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COMPARATIVE METABOLOMICS REVEALS THE INHIBITION OF GLUTAMINE AND PYRUVIC ACID SYNTHESIS CLOSELY RELATED TO PEPPER CYTOPLASMIC MALE STERILITY

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Abstract

To investigate the mechanisms of cytoplasmic male sterility (CMS) in pepper (*Capsicum annuum* L.), cytological observation and metabolomics analysis of different pollen development processes were carried out. The pollen development processes were divided into five stages. Resin semi-thin section analysis indicated an abnormality of the sterile anthers started at the tetrad stage (S2); the cell of the tapetal layer was over-vacuolated. The tapetal began to degrade and lose its function. The tetrad could not get essential energy and materials which resulted in pollen abortion. Anthers of S2, and uninucleate pollen stage (S3), were selected and analyzed by non-target metabolomics. A total of 454 metabolites in S2 and S3 stages of maintainer line were detected, and 201 were annotated. For the CMS line, 459 metabolites in two stages were detected and 211 were annotated. Alanine, arginine, serine, and tryptophan were found in the two stages of maintainer line. A marked decrease of glutamine and pyruvic acid during the pollen abortion process were especially found in the pepper CMS line. The decrease of pyruvate concentration further leads to the abnormal synthesis of various kinds of amino acids and ATP which may ultimately contribute to the phenotype of the CMS line.

Key words: Pepper; CMS; Metabolomics; Glutamine; Pyruvate synthesis; Pollen abortion.

Introduction

Pepper is one of the oldest crops grown by humans. It originated in South America and is now a global vegetable and spice. In recent years, both the sown area and its total yield continue to increase steadily. Asia is a major producer of pepper, and the output of fresh and dried fruits accounts for about 65% of the world's. (https://www.sciencedaily.com/releases/2019/02/190221110508.htm). China is the world's largest producer and consumer of peppers, with the sown area accounting for about 40% of the world's sown area of pepper. The planting area of pepper in China is 2 million hectares, accounting for more than 12% of the vegetable planting area. The output of peppers is 40 million tons, with an output value of 70 billion RMB. Hybrid varieties are widely used; hybrid seed production is complex and requires a lot of labor. Furthermore, the purity of the seed is at risk due to emasculation and isolation. Cytoplasmic male sterility (CMS) lines were developed to efficiently produce hybrids that exhibit a great deal of hybrid vigor (Kim & Zhang, 2018; Tester & Langridge, 2010).

In plants, CMS lines express pollen abortion and maternal inheritance, largely due to the interaction between mitochondrial and nuclear genes, leading to the inability to develop functional anthers, pollen or male gametes (Wang *et al*., 2019; Xu *et al*., 2015). The CMS line, restorer line and maintainer line constitute a three-line hybrid system (Chen & Liu, 2014). CMS lines lack a functional nuclear restorer of male fertility genes whilst the restorer lines have fertile genes and the maintainer lines have an identical nucleus to that of the CMS lines (Bohra *et al*., 2016). So far, many genes that cause CMS have been identified such as *orf456*, *orf507* and *atp6-2* genes in peppers (Ji *et al*., 2015; Ji *et al*., 2013; Kim *et al.*, 2007; Kim & Kim, 2006), *orf79* in rice (Wang *et al*., 2006), *orf138*, and *orf463* in radishes (Iwabuchi *et al*., 1999; Park *et al*., 2013). Although the underlying mechanisms of some CMS lines has been well studied, there are still problems in existing CMS lines such as cytoplasm singularities, poor binding abilities and unstable sterility (Ma *et al*., 2013).

Metabolites are instrumental in the plant response to the environment and development. Therefore, profiling of the changes in metabolites may improve our understanding of some biological mechanisms (Hong *et al*., 2016; Nicholson *et al*., 1999). Following the development of 'omics' technology, metabolomics gives an objective, complete, qualitative and quantitative outline of the metabolites existing in an individual organism (Hall, 2010). Metabolomic research on the CMS during soybean bud development showed that, it was not possible to effectively eliminate reactive oxygen species (ROS) in anthers due to the defect of ROS scavenging systems in soybean CMS lines. Overaccumulation of ROS initiated programmed cell death and, eventually, led to pollen abortion in soybean CMS lines (Ding *et al*., 2019). The biodiversity of Capsicum species was revealed by characterizing pepper accessions and differentiating species based on their metabolic profile using an untargeted metabolomics approach (Aranha *et al*., 2017). Many metabolic processes are well understood at the metabolite level due to metabolomics. For example, the mechanism by which the soybean adapts to drought was explained by metabolomics (Khan *et al*., 2018).

Our research team used two pepper CMS varieties that were introduced from the Centre for Genetic Resources of Dutch. After five years of backcrossing and directional separation, we have developed a stable CMS line (DH-01-1-1A), and a maintainer line (DH-01-1-1B). In this article, the anther development progress of pepper CMS line 'DH-01-1-1A' and maintainer line 'DH-01-1-1B' were

cytologically observed. After determining the first stage of the CMS phenotype, the non-targeted metabolomic was used to analyze the mechanism of CMS.

Materials and Methods

Plant materials: The CMS pepper line (DH-01-1-1A) and its maintainer (DH-01-1-1B) were cultivated at Hangzhou Academy of Agricultural Sciences in Zhejiang, China. The detailed source of the two lines and the anther development stage are described in the former study (Fang *et al*., 2016).

Resin semi-thin section of the anther: The semi-thin section was obtained according to the method used in the study of Pan *et al*. (Pan *et al*., 2019). Briefly, samples (1mm x 3mm) were fixed with a fixation buffer (0.1M potassium phosphate, 2.5% (v/v) glutaraldehyde, pH 7.2), washed twice with the fixation buffer, and then immersed in 1% osmic acid. After 2 hours, samples were again washed with the fixation buffer and dehydrated via a gradient ethanol series. Lastly, samples were embedded in Spurr's resin and polymerized. Semi-thin sections (2μm) were stained using 1% methylene blue and photographed under an electron microscope (Leica DM1000, Germany).

Metabolomic sample treatment: We weighed 40 mg of the sample, then added 800μl of methanol and crushed it in a homogenizer. Next, the vortex was fully swirled for 30 s and the ultrasonic was broken for 30 min. Then, high-speed centrifugation was carried out at 4℃, at 12000 rpm for 15 mins. Lastly, carefully took out the centrifuge tube, transferred 200μl of supernatant and moved it into the vial for injections.

Untargeted metabolomic analysis: Untargeted metabolomic analysis were performed on an Ultra-performance liquid chromatography (UPLC) with an Orbitrap mass spectrometry system. Liquid chromatography separation was performed on Ultimate 3000 UPLC system (Thermo Fisher, USA) equipped with a Hypergod C18 column (100×4.6mm, 3μm, Thermo Fisher, USA). Mobile phase A was watered with 0.1% (v/v) formic acid and B was acetonitrile with 0.1% (v/v) formic acid. Elution gradient at a flow rate of 0.3ml/min was as follows: 0-2 min, from 0 to 5% (B); 2-12 min, from 5 to 95% (B); then 3 minutes to 95% (B) and back to 5% (B) for 2 minutes. The column temperature was set 40℃; the injection volume was 4μl and the temperature of the automatic injector was 4℃. Mass spectrometry scan was performed on Orbitrap elite (Thermo Fisher, USA) in both positive and negative mode. In both modes, heater temperatures were 300℃; sheath, aux and sweep gas flow rates were 45 arb, 15 arb, 1 arb, respectively; capillary temperatures were 350℃. Spray voltage was 3.0KV in positive mode and 3.2KV in negative mode; S-Lens RF Level was 30% in positive mode and 60% in negative mode.

Data analysis: The LC-MS raw data was processed with software SIEVE (Thermo fisher, USA) for pretreatment including peak alignment and feature extraction, and an excel table containing ion information of m/z, $t_{R,}$ and peak intensity in all samples was exported. The data was then normalized to perform PCA and OPLS-DA analysis by SIMCA-P 14.0 (Umetrics, Sweden). Ions with VIP (variable importance in the projection) values were greater than 1.0 proceeded students't-test analysis. The ions with p-values were less than 0.05 and an FC (fold change of maintainer (S3/S2) / CMS (S3/S2))＞1.5 or＜0.67 was considered as to be differential metabolites. Metabolites were identified through database including HMDB (Wishart *et al*., 2007), and LIPMAPS (Sud *et al*., 2007), based on exact mass measurement (＜5ppm), MS/MS fragmentation and retention time. KEGG (Gerlich & Neumann, 2000) pathway enrichment of differential metabolites was processed by MBRole (Javier *et al*., 2016) (http://csbg.cnb.csic.es/mbrole2/).

Results

Cytological observation of pollen development in CMS and maintainer lines: When compared with the maintainer line 'DH-01-1-1B', no mature pollen grain was found in the anther of the CMS line 'DH-01-1-1A' after dehiscence. Cytological observation of the anthers of the two cultivars revealed that there was no pollen in the anther locules of the sterile line (Fig. 1). To determine when pollen abortion began, the anther development process was divided into five stages and observed in sections (Fig. 2). Prior to the pollen mother cell stage (S1), there was no significant difference between the CMS and maintainer lines. During the tetrad period of meiosis (S2), the tapetal layer cells of the CMS line are over-vacuolized. At the uninucleate pollen stage (S3), tapetum of the CMS line started to degenerate into irregular shapes and the tapetal cells began to dissociate (S3). The callose around the tetrad was not decomposed in binucleate pollen (S4). The tapetal cells degenerated much faster, only part of the unfinished vestigial residual remained visible. In the mature pollen stage (S5) there was no pollen grain observed in the anther locule of the CMS line.

Fig. 1. Phenotypes of maintainer and CMS lines.

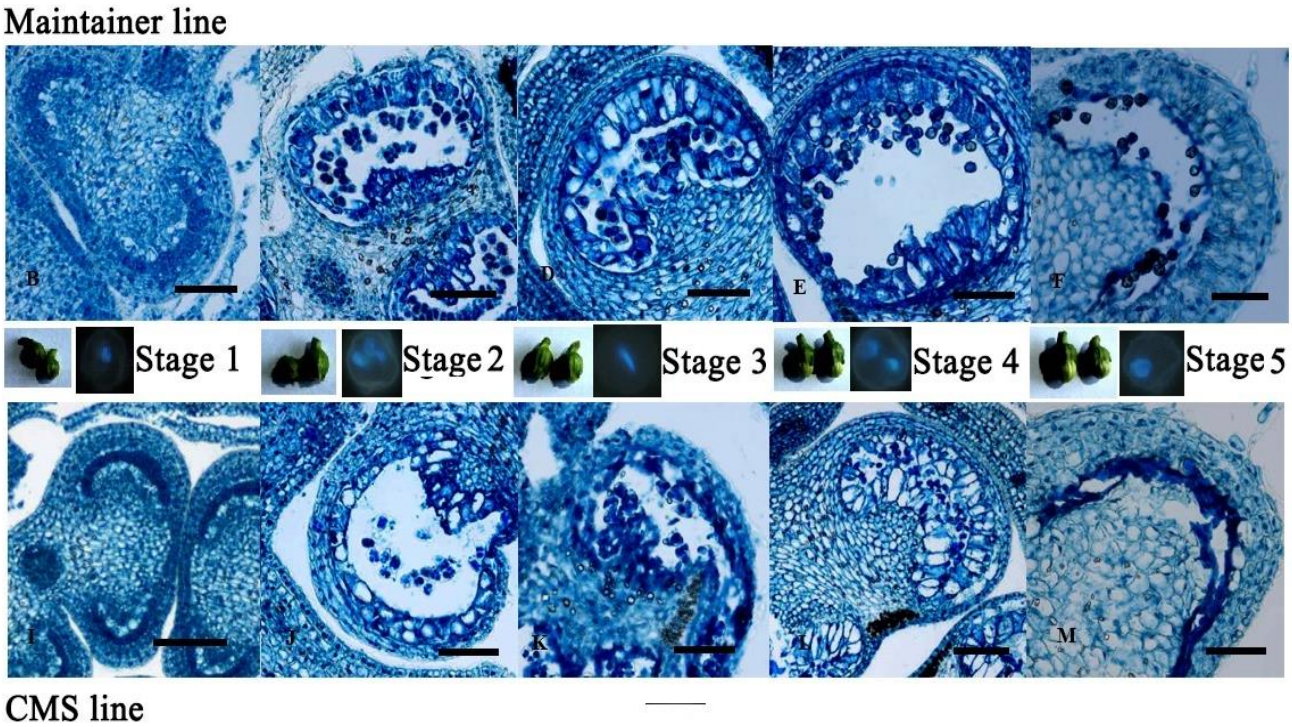

Fig. 2. Cytological observation on anther development of two lines.

Metabolite profiling of the CMS and maintainer lines: The anthers of both lines during stage 2 and stage 3 were analyzed by non-target metabolomics. For the maintainer line, a total of 454 metabolites were detected across the two stages, of which, 201 were annotated. For the CMS line, a total of 459 metabolites were detected across the two stages, 211 of these were annotated (Fig. 3a). Only 51 metabolites were identified in both lines (Fig. 3b). Principal component analysis showed that metabolites in both stages of the two cell lines differed significantly (Fig.3c). These different metabolites were annotated by the KEGG database and the different metabolic pathways were shown in Figure 3d. Several amino acid metabolic pathways such as aminoacyl-tRNA biosynthesis, glutamate metabolism, and pyruvate metabolism were flagged as abnormal by the KEGG.

Abnormal decrease of glutamine during the pollen development process in CMS: The types and expression of amino acids were further analyzed. Compared with the maintainer line, nine amino acids were flagged as abnormal in CMS line. Beta-alanine, arginine, serine, and tryptophan were especially found in the two stages of the maintainer line. Isoleucine, methionine, phenylalanine, and proline were exclusively expressed in the CMS line. Glutamine was present in both lines but its expression was inconsistent. The abundance of glutamine in S2 was significantly increased in the CMS line compared to the maintainer line. In S3, this was reversed and the maintainer line displayed a higher glutamine abundance than the CMS line (Fig. 4).

Pyruvate synthesis impediment in CMS: Pyruvic acid plays a key role in alanine, aspartate and glutamate metabolism, as well as in arginine and proline metabolism and glycine, serine and threonine metabolism. Pyruvic acid is abnormally inhibited in the CMS line (Fig. 5). During the pollen developing process that occurs over S2 and S3, the increase of pyruvic acid in the maintainer line was significantly higher than in the CMS line (Fig. 5).

Discussion

The cytological observation results are similar to that in our former study. The fundamental cytological reason behind the abortion is the abnormal function of the tapetal cells (Fang *et al*., 2016). There is no significant difference between the CMS and maintainer lines at S1. However, notable abnormality was observed in the CMS line at the start of S2: the tapetal layer cells of the CMS line were over-vacuolized. At S3, the callose around the tetrad failed to be decomposed which led to complete male sterility (Luo *et al*., 2010; Qiu *et al*., 2016; Ünal *et al*., 2013).

Glutamine is one of the most abundant free amino acids in plants and it plays a crucial role in the development of pollen. Isolated and in vitro cultured microspores fail to progress into functional pollen grains in glutamine-deficient media (Harada, 1986). Inhibiting the role of rate-limiting enzyme in glutamine biosynthesis, glutamine synthetase, causes male sterility in rice and tomato anthers and the fertility can be restored by supplying glutamine to plants (Kimura *et al*., 2008; Ribarits *et al*., 2010). Our studies showed that L-Glutamine levels increase significantly during the tetrad period to the binucleate pollen stage of pepper pollen development in the maintainer line, whilst the CMS line follows the completely opposite trend, indicating the abnormal inhibition of glutamine biosynthesis in CMS lines. Our results further demonstrate the importance of glutamine in pollen development.

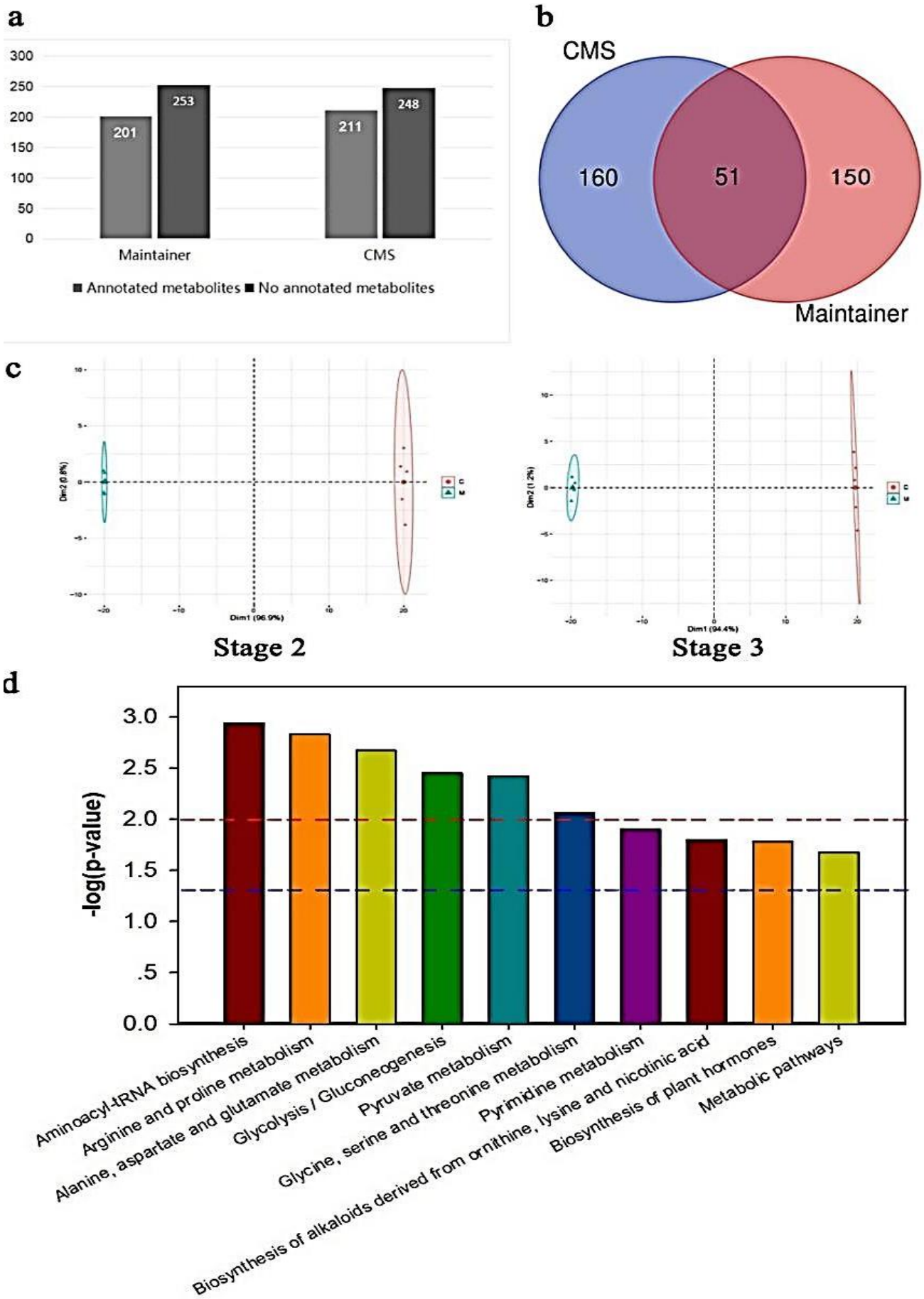

Fig. 3. Metabolomic profiling of two lines. (a) Annotated metabolites and not annotated metabolites. (b) Common and different metabolites. (c) PCA analysis of metabolites. (d) KEGG pathway annotation.

Pollen development is a very energy-consuming process, thus mitochondrial respiration and fermentation is essential to generate the required energy for male gametogenesis (Chen & Liu, 2014; Li *et al*., 2016; Selinski & Scheibe, 2014; Yue *et al*., 2018). Tang et al. found that the tricarboxylic acid (TCA) cycle and glycolysis were very active during anther development through GC-MS based metabolomics (Tang *et al*., 2018). The mitochondrion genome encodes genes that are essential for TCA cycle and ATP generation (Lv *et al*., 2020). Reduced ATP synthase activity in mitochondria could lead to CMS in the chili pepper (Li *et al*., 2012). Temporal and spatial specific accumulation of cytotoxic CMS proteins within the tapetum causes an alteration of mitochondrial function (Touzet & H. Meyer, 2014). Pyruvic acid connects the TCA cycle and glycolytic pathway, both of which are involved in ATP metabolism (Voet *et al*., 2006). Previous studies in rice found that CMS proteins cause mitochondrial dysfunction by impairing ATP production in anther tissues (Luo *et al*., 2013; Wang *et al*., 2013). Begacy *et al*., found that transient heat stress on maize at the tetrad stage also results in pollen sterility (Begcy *et al*., 2019). Additionally, transcriptomic studies showed a dysregulation of energy biosynthesis-related genes whilst metabolomic studies found a reduction of pyruvate in sterile pollen. Our results showed that the concentration of pyruvic acid basically remained unchanged during the tetrad period and binucleate pollen stage and was significantly lower compared to those in the maintainer line. The decrease of pyruvate concentration further leads to the abnormal synthesis of various kinds of amino acids which may ultimately contribute to the phenotype of the CMS line. In general, the change in pyruvate metabolism explains why several amino acids are expressed abnormally in CMS lines and authenticate the results of previous studies (mitochondrial dysfunction). It also provides a novel idea for the precise breeding of CMS lines in the future: we may try to interfere with the expression of pyruvate to obtain CMS lines.

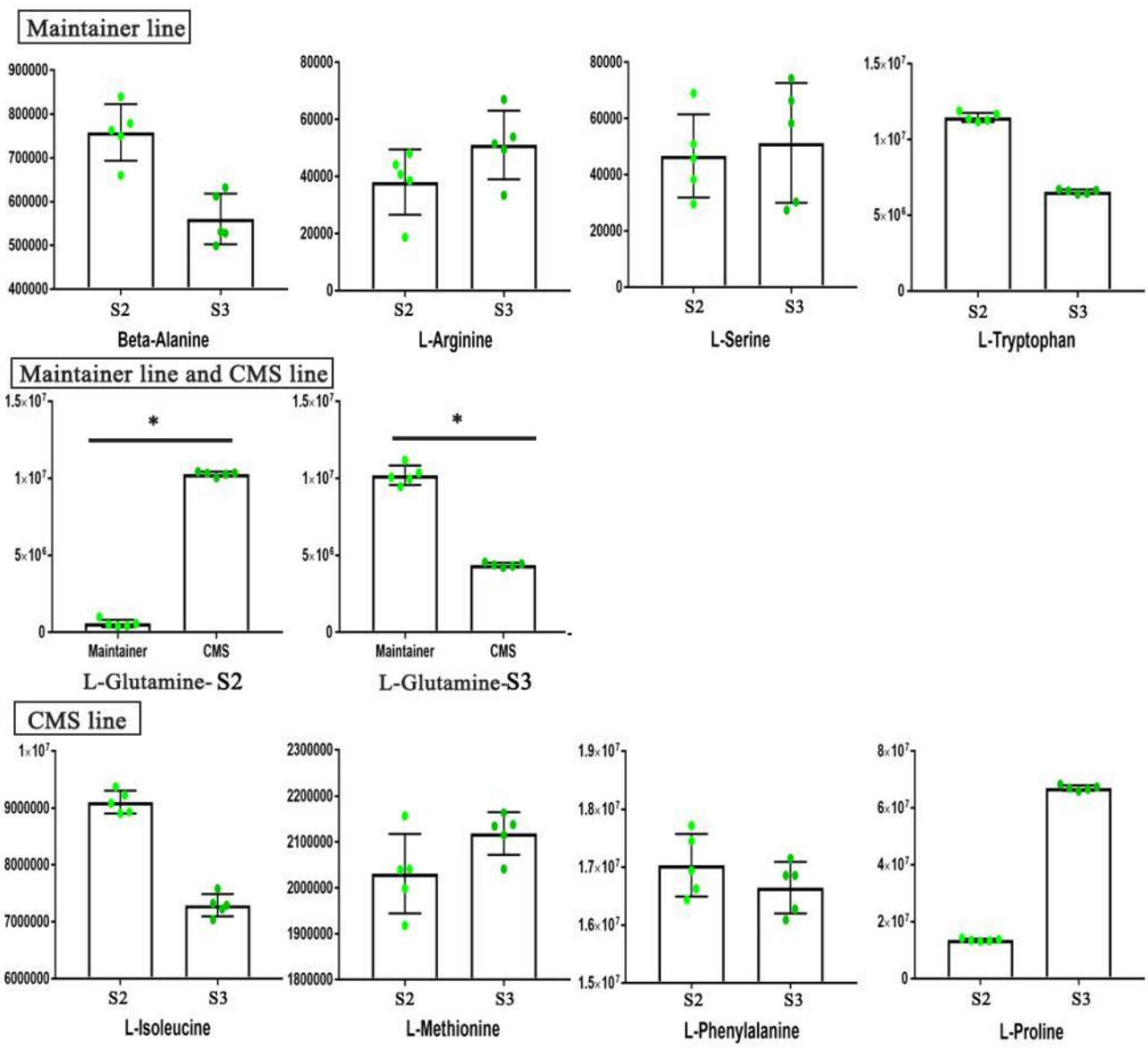

Fig. 4. Changes of various amino acids in two lines.

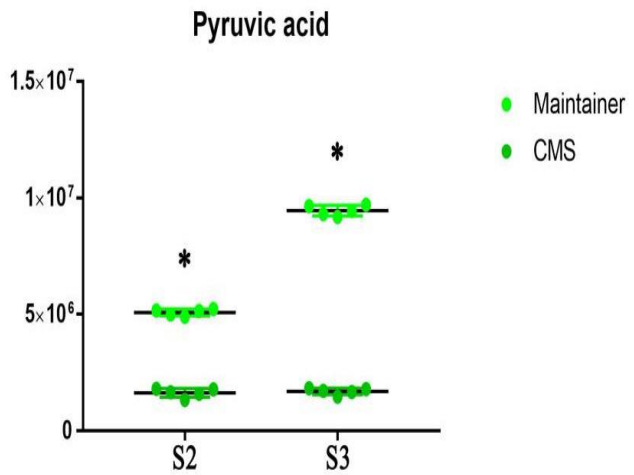

Fig. 5. Changes of pyruvic acid in two lines.

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