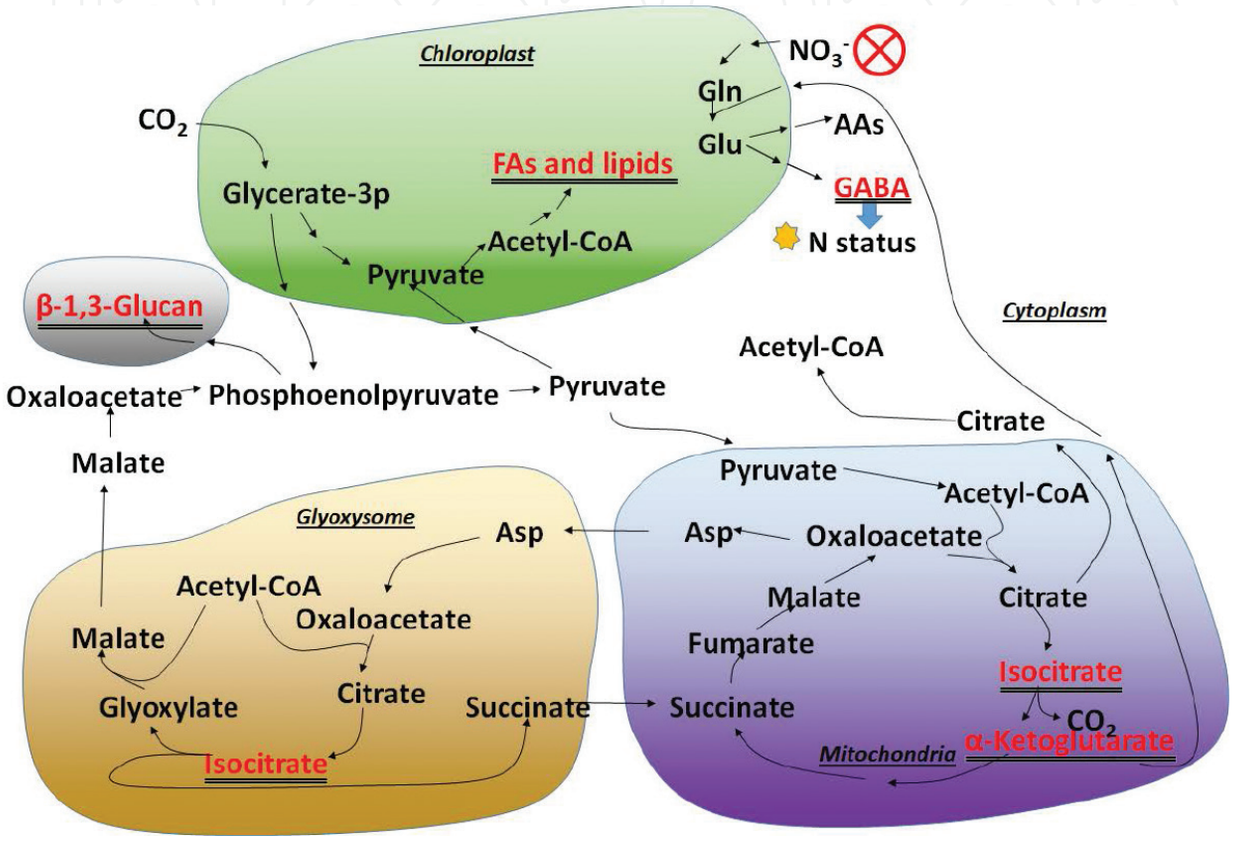

Figure 4. Postulated carbon partition of *I. zhangjiangensis* under during the "golden period." While exogenous nitrogen depleted, the nitrogen assimilation stopped and α-ketoglutarate as the reactant of Gln-Glu cycle had a transitory increase. The synthesis of Gln was reduced, and proteolysis was enhanced, while GABA acted as a transient N buffer as well as a signal to indicating the nitrogen level in cells to stimulate subsequent response. The slowdown of growth without exogenous nitrogen also caused the accumulation of carbohydrates, such as glucose or glucan, which and whose derivants in glycolysis together with α-ketoglutarate inhibited the activities in TCA and glyoxylate cycle. Partial of carbon flowed to pyruvate for further FAs or lipids. Words with doubled underline: increased, others: no significant change. Drafted based on Zhang et al.

4. Conclusion

The AA metabolism changes during the fast ESCs accumulation process of *I. zhangjiangensis* was detailed illustrated by "-omics" analysis. An overall changing model is raised from above data, which will help us to understand the function of different AAs and promote the new regulation methodology for the bioenergy development. A draft global change of AA-related metabolism is shown in **Figure 5**.

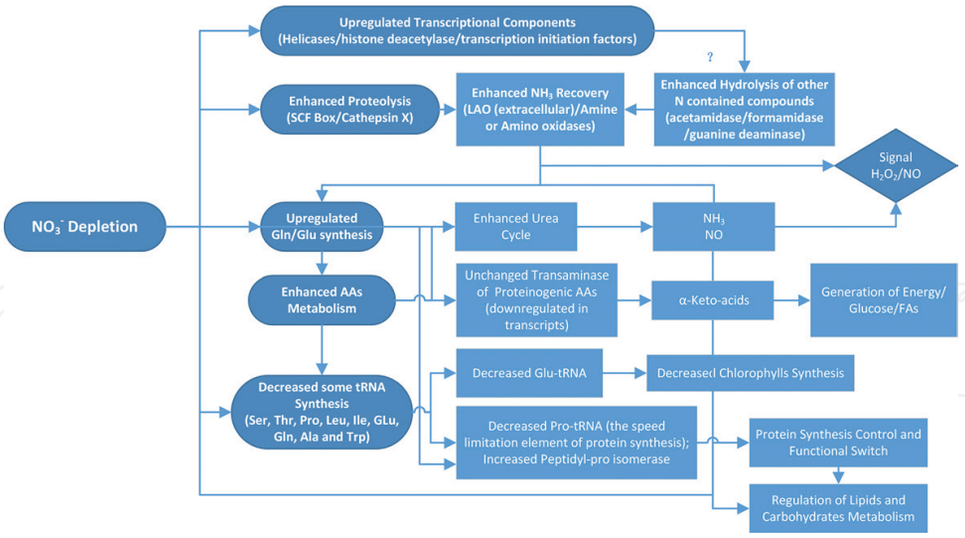

Figure 5. The possible response of AA metabolism of *I. zhangjiangensis* based on omics analysis in the fast ESCs accumulation period.

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