Reducing the nitrate content of burley tobacco by grafting with flue-cured tobacco

Yuqing Feng
Tobacco Harm Reduction Research Center of China Tobacco, Henan Agricultural University
Yuanyuan Zhao
Tobacco Harm Reduction Research Center of China Tobacco, Henan Agricultural University
Geng Li
Tobacco Harm Reduction Research Center of China Tobacco, Henan Agricultural University
Yafei Li
Tobacco Harm Reduction Research Center of China Tobacco, Henan Agricultural University
Jun Zhou
Shanghai Tobacco Group Co., Ltd
Hongzhi Shi (shihongzhi88@163.com)
Tobacco Harm Reduction Research Center of China Tobacco, Henan Agricultural University

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Abstract

Background: Nitrosition of tobacco pyridine alkaloids by nitrate-derived NOx is the origin of tobacco-specifc nitrosamines (TSNAs) formed in tobacco, which are among the most notable toxicants present in tobacco products and smoke. Burley tobacco is particularly susceptible to TSNA formation because the cultivars exhibit a chloroplast-deficient and nitrogen-use-defciency phenotype which results in high accumulation of nitrate. Strategies to lower nitrate levels in tobacco could produce a corresponding decrease in TSNAs accumulation in leaves. Previous studies have showed that grafting with high nitrogen use efficiency rootstock was able to improve the nitrogen utilization of flue-cured tobacco. In this study, a reciprocativel grafting experiment has been conducted with two varieties of different tobacco types (burley tobacco 'Eyan No.1' and flue-cured tobacco 'K326') to investigate whether replacing burley tobacco root with flue-cured tobacco by grafting can enhance pigment biosynthesis and photosynthesis parameters and reduce nitrate content of burley tobacco leaves, and to explore the corresponding mechanism.

Results: The results showed that compared with the self-rooted burley tobacco seedlings, grafting significantly increased the pigment content, net photosynthesis, biomass, total soluble sugar, reducing sugar, nitrate reductase activity, glutamine synthetase activities, NH_4-N and soluble protein content of the leaves of Eyan No.1 while decreased the nitrate content and NO_3-N/TN. Interestingly, transcription level analysis suggested that replacing burley tobacco root with flue-cured tobacco by grafting was able to up-regulate the genes involved in starch and sucrose metabolism, porphyrin and chlorophyll metabolism, carbon fixation in photosynthetic organism metabolism, carotenoid biosynthesis and nitrogen metabolism of burley tobacco leaves. In addition, the PPI network revealed gene_68511, gene_35043 and gene_77508 had higher degrees via replacing burley tobacco root with flue-cured tobacco, which might be hub proteins to reduce nitrate accumulation.

Conclusions: In conclusion, grafting with high nitrogen use efficiency rootstock provided an exceptionally promising means of nitrate reduction in burley tobacco leaf , which is a principle precursor of TSNAs, due to the improvement of photosynthesis and nitrogen metabolism in the scion.

Background

Tobacco (*Nicotiana tabacum* L.) is a nonfood crop that is economically important worldwide and is grown in many countries[1] . Cured leaves of different tobacco market types (flue-cured, burley and Oriental) are typically blended together in the production of cigarettes. Of these market types, burley tobacco is unique in that they display a chloroplast-deficient phenotype and is impaired in nitrogen use efficiency with reduced pigment content[2] . As a result, burley tobacco requires 3–5 times levels of N-fertilization than flue-cured tobacco to achieve comparable yields[3] . Lewis et al.[4] found reduced nitrogen use and utilization deficiencies in burley tobacco are attributed to two homozygous recessive mutant alleles at the Yellow Burley 1 (Yb1) and Yellow Burley 2 (Yb2) loci, which are probably the main explanations for the typically high fertilization level of burley tobacco with greater quantities of nitrogen, to achieve a yield that is commensurate with that of most other tobacco types. Carbohydrates are the material basis for plant metabolism. From the perspective of energy metabolism, an important reason for the increase in nitrate accumulation in burley tobacco was the reduction of carbon assimilation products induced by the decrease in photosynthesis[5] . The increased leaf nitrate nitrogen content is associated with the formation of tobacco-specific nitrosamines (TSNAs), which is a class of toxicants that contribute to cancers formation, in burley tobacco[4] , since nitate could be reduced to nitrite by microbiols during leaf curing and could give rise to gaseous NOx during leaf storage[6-7] , which are important nitrosating agents. Therefore, technologies to decrease nitrate content in burley tobacco should be justified to decrease the risk exposed to the portion of the population that uses tobacco products.

Based on above information, parameters such as photosynthesis, pigment content, nitrate reductase acivity (NRA), glutamine synthetase acivity (GSA) and total nitrogen content are highly related to nitrate content, and all these traits should be taken into account when considering nitrate decrease. In recent years, many strategies including molecular breeding[8] , chemical regulation[9] and agronomic practises[10] have been conducted to reduce nitrate content in burley tobacco. Nevertheless, such approaches still have some limitations. For instance, the potential of chemical and agronomic regulation to reduce nitrate content in buley tobacco leaves is very limited. Overexpression or knockout of the key genes related to nitrate content can effectively alleviate

nitrate accumulation in burley tobacco leaves, but it is costly and difficult to operate owing to the impact on quality and restrictions on genetically modified organisms[11-12] .

Grafting, as an ancient cultivation technique with a long history, has been reported as an effective strategy to enhance plant resistance[13 , increase yield[14 , improve photosynthesis[15 and reduce the alkaloid synthesis[16 . In particular, grafting has a significant impact on improving the absorption of nutrients by using cultivar rootstocks with more advantageous nitrogen-efficient absorption and enhanced nitrogen- use efficiency, thus alleviating the nitrate accumulation in tobacco[17 . Another described effect of grafting is the alteration of the hormonal balance between rootstock-scion[18-19 . Beyond that, grafting also plays a significant part in facilitating plant photosynthesis performance[20 . However, no data relating the impacts of grafting on nitrate content of burley tobacco leaves was publicly available. In our previous study, we observed that nitrogen use efficiency and carbon assimilation differed significantly between flue-cured and burley tobacco with flue-cured tobacco exhibiting much higher levels of nitrogen use efficiencu than burley tobacco [5 . The lower pigment content, week carbon fixation and nitrogen assimilation were the main causes of abnormally high levels of nitrate accumulation in burley tobacco.

Taken above information into consideration, we hypothesized that grafting burley tobacco onto flue- cured tobacco can improve the photosynthesis and nitrogen utilization efficiency of burley tobacco, thus decreasing the nitrate content of burley tobacco leaves. Therefore, a pot experiment using burley tobacco as scion and flue-cured tobacco as rootstock was conducted. The physiological and transcriptome differences were determined aiming to clarify whether the replacement of burley roots by flue-cured tobacco roots is able to reduce the nitrate accumulation in burley tobacco leaves and to investigate the mechanism of this decrease.

Results

Differences in pigment content and photosynthesis traits

The pigment content, net photosynthetic rate, biomass accumulation and carbohydrate content are displayed in Figure 1. There were significant differences in the pigment content and photosynthesis between different grafting groups. Contents of chlorophyll a, chlorophyll b, carotenoid and pigment were significantly lower in E/E than those in K/K (Figure 1.a-d). Also, the net photosynthesis rate, biomass accumulation and carbohydrate content were always lower in E/E than those in K/K (Figure 1.e-h). Lower pigment content may have an influence on carbon fixation and lead to low biomass and carbohydrate accumulation in the leaves of E/E. The pigment content and photosynthesis were significantly improved in the grafting combination, chlorophyll a, chlorophyll b, carotenoid, pigment contents and net photosynthesis rate increased by 20.17%, 22.43%, 48.38%, 25.44% and 21.89% in the leaves of E/K in comparision to E/E (Figure 1.a-d). In addition, burley tobacco plants grafted onto ‘K’ rootstocks showed higher total soluble sugar and reducing sugar content of 21.05% and 79.44% of the leaves (Figure 1.g-h). Regardless of the leaves, stem or roots, the biomass accumulation in E/K plants was higher than that in E/E. More nitrogen could be absorbed by the roots of flue-cured tobacco, thus resulting in the increase of pigment content which contributed to the increase of photosynthesis and carbohydrate accumulation.

Difference in enzyme activities related to nitrogen metabolism and nitrogen compounds

Two key enzyme activities, namely, NR and GS, and nitrogen compounds were measured in tobacco seedling tissues, as shown in Figure 2. The NRA and GSA in the leaves of ‘K/K’ treatment was higher by 33.07% and 32.95% compared with E/E, respectively (Figure 2.a-b). Also, the NH_4-N and soluble protein content all showed higher levels in the leaves of ‘K/K’ while lower levels in NO_3-N, TN and NO_3-N/TN compared with E/E (Figure 2.c-g). However, the nitrogen accumulation of leaves, stems and roots of ‘K/K’ were all higher than those of E/E (Figure 2.h), which are consistent with the lower biomass accumulation in E/E. Grafting significantly influenced enzyme activities related to nitrogen metabolism and nitrogen compounds of E/E. The NRA, GSA, NH_4-N and soluble protein content in the leaves of ‘E/K’ treatment increased by 13.29%, 14.58%, 16.06% and 11.07% compared with E/E, while NO_3-N content and NO_3-N/TN decreased by 15.28% and 15.31% (Figure 2.a-f). In addition, a significant increase in the nitrogen accumulation in leaves, stems and roots of ‘E/K’ was recorded compared with E/E (Figure 2.g-h).

Data processing

Nine cDNA libraries were established for RNA-seq analysis. A total of 131.07, 128.42, and 128.62 million raw reads were identified in the K/K, E/E and E/K samples, respectively. After data processing, 129.38 (98.71%), 126.30 (98.35%) and 126.79 (98.58%) million clean reads were obtained for further analysis (Table 1). More than 94% of the clean reads across all the samples were mapped to the tobacco reference genome using Hisat2[21] , most of which had unique location in that genome. And more than 92.05% of cDNA sequencing quality was ≥ 30 (Q30), implying successful library construction and reliable for subsequent bioinformatics analysis.

Gene expression and correlation analysis between samples

As shown in Figure 3.a, the FPKM expression levels for each sample were calculated. In addition, Pearson's correlation coefficient and principle component analysis (PCA) of the data profiles from all 9 samples revealed high correlation among all the samples (Figure 3.b-c). These analysis demonstrated that the sequencing data in this study was adequately representative and valid.

Differentially Expressed Genes (DEGs) selection and functional classification of differentially expressed genes among the libraries

A total of 8443 DEGs (4654 down- and 3789 up-regulated) were observed in E/E-vs-K/K while 5777 (3472 down- and 2305 up-regulated) DEGs were observed in E/K-vs-E/E (Figure 4.a). The differentially expressed genes (DEGs) were identified using DESeq2 following the criterion of adjusted *p*-value < 0.05 and |log2 Fold Change| ≥1. Hierarchical clustering analysis and volcano map revealed that the DEGs could be used to distinguish samples between different grafted combinations(Figure 4.b-d). In E/E-vs-K/K, the down-regulated DEGs were mainly enriched in pigment biosynthetic process (GO:0046148), thylakoid (GO:0009579), photosystem II assembly (GO:0010207), photosystem I assembly (GO:0048564), photosystem II oxygen evolving complex (GO:0009654) and starch metabolic process (GO:0005982), while the up- regulated DEGs were mainly enriched in membrane part (GO:0044425), plasma membrane (GO:0005886) and calcium ion binding (GO:0005509) (Figure 4.e-f). In E/K-vs-E/E, the down-regulated DEGs were mainly enriched in phosphate-containing compound metabolic process (GO:0006796), response to stimulus (GO:0050896), membrane part (GO:0044425) and catalytic activity (GO:0003824), while the up-regulated DEGs were mainly enriched in chloroplast part (GO:0044434), plastid part (GO:0044435), carbohydrate metabolic process (GO:0005975) and plastid part (GO:0044435) (Figure 4.g-h). Further KEGG pathway analysis showed that the DEGs involved in starch and sucrose metabolism, porphyrin and chlorophyll metabolism, carbon fixation in photosynthetic organism metabolism and carotenoid biosynthesis were down-regulated in E/E-vs-K/K while up-regulated in E/K-vs-E/E, which were consistent with gene ontology results.

GO enrichment and KEGG pathway analysis of DEGs Response to Grafting

To explore the DEGs in E/E plants improved by using the root of flue-cured, venn digram analysis between E/K_E/E_up and E/E_K/K_down was conducted (Figure 5.a). GO and KEGG were then conducted to examine the potential gene function and metabolism pathway of DEGs. Venn analyses showed that 1590 DEGs were enriched between E/K_E/E_up and E/E_K/K_down. The GO and all KEGG pathways are presented in Figure 5.b-c. The genes were significantly enriched in the pathway of starch and sucrose metabolism, porphyrin and chlorophyll metabolism, carbon fixation in photosynthetic organism metabolism and carotenoid biosynthesis.

DEGs Involved in Carbon and Nitrogen Metabolism

Transcriptome sequencing technology provides a large amount of information regarding the DEGs that are involved in specific biological responses. To further understand how the nitrogen and carbon metabolism-related genes in E/K plants changed compare to E/E tobacco seedlings, we extracted the DEGs involved in starch and sucrose metabolism, porphyrin and chlorophyll metabolism, carbon fixation in photosynthetic organism metabolism, carotenoid biosynthesis and nitrogen metabolism (Figure 6.a-e).The results showed that DEGs were down-regulated in E/E tobacco than in K/K tobacco while up-regulated in E/K tobacco seedlings, which might also be the cause for lower nitrate content in E/K-treated tobacco.

PPI network construction and network degree analysis

The PPI networks were constructed (Figure 7.a-c).There were 283 protein nodes and 300 protein pairs in the PPI network. After network degree analysis, gene_68511 had highest degrees in networks, followed by gene_35043 and gene_77508, which might be hub proteins.

Discussion

It has long been reported that the growth and yield of nitrogen-efficient genotypes was higher than those of nitrogen-inefficient genotypes under normal or low nitrogen conditions[22-23] . Studies by lewis et al.[4] and Shi et al.[5] indicated that burley tobacco had a weak nitrogen utilization efficiency and nitrogen assimilation capacity, thus leading to lower biomass and higher nitrate accumulation. In this study, we found that grafted burley tobacco with a flue-cured tobacco rootstock had reduced levels of nitrate accumulation in leaves (Figure 2.e). Our study also showed that the root, stem and leave biomass were lower in self-grafted burley tobacco (E/E) compared with self-grafted flue-cured tobacco (K/K) (Figure 1.f). However, grafting burley tobacco onto flue-cured tobacco (E/K) improved the growth of burley tobacco, indicating rootstock indeed had significant influence on scion growth to some extent. The results are in line with that of Huang Yuan[24] , who reported that grafting with wild watermelon rootstocks with high nitrogen use efficiency substantially improved whole plant growth.

Chlorophyll content is used as an indicator of photosynthesis which functions to provide both energy and carbon skeletons for plant growth and N assimilation[25,8] . Burley tobacco is a chloroplast- deficient mutant with reduced pigment content and lower photosynthetic capacity[2] . In this study, it was concluded that grafting burley tobacco onto flue-cured tobacco enhanced pigment content, net photosynthesis rate and photosynthesis products of burley tobacco (Figure 1.a-h), and also promoted the total nitrogen and nitrogen accumulation in root (Figure 2.g-h). This finding suggests that the flue-cured tobacco rootstocks has an increased nutrient acquisition capacity, which was translated into higher leaf chlorophyll content and photosynthesis products[26-27] . These results are similar to those found in other field crops and tomato, in which an increase of absorption and utilization of nitrogen and photosynthesis was observed in grafted plants[28,20] . In addition, studies have also shown that grafting significantly affected the content of endogenous hormones in plant leaves, thereby having regulatory effect on photosynthetic capacity of plant leaves[29-30] , which need to be verified in our study. Furthermore, transcriptome analysis revealed 5777 DEGs between burley tobacco/flue-cured tobacco (E/K) and burley tobacco self-grafted combination (E/E), which were significantly enriched in the pathways of starch and sucrose metabolism, porphyrin and chlorophyll metabolism, carotenoid biosynthesis, carbon fixation in photosynthetic organism, and nitrogen metabolism (Figure 4.a, k-i). Further investigation of down-regulated DEGs between burley tobacco self-grafted and flue-cured tobacco selfed-grafted combination and up-regulated DEGs between burley tobacco/flue-cured tobacco and burley tobacco self-grafted combination suggested flue- cured tobacco rootstock promoted pigment biosynthesis and photosynthesis of burley tobacco leaves (Figure 5.a-c). Expression of genes of POR (gene_13949), PBG (gene_76264) and GSA (gene_69664) were greatly up-regulated by grafting burley tobacco onto flue-cured tobacco (Figure 6.b). Beyond that, the genes related to carbon fixation in photosynthetic organism and starch and sucrose metabolism were all up-regulated by grafting burley tobacco onto flue-cured tobacco, including SBP (gene_47109), which encodes a key enzyme in ribulose-1,5-bisphosphate (RuBP) regeneration and Calvin cycle process [31] , FBA (gene_57322) , which has a major influence on the growth and photosynthesis of tobacco [32-33] .

Nitrogen metabolism needs energy and carbon skeleton provided by photosynthesis and carbon fixation[34 ,8 . In this study, nitrate content and NO_3-N/TN of burley tobacco was signifcantly decreased by using an flue-cured tobacco as rootstock, indicating that the replacement of root system reversed the disadvantage of scion to some extent. Nitrogen metabolism is one of the most important and basic metabolisms in plants and nitrate content is highly correlated with the capacity of nitrogen assimilation[35,5] . Nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS) are considered to play key roles in nitrogen assimilation[36-37] . Previous work published elsewhere has indicated that grafting improved tomato yield by enhancing the activities of GS[28] . Our results demonstrated that grafting burley tobacco onto flue-cured tobacco increased the activities of NR and GS in burley tobacco. The greater efficiency in NO_3^- reduction and assimilation in grafted plants was confirmed by the results for NH_4-N, proteins and total N, which were all higher in grafted burley tobacco with an flue-cured tobacco rootstock compared with self-grafted burley tobacco. Moreover, expression of genes NiR (gene_84305) and GS (gene_61180) were

significantly up- regulated, indicating that nitrogen metabolism was markedly stimulated in burley tobacco with a flue-cured tobacco rootstock.

Conclusion

In conclusion, in this experiment, we found that reduction in nitrate was accompanied by significant increases in pigment content, photosynthesis, carbohydrate and nitrogen assimilation capacity, indicating rootstocks from plants with better NUE traits can be used in grafting to improve this characteristic in less efficient shoots. In a word, grafting in tobacco plants can be defined as a rapid and effective alternative for enhancing pigment biosynthesis and photosynthesis, thereby increasing yield and decreasing nitrate and subsequent TSNA accumulation in tobacco.

Methods

Plant material and study design

A pot experiment was carried out on substrate culture in the greenhouse located in National Tobacco Cultivation & Physiology & Biochemistry Research Center of Henan Agricultural University. The flue-cured tobacco variety 'K326' and burley tobacco variety 'Eyan No.1' were used in the present study. In order to ensure the success rate of grafting, the tobacco seedlings was maintained unwatered. The 'split grafting' was used as the grafting method when the seedlings had six to eight true leaves. And when the new leaves emerged in grafted plants, the plants were transplanted into plastic pots and cultivated with nutrient solution which was provided with MS medium. The plants were grown at a temperature of 25 ± 2 ◦C under a 12h light/12h dark cycle with an average photosynthetic photon flux density of 400 μmol m^{-2} s^{-1} and relative humidity ranged from 65% to 85%. There were three graft groups: (1) K/K, both scions and rootstock were K326; (2) E/E, both scions and rootstock were Eyan No.1; (3) E/K, K326 as rootstock and Eyan No.1 as scion. The Eyan 1 and K326 seeds used in this study were provided by Hubei Tobacco Company and Henan Agricultural University, respectively. And the collection of the plant material complied with relevant institutional, national and international guidelines and legislation.

Sampling

After 3 weeks of transplantation, samples were taken for physiological and transcriptome analysis. Each treatment were performed with eighteen uniform and were divided into two groups. Fully expanded leaves of 12 plants were sampled in liquid N_2 for test of transcriptome, NRA, GSA, NH_4- N, pigment content and soluble protein content. The remaining plants were used for the photosynthesis measurement, before being divided into root, stalk and leaves for chemical measurements. Plant tissues were heated at 105 ◦C for approximately 15 min, dried to constant weight at 65 ◦C, and then pass through a 60-mesh screen. Dried powder was analyzed for total nitrogen content, nitrate, total soluble sugar and reducing sugar content in a sample.

Measurement of pigment, net photosynthesis rate and enzyme activity

Pigment content and NRA were determined by the method described by Li[38] . GSA was tested as per O'Neal and Joy[39] . Net photosynthesis rate (Pn), stomatal conductance (Gs), transpiration rate (Tr) and intercellular CO_2 concentration (Ci) were performed by a portable photosynthetic system (LI-COR Biotechnology, 6400XT, Lincoln, NE, USA) at 9:00 - 11:00 a.m..

Measurement of dry matter and carbonitride content

Nitrate content was determined by the method described by Cataldo[40] . About 1.0g of each samples was frozen in liquid N_2 and used to assay the soluble protein content according to Li[41] . About 0.5g of each samples was frozen in liquid N_2 and used to investigate the NH_4-N content according to Fan[42] . Total nitrogen, total soluble sugar and reducing sugar content were determined according to methods modifed from the Chinese Tobacco Industry standard (YC/T 161—2002 and YC/T 161, 159-2002).

Statistical Analysis

Figures were processed using GraphPad Prism (v. 8.0.1, GraphPad Software Inc., CA, USA) and correlation analysis and variance between treatments were all processed using SPSS 20.0 (IBM, Palo Alto, CA, USA). Treatments were compared by LSD multiple range test. All presented data is mean of three biological replicates (n = 3).

RNA Extraction, Preparation of cDNA Library, and Sequencing

Samples were collected form the leaves of different grafting groups and total RNA was isolate d using the mirVana miRNA Isolation Kit (Ambion, Waltham, MA, USA). Then the cDNA libr aries were synthesized using TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA). After that, the established cDNA libraries was quantified and qualified by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Quality control was perform for yielding clean datas as previous descripted[43] . The tobacco genome K326 (ftp://ftp.solgenom ics.net/genomes/Nicotiana_tabacum/assembly/Ntab-K326_AWOJ-SS.fa.gz) was used as reference g enome. And sequencing reads were mapped to the reference genome sequenc using bowtie2 or Tophat software (http://tophat.cbcb.umd.edu/)[46-47] .

Correlation analysis and PCA between samples

Correlation function (https://stat.ethz.ch/R-manual/Rdevel/library/stats/html/cor.html) in R3.4.1 was used to calculate the Pearson correlation coefficient (P). The closer the P to 1, the higher the similarity in the expression patterns between samples. PCA aims to transform the multi-index into a few comprehensive indexes using dimension reduction. In this study, the prcomp function was used (https://stat.ethz.ch/R-manual/Rdevel/library/stats/html/prcomp.html) to perform dimension reduction, and the ggfortify (Version: 0.4.5,https://mirrors.tuna.tsinghua.edu.cn/CRAN/bin/windows/contrib/3.4/ggfortify_0.4.5.zip) package was used to construct the PCA diagram.

GO and KEGG Pathway Enrichment Analysis of Differentially Expressed Genes (DEGs)

Data normalization was performed using the read fragments per kilo base per million mapped reads (FPKM) and the read counts of each gene were obtained by htseq-count[48-49] . DEGs were screened out using the DESeq (2012) [50] . The threshold for significantly differential expression were P-value < 0.05 and fold change > 2 or fold change < 0.5. Then, GO and KEGG pathway enrichment analyses for DEGs were performed using R package.

Abbreviations

Pn, Net photosynthesis rate; Gs, stomatal conductance; Tr, transpiration rate; Ci, intercellular CO_2 concentration; GS, glutamine synthase; KEGG, Kyoto Encyclopedia of Genes and Genomes; NR, nitrate reductase; PCA, principal components; PCA: Principal component analysis; DEGs: Differentially expressed genes; GO: Gene ontolog; KEGG: Kyoto Encyclopedia of Genes and Genomes; TSNA: Tobacco-specific nitrosamine; FC: Fold change;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The sequencing data were deposited in National Center of Biotechnology Information database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA720751). The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have non-financial competing interests.

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Authors' contributions

H.S. performed the conception and design of the research. Y.F., G.L. and Y.Z. conducted the research and investigation process. J.Z. and Y.L. analyzed the data and prepared the figures1-6. J.Z. and Y.F. checked the data. Y.F. drafted the manuscript. H.S. completed the revision of manuscript for important intellectual content. All authors read and approved the final manuscript.

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Table

Table 1. Statistics of transcriptome sequencing data.

Sample	Raw reads	Clean reads	Total mapped	Uniquely mapped	Error rate(%)	Q20(%)	Q30(%)	GC content(%)
K/K_1	44541564	43969238	42055032(95.65%)	39178341(89.1%)	0.0266	97.43	92.53	44.27
K/K _2	42918672	42447710	40460508(95.32%)	38782168(91.36%)	0.0263	97.56	92.79	43.52
K/K _3	43608662	42958374	40976944(95.39%)	36942872(86.0%)	0.0265	97.48	92.67	44.71
E/E_1	42229730	41506252	38963866(93.87%)	35437386(85.38%)	0.0272	97.19	92.05	44.37
E/E _2	43825134	43126072	40946360(94.95%)	37654122(87.31%)	0.0267	97.4	92.47	44.19
E/E _3	42369488	41663348	39268612(94.25%)	37893543(90.95%)	0.0271	97.25	92.12	43.26
E/K_1	42487236	41888980	39710223(94.8%)	38396153(91.66%)	0.027	97.3	92.23	43.5
E/K _2	44021296	43430390	41237990(94.95%)	39848092(91.75%)	0.0267	97.41	92.45	43.59
E/K _3	42107858	41469528	39251686(94.65%)	37635313(90.75%)	0.0265	97.48	92.64	43.5

Figures

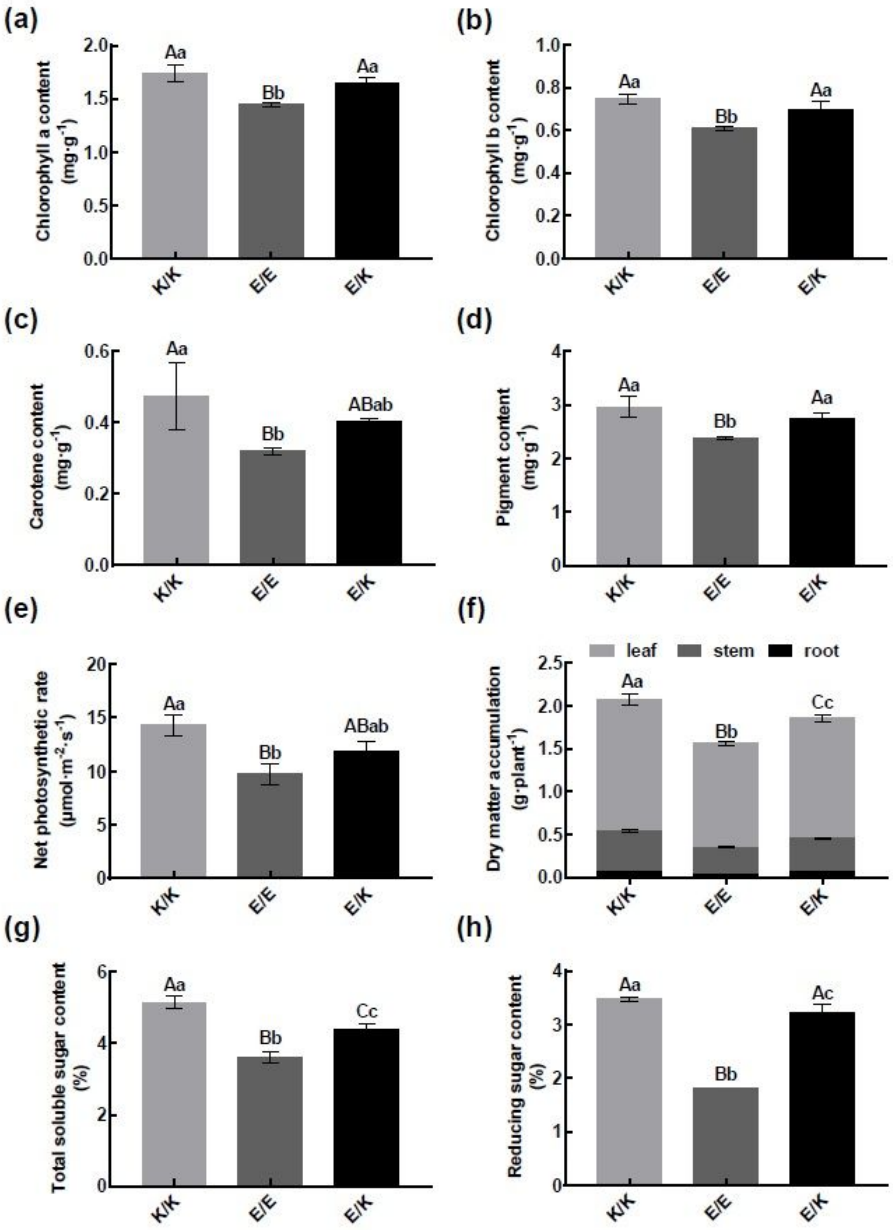

Figure 1

Differences in pigment content and photosynthesis traits. (a) Chlorophyll a. (b) Chlorophyll b. (c) Carotenoid. (d) Pigment. (e) Net photosynthesis rate. (f) Biomass accumulation. (g) Total soluble sugar. (h) Reducing sugar. Error bars indicate standard error of the means (N =3, “N” means the number of individuals). Symbols capital letters and little letters indicate that the signifcant differences between treatments are at 0.01 and 0.05, respectively. The same as belows.

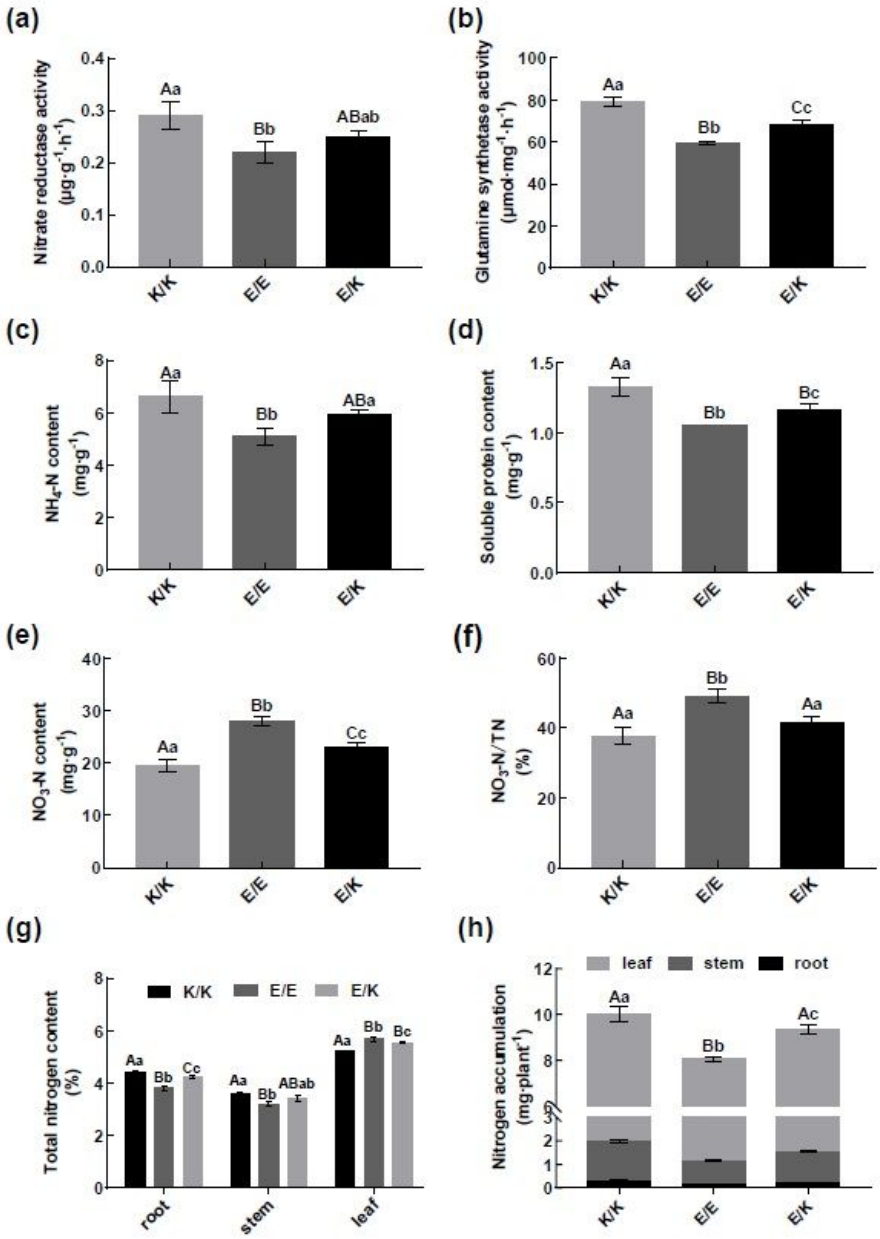

Figure 2

Differences in enzyme activities related to nitrogen metabolism and nitrogen compounds. (a) Nitrate reductase activity. (b) Glutamine synthetase activity. (c) NH4-N content. (d) Soluble protein content. (e) NO3-N content. (f) NO3-N/TN. (g) Total nitrogen content. (h) Nitrogen accumulation. Error bars indicate standard error of the means (N =3, “N” means the number of individuals).

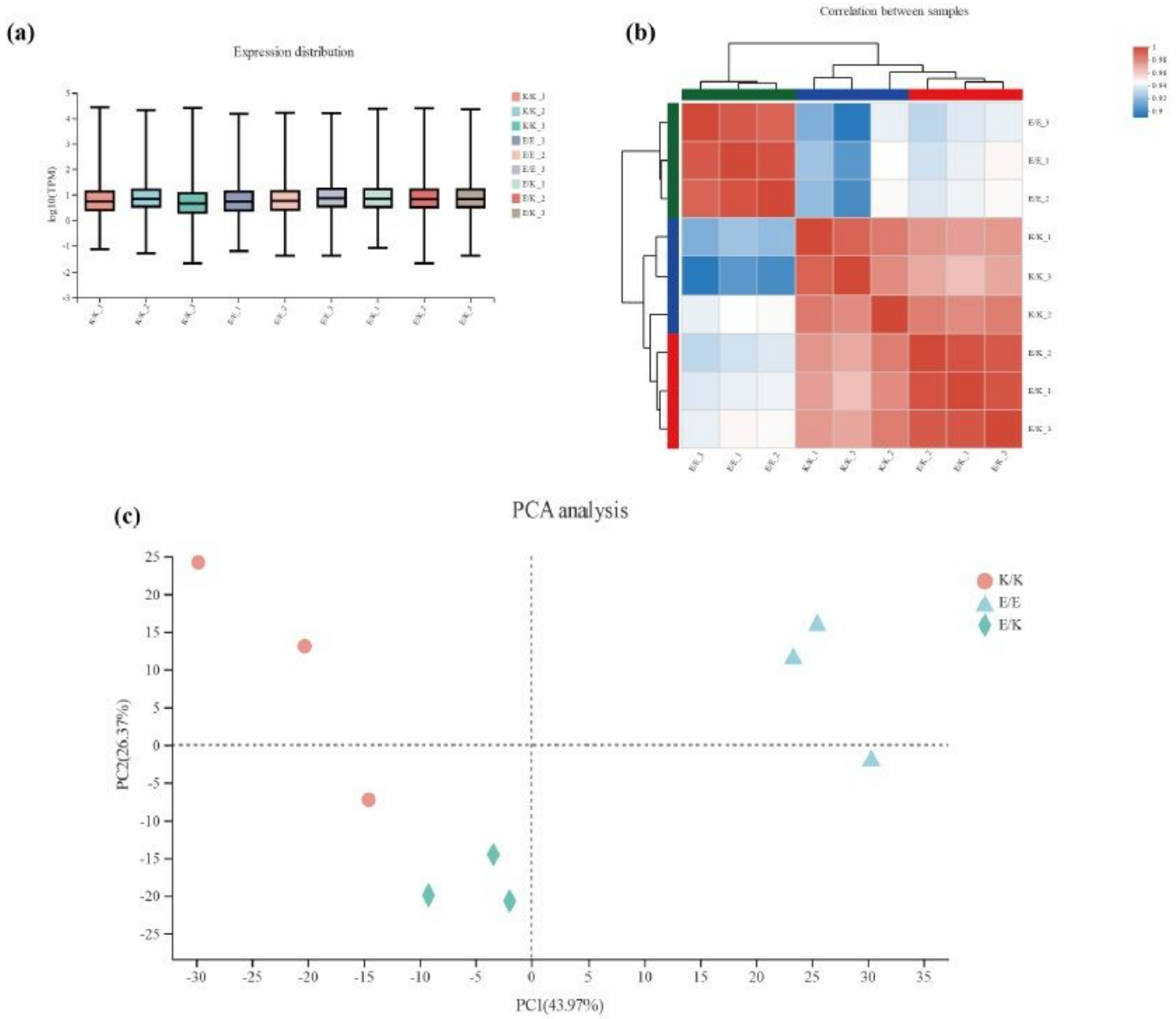

Figure 3

(a) Distribution of gene expression levels in samples. (b) Heatmap of correlation between samples based on gene expression abundance. (c) The results of principal component analysis. X and y axes represent PC1 and 2, respectively. 1-3 represents three biological replications.

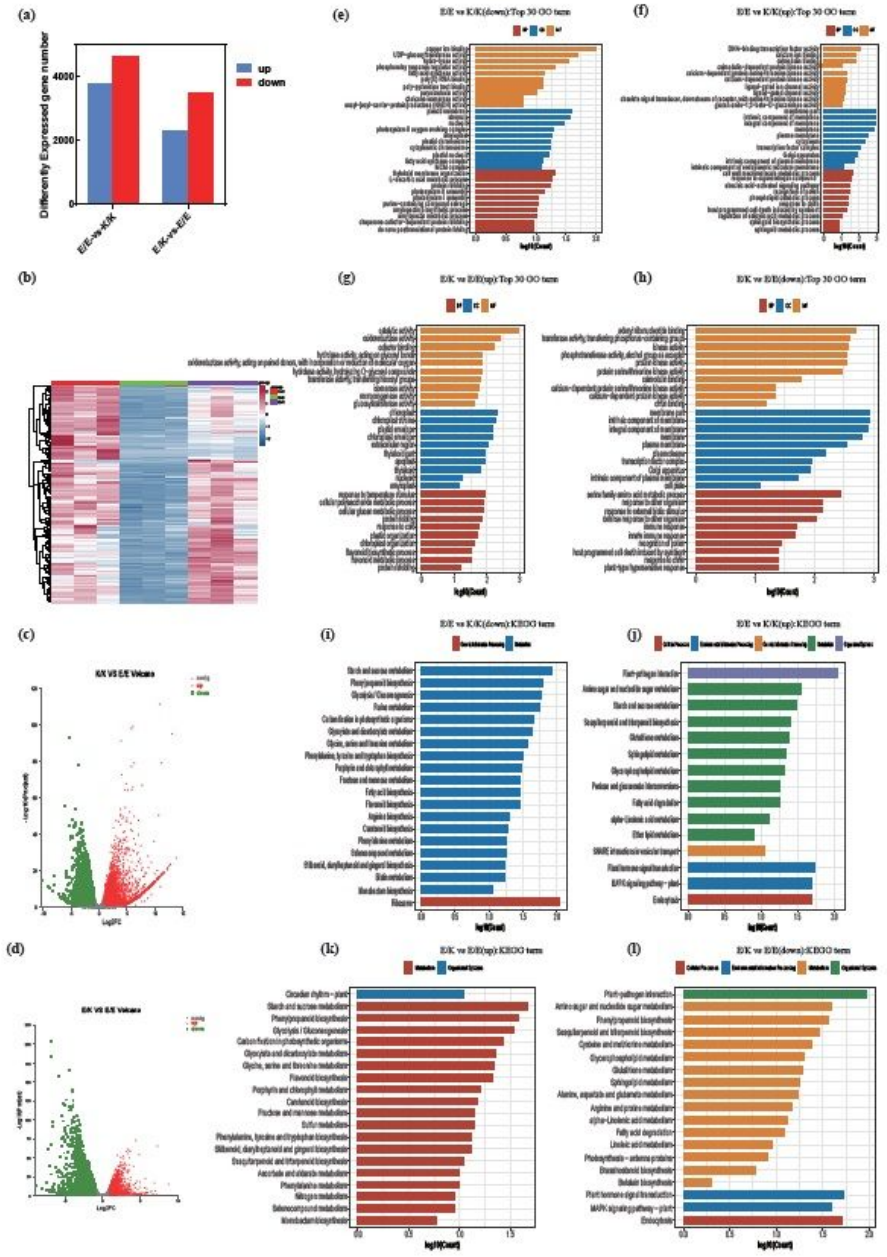

Figure 4

Differentially Expressed Genes (DEGs) selection and founctional classification of differentially expressed genes among the libraries. (a). Number of up- and down-regulated DEGs. (b). Cluster analysis of DEGs among 9 samples using hierarchical clustering method. Expression of the same gene among different samples was shown in the horizontal direction. Red color represents high expression and blue color represents low expression. (c). Volcano of DEGs between E/E and K/K. Red color represents up-regulated DEGs and green color represents down-regulated DEGs. (d). Volcano of DEGs between E/K and E/E. (e-f). GO enrichment in up-and down-regulated DEGs between E/E and K/K. (g-h). GO enrichment in up-and down-regulated DEGs between E/K and E/E. (i-j). KEGG pathway in up-and down-regulated DEGs between E/E and K/K. (k-l). KEGG pathway in up-and down-regulated DEGs between E/K and E/E.

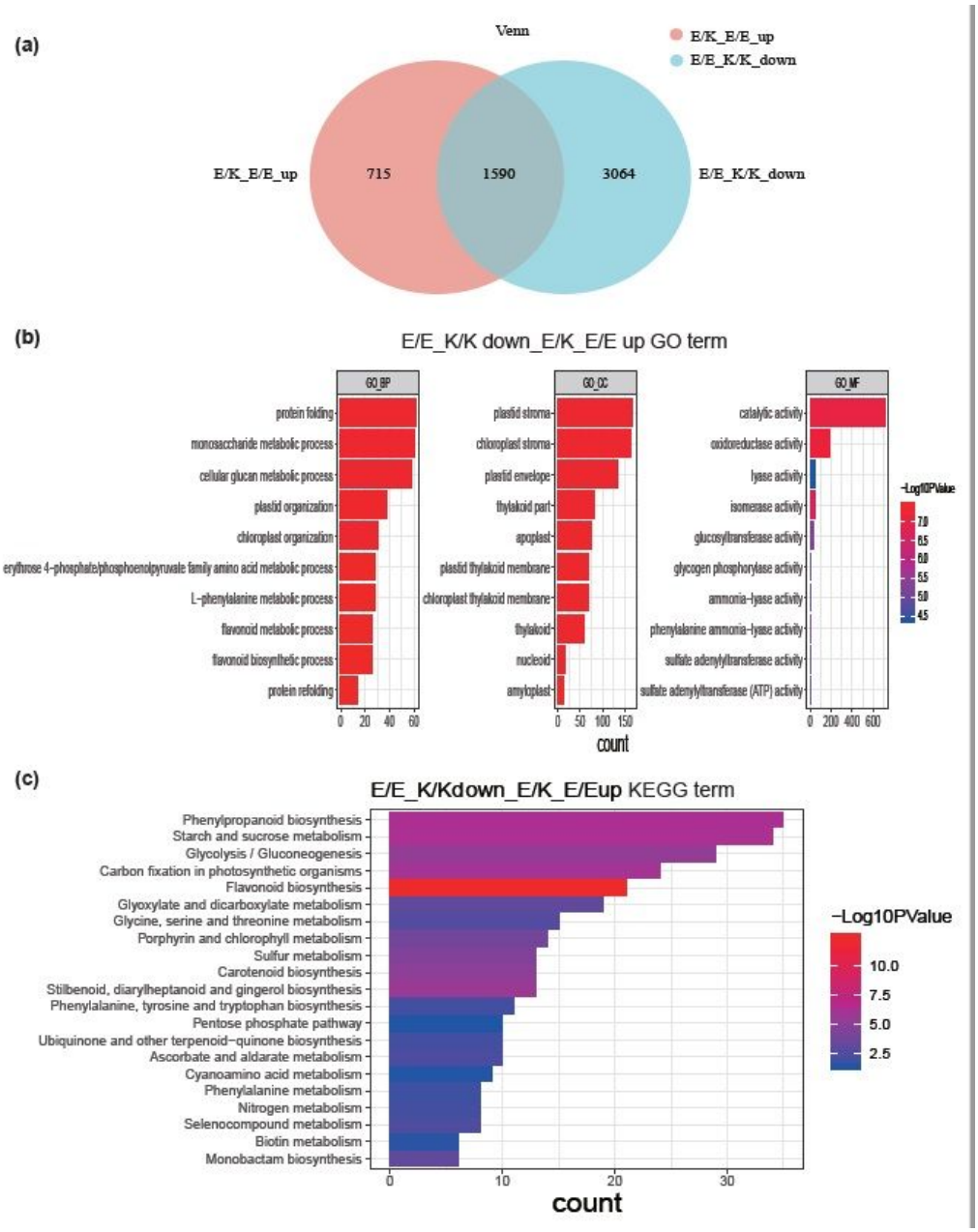

Figure 5

Go enrichment and KEGG pathway analysis of DEGs Response to Grafting. (a). Venn diagram of DEGs between E/E_K/K down and E/K_E/E up. (b). GO enrichment in DEGs response to grafting. (c). KEGG pathway in DEGs response to grafting.

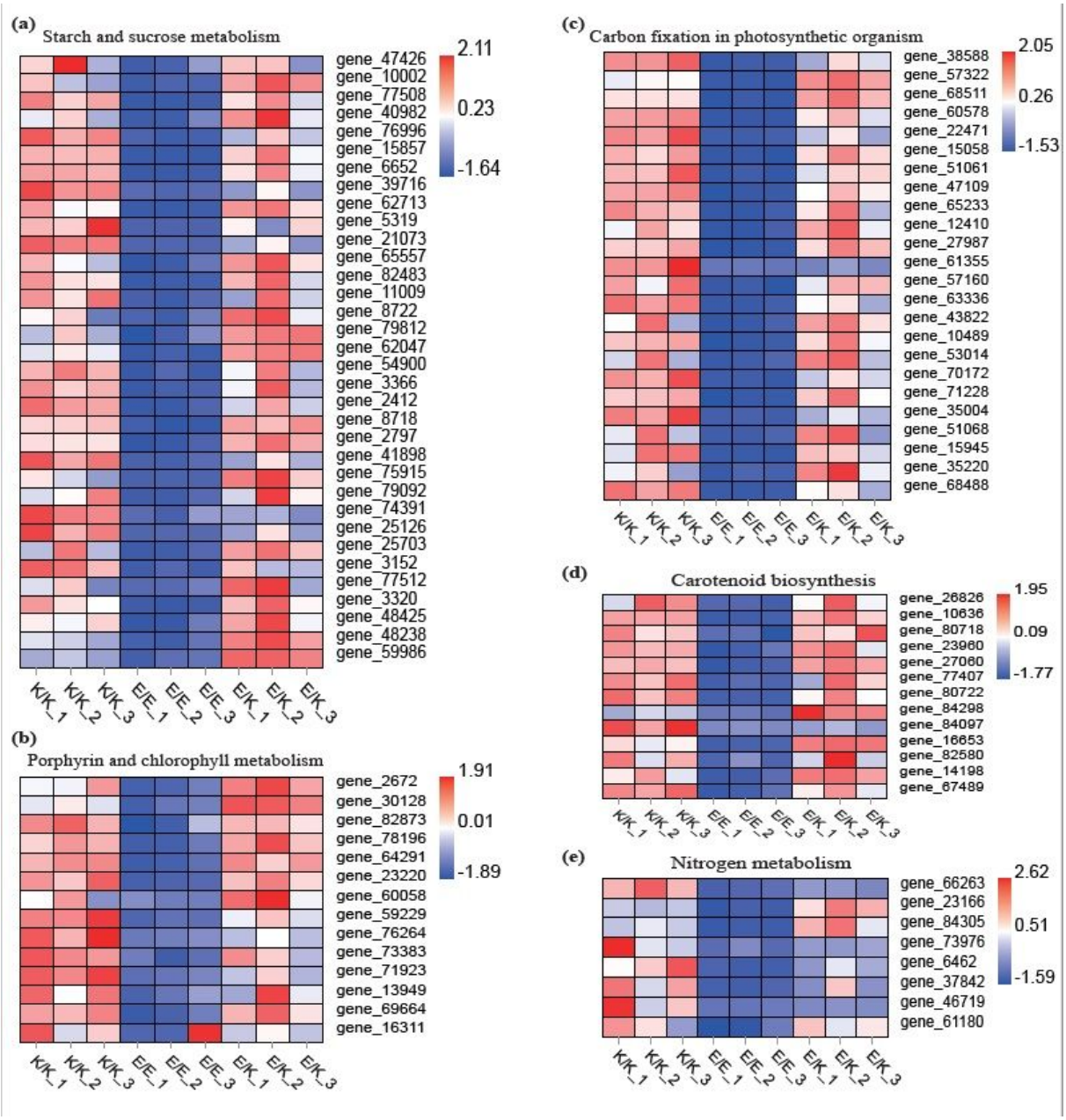

Figure 6

DEGs Involved in Carbon and Nitrogen Metabolism. (a). DEGs Involved in starch and sucrose metabolism. (b). DEGs Involved in porphyrin and chlorophyll metabolism. (c). DEGs Involved in carbon fixation in photosynthetic organism metabolism. (d). DEGs Involved in carotenoid biosynthesis. (e). DEGs Involved in nitrogen metabolism.

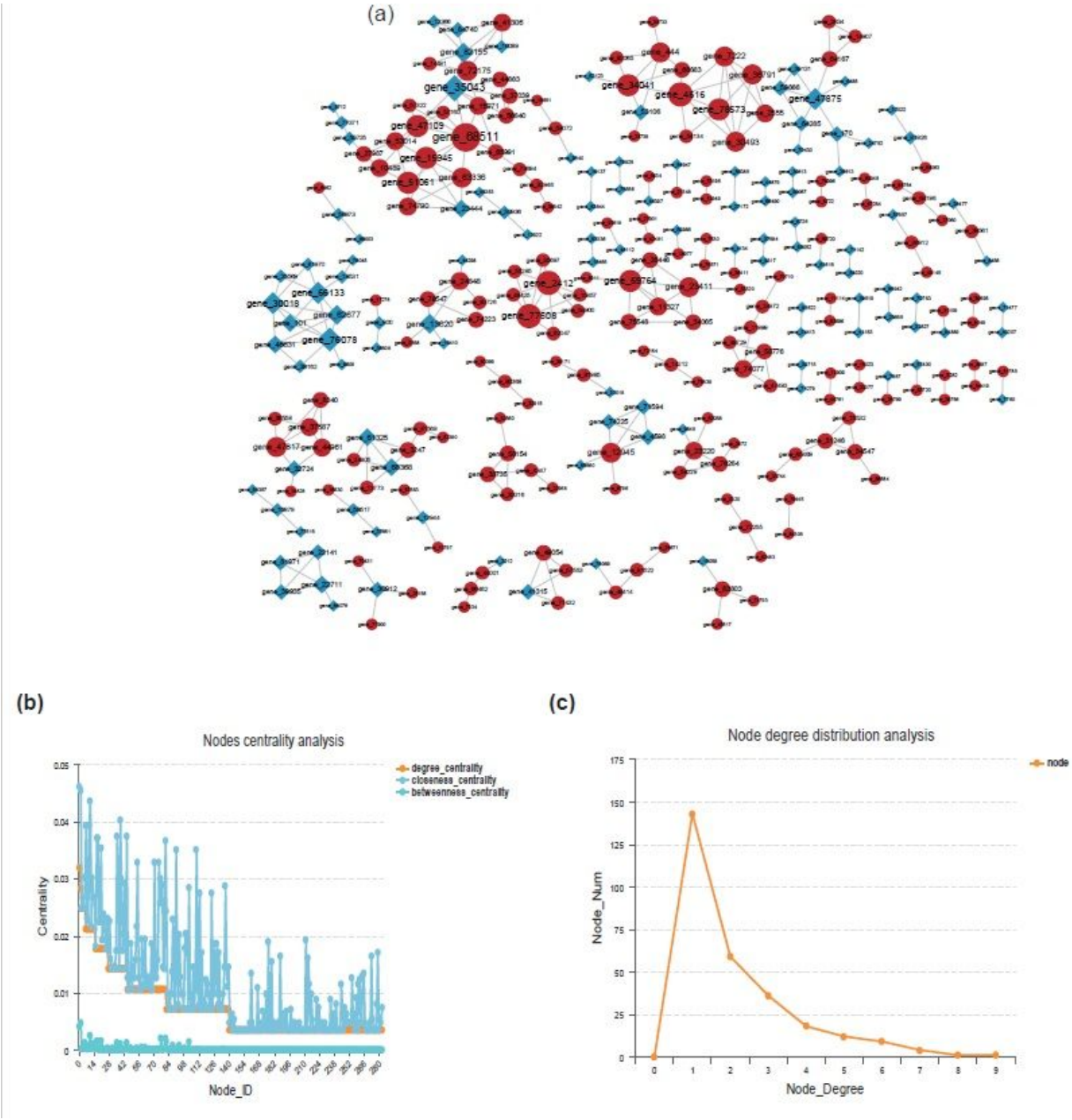

Figure 7

PPI network construction and network degree analysis. (a). The protein interaction relationship diagram of E/K VS E/E. Red circles indicate up-regulated DEGs, blue circles indicate down-regulated DEGs, connections indicate protein interaction relationships, and node size indicates the degree of connection. (b-c). Analysis of topological properties of PPI network nodes. Y-axis represents the importantance of nodes in diagram b. Y-axis represents the number of nodes on the corresponding degree in diagram c.