Transcriptomic analysis reveals the mechanism of leaf yellowing in Nicotiana tabacum L.

Rongli Jia [1,2]. Pingsong Wang [1,3]. Lili Duan [1,3]. Zejun Mo [1,3]. Yuanhui Que [1,2].Ting Liang [1,3]. Ying Huang [3].Renxiang Liu [3,*]

[1] College of Tobacco, Guizhou University, Guiyang 550025, P.R.China

[2] Guizhou Key Laboratory for Tobacco Quality Research, Guizhou University, Guiyang 550025, P.R.China

[3] College of Agriculture, Guizhou University, Guiyang 550025, P.R.China

[*] Corresponding author: Renxiang Liu; rxliu@gzu.edu.cn

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Authors' contributions R L and P S participated in data analysis and manuscript writing; WPS constructed the population material and obtained phenotypic data with L L, Z J, Y H, and T L; R L, P S, Y H and R X conceived the This study; edited by R X.

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Declarations

Competing interests The authors declare that there is no confict of interest.

Declaration of Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abstract

Background Leaf color affects the yield and quality of flue-cured tobacco. At present, the cost of labor for the harvesting of tobacco in batches is high. It is difficult to realize mechanized operations. The yellow leaf mutant of tobacco discovered by the research group can promote the maturity of tobacco leaves and improve the centralized maturity of tobacco leaves. However, its yellowing mechanism remains poorly understood.

Objective This study providing a new insights into both physiological and molecular aspects of the leaf yellowing mechanism.

Methods We compared the yellow leaf mutant (Y), the normal green plant K326 (K) and backcross material yellow leaf K326 (KY) in physiology, cytology, and transcription spectrum.

Results In the present study, we found that the total chlorophyll content of the two yellow leaf plants before maturity was significantly lower than that of normal plant, and the thylakoid structure was incomplete. A total of 3551 differentially expressed genes were identified in the transcriptome. The synthesis of Mg-Proto IX, abnormal thylakoid structure and the chlorophyll content in leaves were significantly reduced, which was due to the significant reduction in the expression of chlorophyll synthesis genes ChlH and ChlD and chloroplast development gene PPR. We speculate that the expression changes of the zeatin synthesis gene CISZOG and the degradation gene CKX in the yellow leaves, and inhibit chlorophyll synthesis by reducing the zeatin content and the expression of ChlH. FPGS gene, folB gene and metabolic pathway GGH gene expression are altered in yellow leaves to inhibit chlorophyll synthesis by reducing folate content. The above is the main reason for the yellowing of tobacco leaves.

Conclusion By comparing yellow leaf and green leaf tobacco varieties, this study not only confirms the important role of chlorophyll and chloroplasts in yellow leaf leaf coloration, but also provides new insights into the transcriptome resolution mechanism of leaf color mutation.

Keywords Nicotiana tabacum L.· Leaf yellowing · Physiological feature · transcriptomic

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Introduction

Leaf coloration is a common but essential biological process, especially for ornamental plants and eco-nomic crops. Leaf color mutants are not only ideal materials for the study of chlorophyll synthesis, but also for the study of plant photosynthesis, photomorphogenesis, chloroplast differentiation, and development (He et al. 2006). There are two main reasons for the formation mechanism of leaf color mutants, namely the mutation or abnormal expression of genes related to the chlorophyll synthesis and metabolism pathway and the chloroplast development pathway. Chlorophyll anabolism involves 15 steps of enzymatic reactions and 27 coding genes, starting from the formation of glutamyl-t RNA and finally generating Chl a and Chl b (Beale 2005). There are three important control points in chlorophyll synthesis: 5-aminolevulinic acid

synthesis, magnesium chelatase-catalyzed reaction, and chlorophyll ester synthesis, which can directly affect chlorophyll synthesis. The content of 5-aminolevulinic acid (ALA) determines the total amount of downstream precursors in the chlorophyll synthesis pathway. The activities of glutamyl-tRNA reductase (Glu-TR) and 1-semialdehyde-glutamate aminotransferase (GSA) directly affect ALA synthesis (Meskauskiene et al. 2001). Heme negatively regulates ALA synthesis, and the exogenous application of heme inhibits Glu-TR activity (Pontoppidan and Kannangara 1994). Magnesium chelatase is composed of ChlH, ChlI, and ChlD subunits. Each factor affects the activity of magnesium chelatase by regulating the transcription level of each subunit or by regulating the interaction between the three subunits.

Chlorophyll biosynthesis is performed in the chloroplasts. Many key enzymes involved in the biosynthesis of chlorophyll are combined with thylakoids. Chloroplast development directly affects chlorophyll synthesis (Zhao 2020). APG1 encodes a chloroplast inner membrane protein precursor, and chloroplast numbers and chlorophyll levels are reduced in apg1 mutants (Motohashi et al. 2003). Some studies have also found that chloroplast protein mutations can lead to defects in the development of the inner membrane structure of the chloroplast, resulting in changes to the content and ratio of photosynthetic pigments in chloroplasts (Chen et al. 2005).

Transcriptome sequencing technology is widely used in the study of leaf color mutants and is of great significance for the study of pigment metabolism and molecular regulation, as well as the discovery of new genes. The transcriptome sequences of the green and white leaves of Pseudosasa japonica f.Akebonosuji have been previously sequenced. Through co-expression analysis, a regulatory network involving chlorophyll deficiency was found, wherein ELIP genes may be responsible for inducing leaf color variation. At the same time, the genes highly expressed in the white leaves were found to be mainly involved in the stress response and tetrapyrrole synthesis, indicating that the formation of white leaves is related to chlorophyll deficiency (Yang 2015). A transcriptome analysis of the barley albino glume mutant and normal glume showed that the differential genes were mainly annotated into chloroplasts, carbon metabolism, and photosynthesis. Therefore, the downregulation of genes related to chloroplast development resulted in wheat glume albinism (Luan et al. 2017).

Tobacco is an important foliar cash crop, with leaf color affecting both the yield and quality of tobacco. The batch harvesting of flue-cured tobacco has always represented a bottleneck within the mechanized operation of tobacco production. If the leaves of the whole plant mature and yellow consistently, the number of harvesting can be reduced and the labor cost can be reduced. Improving the “wild” with inconsistent maturity of crops through breeding is the main goal of human domestication and the transformation of wild plants. Our research group previously discovered tobacco yellow leaf mutants that have the effect of promoting tobacco leaf maturation and increasing the concentration of tobacco leaf maturation (Chen et al. 2011). However, the

etiolation mechanism of this mutant remains unclear. To understand how the etiolation characteristic of this mutant is formed, we used cytology, physiology, and transcriptomics to compare the characteristic differences between yellow leaves and normal leaves, which enriched the formation mechanism of leaf color mutation and provided rich germplasm resources for the breeding of tobacco varieties with concentrated maturity and the rapid identification of variety purity by using yellowing traits.

Materials and methods

Plant materials

The tobacco yellow leaf mutant (Y) was obtained by backcrossing flue-tobacco species G28 × NC2326 hybrid F1 plants. Additionally, normal green leaf K326 (K) and yellow leaf K326 (KY) varieties were used. The yellow leaf mutant (Y) was selected and bred from a yellow leaf plant discovered in the DH line, obtained through in vitro anther culture when the research team used the green leaf flue-cured tobacco varieties G28 and NC2326 to perform haploid breeding. A yellow leaf mutant (Y) was used as the donor parent of yellow leaf K326 (KY), and green leaf flue-cured tobacco variety K326 was used as the recurrent parent. The yellow leaf strain BC5 was obtained after five generations of backcrosses.

The experiments were performed at the tobacco scientific research base of Guizhou University. A floating tray was used to raise seedlings; a complete random experimental design was used in the seedling raising stage, and a random block experimental design was used in the field experiment, repeated in triplicate. Samples were taken 10, 20, 40, 60, 80, 100, and 120 days after emergence to evaluate photosynthetic pigments, observe the chloroplast ultrastructure, determine intermediate products of chlorophyll synthesis, and for transcriptome sequencing. The samples were immediately placed in liquid nitrogen and stored in a −80°C ultra-low temperature refrigerator until further use. The TEM samples were placed in 4% glutaraldehyde fixed solution, pumped by a vacuum pump, and stored at 4°C until further use.

Determination of photosynthetic pigment content

The seedling at 10, 20, 40, 60, 80, 100, and 120 days were determined. Take Fresh leaves 0.2 g, cut it into pieces, and 80% acetone was added to extract the pigment. During extraction, the leaves were shaken slowly in the dark for 48h until they turned completely white. The absorbance values D at 470 nm, 663 nm, and 645 nm were measured using a spectrophotometer(Sun et al. 2022) .

Chloroplast transmissive electron microscope observation

Using seedlings sampled at 20 days (the maximum period of photoraximin content change), leaves from normal green K and yellow leaf KY and Y were cut into 1 × 1 cm pieces and placed

in a 4% glutaraldehyde fixation until the blade was able to sink into the tube. After storing overnight at a low temperature, the samples were rinsed with phosphoric acid buffer, and 1% osmic acid was added for secondary fixation for 1 h. This was followed by dehydration using ethanol, which was subsequently replaced with acetone. Next, the sample was immersed in the resin at 60°C and polymerized for 2-3 days to complete embedding. Lastly, sections were obtained using a slicer (Leica microsystems GmbH, Wetzlar, Germany), followed by staining with uranyl acetate (UA) for 20-25 min. The samples were observed and photographed under a transmission electron microscope (JEM 1230; Jeol, Tokyo, Japan)(Zhang et al. 2019).

Determination of chlorophyll synthesis precursorsampled at 20 days after emergence were used to identify the chlorophyll synthesis precursors in the experimental plants. A method previously reported in the literature was used to determine the relative content of ALA and PBG, with some modifications (Lew and Tsuji 1982). Briefly, the sample (1.0 g) was firstly ground in liquid nitrogen, followed by the addition of acetic acid-sodium acetate buffer. The sample was boiled in a water bath before centrifuging. The resulting precipitate was washed before repeating centrifugation. After combining the supernatant and centrifuge, ethyl acetoacetate was added in a boiling water bath and centrifugation was repeated. Equal volumes of Ehrlich reagent were added and the absorbance was measured at 553 nm to determine the ALA content. The leaves were extracted with phosphoric acid buffer and treated using the method described above. The absorbance was measured at 553 nm for the analysis of the PBG content.

The relative content of Urogen III was determined according to Sun Jieyin's method, with some modifications (Sun et al. 2007). Pretreatment was the same as that for ALA and PBG. The precipitate was washed with phosphate buffer and centrifuged, the supernatant was combined, and PVPP was added for centrifugation. The extract was then added to a boiling water bath containing ethyl acetoacetate. After cooling, an equal volume of ether was added and allowed to stand to aspirate the ether. The absorbance of the aqueous phase was measured at 405.5 nm, and the relative content of Urogen III was calculated. The above-mentioned ether extract was extracted with hydrochloric acid again, and the light absorption value at 399.5 nm of the hydrochloric acid phase was measured after the merger to determine the relative content of CoprogenIII.

The determination of the Proto IX, Mg-Proto IX, and Pchlide content was performed according to Chen Yougen's method (Chen et al. 2013). The leaves (1.0 g) were ground using alkaline acetone. After centrifugation, the supernatant was diluted in acetone and the absorbance values of A575, A590, and A628 were measured to calculate their contents.

RNA extraction, cDNA library preparation, RNA sequencing and transcriptome analysis

Total RNA was extracted from the leaves 20 days after emergence by Trizol up plus RNA Kit Method of all gold RNA extraction kit(Chen, et al. 2020). The concentration and quality of

RNA were determined using an Agilent 2100 biological analyzer (Agilent Technologies, Santa Clara, CA, USA), and purified poly-(a) mRNA from the total RNA by magnetic beads with oligo DT. The mRNA was mixed with fragment buffer to obtain short fragments. A library was constructed using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). An Agilent 2100 biological analyzer (Agilent Technologies, Palo Alto, CA, USA) was used to evaluate the quality of the sample library, and Illumina pe-100bp was used to sequence the whole transcriptome. These tests were completed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. The correlation of gene expression levels between samples is an important indicator for testing the reliability of experiments and the rationality of sample selection. Before performing differential expression analysis, it was necessary to examine the correlation of gene expression levels between the samples. The Pearson correlation coefficient was used to express the correlation between gene expression levels, wherein a correlation coefficient between 0.8 and 1.0 is considered to be relatively strong (Fig. S2, S3).

Analysis of differential geneexpression and enrichment of GO and KEGG

Differential gene analysis between samples reflected the overall differences in gene expression between samples. To identify differentially expressed genes, we used fold change (FC) ≥ 2 and FDR < 0.05 01 as screening criteria. FC represents the ratio of expression between the two samples (groups). FDR (false discovery rate) is the false discovery rate, which is obtained by correcting the p-value of difference significance. FDR was used as the key index of differential expression gene screening. To further explore the biological functions of differentially expressed genes, the software Goatools was used to perform GO enrichment analysis on the genes in the gene set, using Fisher's exact test. To identify the function of differentially expressed genes in specific biological metabolic pathways, we used KOBAS software to detect the statistical enrichment degree in the KEGG pathway.

RT-qPCR analysis

RT-qPCR test was mainly used to determine the expression level of candidate genes, and total RNA was extracted from the leaves of normal green leaf K, yellow leaf material KY, and Y 20 days after emergence, and extracted with the RNAprep pure Kit (Tsingke, China). The NCBI primer BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design gene-specific primer pairs with the tobacco L25 gene as the internal reference gene. The PCR program used was the Bioradcfx system (Bio-Rad, USA), using the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression level of the target gene. Each experiment was repeated three times. A list of the RT-qPCR primers used in this study is provided in table S2.

Results

Analysis of phenotypic characteristics of yellow leaf mutant

In Fig. 1a, the normal green leaf plant K is shown on the left and the yellow leaf plant KY is shown on the right. The yellow leaf plant KY shows leaf yellowing at different growth stages, which is significantly different from the normal plant K. KY can grow and blossom normally, which is a non-lethal yellowing mutation. We investigated and analyzed the agronomic characteristics of two yellow leaf plant KY, Y, and normal plant K, grown in the field environment at different stages. We found that the plant height, middle leaf length, and middle leaf width of the two yellow leaf plant were greater than those of normal plants (Fig. 1b, d, and e, Table S1)with the development of plants in the later stage of growth. The number of leaves (Fig. 1c, Table S1) showed little difference in other stages, except in the mature stage, indicating that the growth of this mutant was not affected.

Color changes in the leaves of plant mainly occur as a result of changes in the pigment contents and types in leaves. Therefore, we measured the photosynthetic pigment content of two yellow leaf plant, KY, Y, and K, of normal plants at different growth stages (Fig. 2, Table S2). In each growth stage, the contents of chlorophyll a, chlorophyll b, and carotenoids in the leaves of two yellow leaf plant, KY and Y, at different stages were significantly lower than those of normal plant K, and the overall change trend first increased and then decreased. At maturity, the content of chlorophyll a, chlorophyll b, and carotenoids in the three materials showed little difference. After entering the mature stage, the chlorophyll is degraded and the leaves begin to turn yellow. In general, the significant decrease of total chlorophyll content is one of the main causes of leaf yellowing. There was almost no difference in the photosynthetic pigment content during the mature stage, indicating that the rate of photosynthetic pigment degradation was accelerated in the yellow leaves during this period, leading to reduced pigment accumulation.

Chloroplast development in yellow leaf material is defective

Since the chlorophyll content in the leaves of the yellow leaf material decreased significantly, to determine whether the chloroplast structure of the mutant changed, we evaluated the chloroplast development of the three materials using transmission electron microscopy (TEM) (Fig. 3a-f). The structure of chloroplasts in normal plants K was complete, spindle-shaped, close to the cell wall, and had a highly differentiated thylakoid system. The grana lamellae of the chloroplasts were clearly and orderly arranged. However, the chloroplasts in the leaves of yellow leaf plant KY and Y were swollen, the chloroplast membrane was damaged and dissolved, the matrix was leaking out, the grana lamellae of thylakoids were not clearly visible, and the surface shape of starch grains in Y was abnormally small. Based on these observations, we speculate that the mutant gene affects the formation and development of the thylakoid membrane and grana lamella.

Hindered synthesis of chlorophyll precursor

The decrease in chlorophyll content in the yellow leaf plant and abnormal chloroplast development usually involve chlorophyll synthesis and metabolism, wherein the obstruction of any step in the reaction may affect subsequent reactions. Therefore, we evaluated several key intermediates involved in chlorophyll synthesis. The analysis showed that there was little difference in ALA and PBG content between yellow and green leaves. The content of Urogen III in yellow leaf plant increased significantly; the increase in KY was 166%, while the increase in Y was 157%. Furthermore, the contents of ProtoIX, Mg-ProtoIX, and Pchlide were significantly lower than those in the normal leaves (Fig. 3g, Table S3). We infer that PBG to UrogenIII synthesis accumulates in yellow leaves and degradation is accelerated during UrogenIII to CoprogenIII synthesis. The CoprogenIII to Pchlide process in yellow leaves has been accompanied by excessive degradation, so the amount of chlorophyll formed eventually decreases due to the accumulation of precursor material.

Transcriptome analysis of yellow leaf plant to screen significantly different genes

Based on the results of phenotypic and physiological changes in the yellow leaf plant, we speculated that the expression patterns of genes related to chloroplast differentiation, development, and pigment synthesis in yellow leaf plant had changed. Transcriptome analysis was performed to verify these inferences. Total RNA of three biological replicate samples was extracted from yellow leaf material KY and Y and normal plant K, and RNA sequencing was performed using Illumina Hiseq. 2500. A total of 110.14 Gb of clean data was obtained; the clean data of each sample was over 10.58 Gb, and the percentage of Q30 bases was over 94.53%. The clean reads of nine samples were compared with the designated tobacco reference genome (https://solgenomics.net/organism/Nicotiana_tabacum/genome), the results of which showed that the comparison rate varied from 95.85% to 96.49% (Table S4). These results indicate that the amount of data obtained from sequencing was sufficient, the sequencing quality was good, and the selection of the reference genome was appropriate. The results of the subsequent differential gene analysis with clean reads were reliable.

The gene expression profiles of the three libraries were analyzed using the fpkm method. A total of 3551 differential genes were detected in normal plant K and yellow leaf material KY and Y (p-adjust < 0.05%, $|\log2fc| \geq 1$). The differentially expressed genes detected in the K_vs_KY group were upregulated (317) and downregulated (378), the differentially expressed genes detected in the K_vs_Y group were upregulated (762) and downregulated (1150), the differentially expressed genes detected in the Y_vs_KY group were upregulated (438) and downregulated (506) (Fig. 4a, Table S5), and K and KY genetic background differences were small. In K_vs_KY and K_vs_Y, 406 genes were differentially co-expressed in the two

comparison groups, which proves that these 406 genes may be closely related to leaf yellowing (Fig. 4b).

Enrichment analysis of the differentially expressed genes

The results of GO enrichment analysis showed that these DEGs fall into three categories, including biological processes, molecular functions and cellular components. DEGs were significantly enriched ($P < 0.05$) in the "carbohydrate derivative catabolic process","carbohydrate binding","defense response","ADP binding" and "tetrahydrofolyl poly glutamate metabolic process"(Fig. 5a). For the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment anal-ysis, DEGs were significantly enriched ($P < 0.05$) in the "Folate biosynthesis" and "Zeatin biosynthesis"(Fig. 5b).

DEGs involved in chloroplast development and chlorophyll synthesis

The structural development of chloroplasts has an important impact on the color of plant leaves. Based on the data of GO term enrichment analysis (Table S6), the PPR gene was significantly downregulated in yellow leaf plant. PPR protein family members are mostly located in mitochondria and chloroplasts. Loss of function may lead to the abnormal development of the chloroplast structure (Rovira and Smith 2019; SCHMITZLINNEWEBER and SMALL 2008). Therefore, the PPR gene was significantly downregulated in yellow leaves, resulting in abnormal chloroplast development.

The biosynthesis of chlorophyll play crucial roles in leaf yellowing. Chlorophyll biosynthesis involves the catalytic reactions of 15 enzymes. Mutations in these enzyme genes or the inhibition of their activity by inhibitors may lead to changes in chlorophyll content (Li et al. 2019). We found two chlorophyll-related DEGs, ChlH and ChlD, in the KEGG pathway analysis. ChlH and ChlD were significantly downregulated in yellow leaves (Table S7, Fig. S1). ChlH and ChlD are subunits of magnesium chelate (MgCh), and ChlH has a catalytic effect. Therefore, the significant downregulation of ChlH and ChlD expression in yellow leaves affected the activity of magnesium ion chelatase MgCh, and the synthesis of chlorophyll in yellow leaves was blocked.

DEGs involved in folic acid and zeatin synthesis

For K_vs_KY and K_vs_Y, the KEGG pathway with the highest significance was folate biosynthesis(Fig. 6). Folic acid accumulation may be the main cause of tobacco leaf yellowing. The number of genes enriched to the pathway was 5. Among them, FPGS gene and folB gene were significantly downregulated in yellow leaf materials KY and Y, while the expression of GGH gene was significantly increased. The above genes were genes in folic acid biosynthesis and metabolism pathway, and abnormal expression would affect the accumulation of folic acid. The content of folic acid affects the efficiency of chlorophyll synthesis (Zhang et al. 2017). Therefore, significant changes in the expression of FPGS gene, folB gene, and GGH gene in the folate

anabolic pathway reduce the accumulation of folate in yellow leaves, and chlorophyll synthesis is affected.

An additionally enriched pathway was zeatin biosynthesis, and the number of genes annotated to this pathway was also five. Among them, CISZOG gene was significantly downregulated in yellow leaf material KY and Y, and CKX gene in yellow leaf material KY and Y. Zeatin is the most common and first discovered class of cytokinins, CISZOG gene and CKX gene finely regulate the anabolic process of cytokinin, cytokinin has a promoting effect on chloroplast ultrastructure and chlorophyll synthesis(Letham 1963). Therefore, significant changes in CISZOG and CKX genes resulted in reduced zeatin content in yellow leaves and inhibited chlorophyll synthesis.

Validation of gene expression by RT-qPCR

We selected seven important genes in chlorophyll synthesis and related to chloroplast development, and analyzed their expression levels and patterns to verify the accuracy of the transcriptome data. The gene expression patterns obtained via RNA-seq and RT-qPCR analyses were similar (Fig. 7), confirming the accuracy and reliability of the transcriptome results.

Discussion

Yellow leaf plant grow normally and can blossom and bear fruit

In the early research of our research group, the yellow leaf mutant has a high light saturation point and strong light energy utilization under high light intensity (Duan et al. 2014). This study shows that the two yellow leaf plant grew and developed normally throughout the growth period. We speculate that there may be some compensation mechanism, the photosynthetic pigment content of yellow leaf plants is significantly reduced, the chloroplast structure is abnormal, but the yellow leaf plants can develop normally.

Chlorophyll synthesis was blocked and pigment content decreased

Many plant mutants exhibit a yellowing or yellowish-green phenotype because of the low chlorophyll content in their leaves. In wheat, the chlorophyll and carotenoid contents in the leaves of albino mutants have been found to be significantly lower than those of the wild type. The obstruction of chlorophyll synthesis is one of the main reasons for albinism in mutants (Su et al. 1990). In the present study, KY and Y were found to show leaf yellowing throughout the growth process. Indicating that a decrease in total chlorophyll content is the main reason for leaf yellowing.

The process of chlorophyll synthesis is complex and includes 15 steps of enzymatic reaction (Tanaka and Tanaka 2006). Abnormal accumulation or reduction at any step affects subsequent chlorophyll synthesis. Protoporphyrin IX (ProtoIX) is a key intermediate metabolite. In a study

on Euonymus japonicus L. var. aureomarginatus and E. japonicus L. var. aureovariegatus, the content of ProtoIX, an intermediate product of chlorophyll synthesis in yellow tissue, was found to decrease sharply, while the contents of Mg protoIX and Pchlide increased significantly. The decrease in chlorophyll content in yellow tissue may be caused by an obstacle in the synthesis of ProtoIX from Coprogen III (Zhu et al. 2017). Intriguingly, our results indicate that while the content of CoprogenIII in yellow leaf plant KY and Y was significantly reduced, it similar with K. This is different from previous research results, and indicates that although the content of CoprogenIII decreased, it was not the cause of the decrease in the chlorophyll content. Transcriptome results also confirmed this finding. Expression of the synthetic CoprogenIII-related gene was not abnormally altered. The ProtoIX, Mg-ProtoIX, and Pchlide contents were significantly lower than those of normal plants. This phenomenon indicates that the synthesis of ProtoIX was abnormal, resulting in the low chlorophyll content of yellow leaves. The corresponding mutants could be used as a high-quality material to study leaf color formation and chlorophyll metabolism.

Chloroplast dysplasia

Another possible cause of leaf yellowing is the structure and development of the chloroplasts. In the millet light green leaf mutant, the grana thylakoids in the chloroplast matrix have been reported to be significantly reduced, with a thylakoid structure that was incomplete (Li 2015). The results of this study showed that the density of KY and Y thylakoids was lower than that of normal plant K, and the chloroplast thylakoids had abnormal structure, which affects chlorophyll synthesis and yellowing of the leaf color.

Transcriptome analysis of leaf etiolation

In previous studies, the deletion of the PPR protein in rice was found to affect the splicing of multiple class II introns; chloroplast development was defective, chlorophyll synthesis was blocked, and the mutant showed a yellowing phenotype (Lv et al. 2020). In our study, PPR gene is significantly downregulated in yellow leaves, resulting in abnormal thylakoid structure in yellow leaves, which could be indicated by chloroplast ultrastructure and accompanied by blocked chlorophyll synthesis.

Folic acid is a water-soluble vitamin(Fabrice et al. 2006; John et al. 2000). Plants with green leaves contain large amounts of folic acid. Folic acid influences chlorophyll synthesis(Douce et al. 2001; Hanson and Roje 2001). Through a synthesis process requiring a methylation step, chlorophyll accumulates in the thylakoid membrane (Block 2002). In etiolated leaves, the synthesis rate of chlorophyll was found to decrease by 2.5% after treatment with MTX, which inhibited the activity of dihydrofolate reductase. Under this treatment, the folate pool was reduced by 25%, accompanied by damage to the methylation cycle, resulting in a three-fold reduction of Mg protoporphyrin IX methyltransferase (ChlM) activity involved in chlorophyll

synthesis (Van Wilder et al. 2009). The results of this study showed that folate synthesis pathway FPGS and folB genes and GGH genes increased in folate leaf materials KY and Y. Less folate synthesis in yellow leaves is accompanied by a faster degradation process, so we speculate that folate accumulation is reduced in yellow leaves. We measured the accumulation of folate in yellow and green leaves that was very low to read, which we believe was caused by the rapid degradation of folate. Folate content can affect the efficiency of chlorophyll synthesis . Chlorophyll synthesis is inhibited and leaf color is yellowing.

Gene expression of CISZOG was significantly downregulated, CKX gene expression in the degradation pathway, and slow synthesis and accelerated degradation resulted in less accumulation of zeatin in the yellow leaves. We determined the content of zeatin in yellow and green leaves and showed that it decreased substantially in yellow leaves(Fig. S4). Studies have shown that the zeatin content in the dark green part of lettuce is higher than that in the light color, with a positive correlation between zeatin content and chlorophyll content (Zhu et al. 2021). Therefore, the accumulation of zeatin in yellow leaves is reduced, resulting in a decrease of chlorophyll content. Cytokinin increases the expression of ChlH in etiolated seedlings (Ryouichi and Ayumi 2007). We believe that the significant decrease in yellow leaves is caused by the abnormal expression of CISZOG gene and CKX genes. It also affected the expression level of ChlH subunit of magnesium ion chelatase, and the transcriptome data indicated that ChlH gene expression was significantly downregulated. It is concluded that the abnormal expression of CISZOG gene and CKX gene, which together leads to the reduction of chlorophyll content in yellow leaves, so the yellowing occurs in tobacco leaves.

Conclusion

In the present study, the synthesis of Mg-Proto IX, abnormal thylakoid structure and the chlorophyll content in leaves were significantly reduced, which was due to the significant reduction in the expression of chlorophyll synthesis genes ChlH and ChlD and chloroplast development gene PPR. Altered expression of zeatin synthesis gene CISZOG and degradation gene CKX in yellow leaves inhibited chlorophyll synthesis by reducing zeatin content and ChlH expression. FPGS gene, folB gene and metabolic pathway GGH gene expression are altered in yellow leaves to inhibit chlorophyll synthesis by reducing folate content. The above is the main reason for the yellowing of tobacco leaves. This study providing a possible explanation of both physiological and molecular aspects of the leaf yellowing mechanism, which lays the foundation for further functional research on leaf yellowing.

Fig. 1 Phenotypic of yellow leaf material and normal green plants. (a) Phenotypic of K and KY at different emergence stages, K on the left and KY on the right. (b) Plant height of yellow leaf material Y and KY and normal green plant K at different emergence stages. (c) The number of leaves of Y, KY, K at different emergence stages. (d) Middle leaf lengths of Y,KY,K at different

emergence stages. (e) Middle leaf width of Y,KY,K at different emergence stages. (*) P⩽0.05, (**) P⩽0.01.

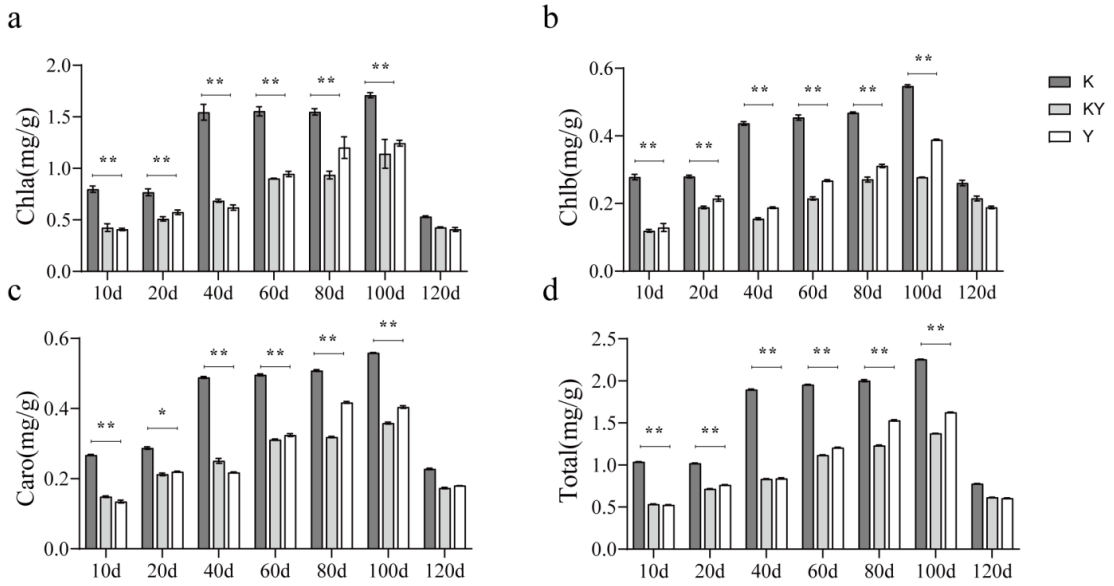

Fig. 2 Determination of leaf pigment content in yellow leaf material and normal green plants. (a) The content of chlorophyll a at different times, (b) The content of chlorophyll b at different times, (c) The content of carotenoids at different times, (d) The content of total chlorophyll at different times. (*) P⩽0.05, (**) P⩽0.01.

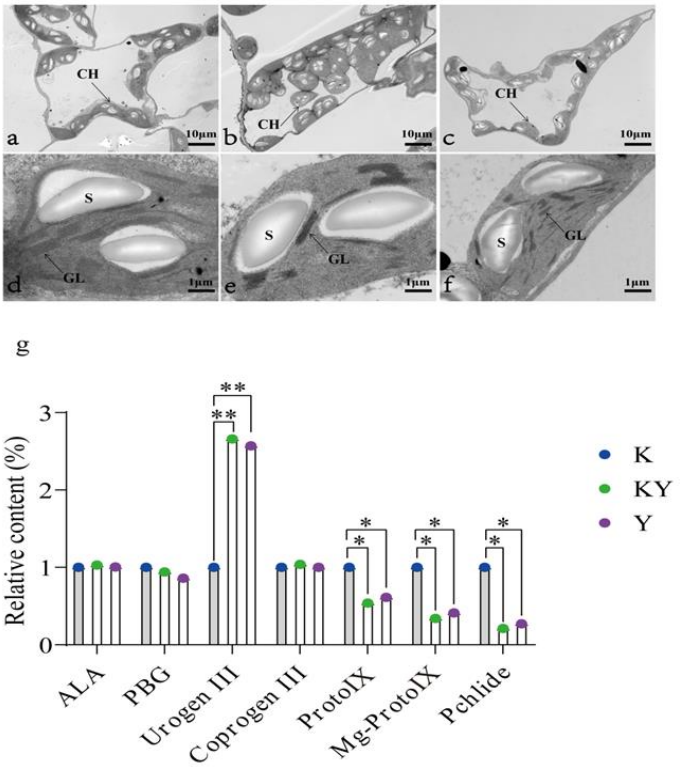

Fig. 3 Chloroplast ultrastructure on yellow and normal green leaves. (a,d) Normal green leaf K. (b,e) Yellow leaf K326 (KY). (c,f) Yellow leaf mutant Y. Ch, chloroplast; S, starch granule; GL grana lamella. (Scale bars: 10 μm in (a,b,c); 1 μm in (d,e,f)). (g) Intermediate yield of chlorophyll synthesis in yellow leaves and green leaves. (*) P⩽0.05, (**) P⩽0.01.

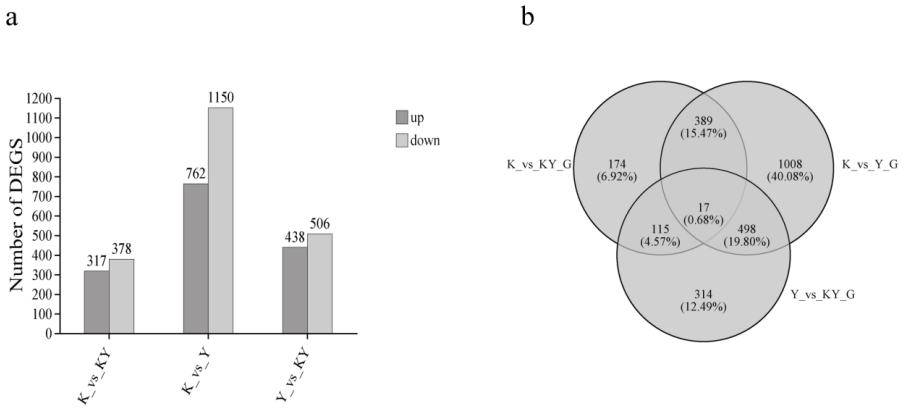

Fig. 4 Differential gene expression in different groups, the three materials were divided into three control groups. (a) The number of differential genes and the number of up-regulated genes in the

three control groups, K_vs_KY, K_vs_Y, and Y_vs_KY. (b) Venn diagram representing the number of differential genes in the three groups.

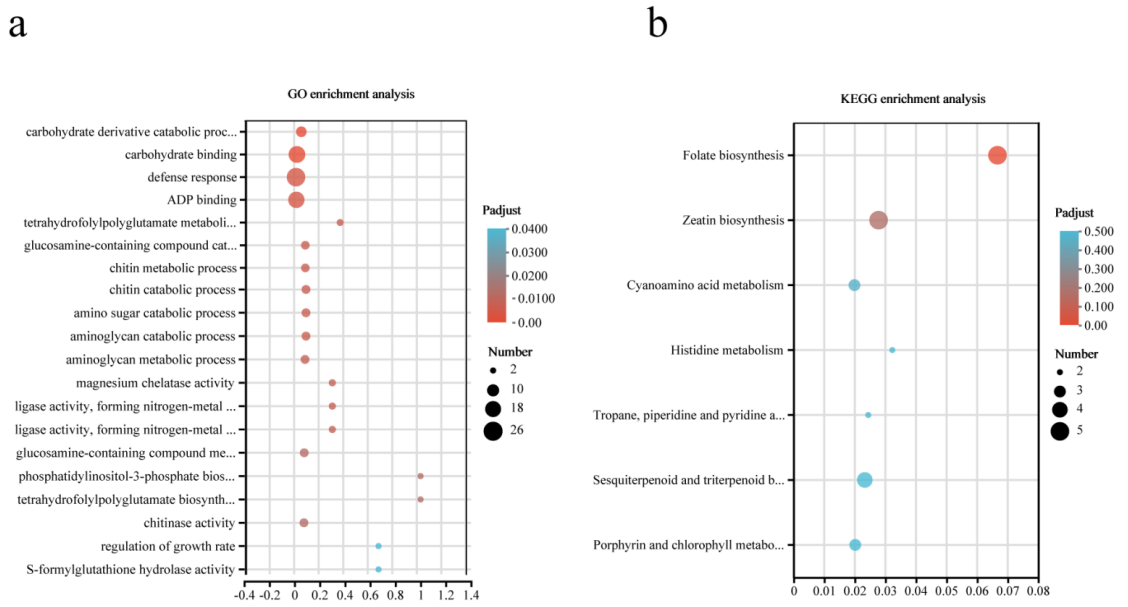

Fig. 5 (a) The GO enrichment analysis of the DEGS. (b) The KEGG enrichment analysis of the DEGS.

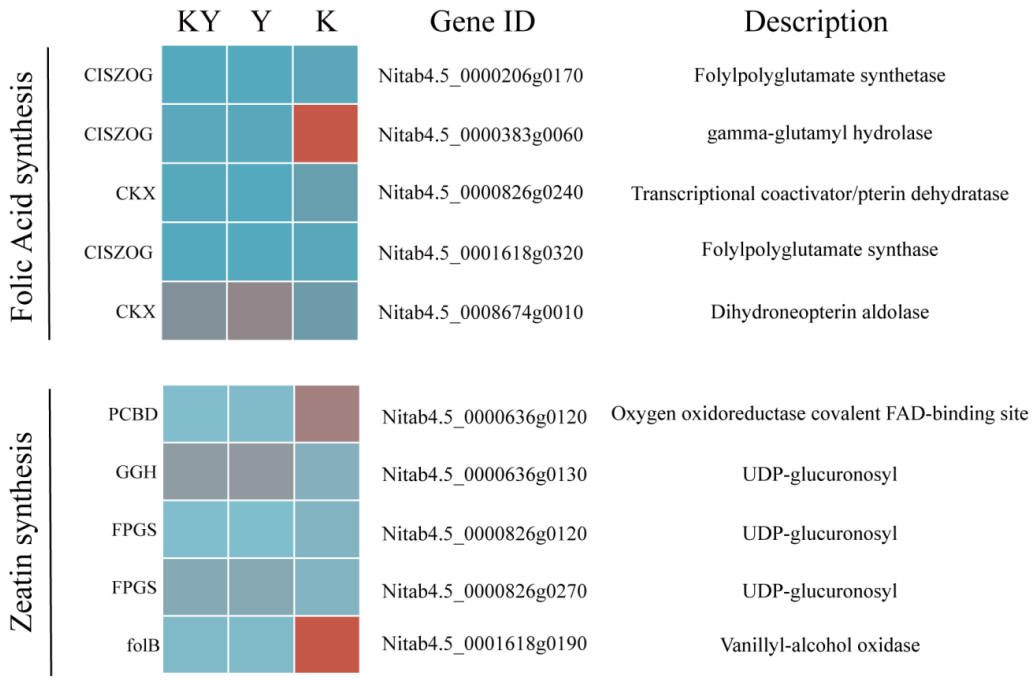

Fig. 6 Expression profiles of different expressed genes (DEG) involved in zeatin and folate synthesis between wild-type (K) and yellow leaf (KY) (Y). The expression level was calculated

from three biological replications at each stage and scaled using DESeq2. The color bar indicates an increasing expression level from blue to red.

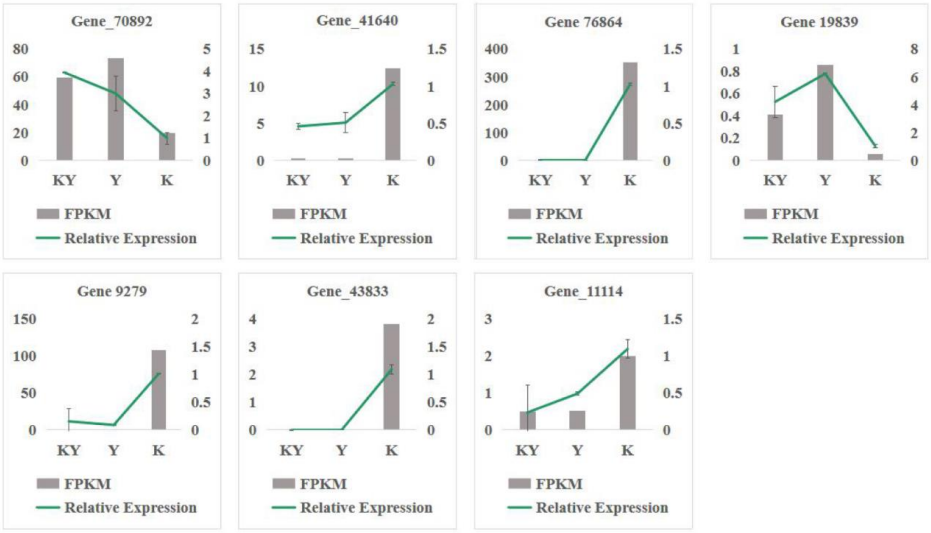

Fig. 7 Select gene expression in transcriptome data. The expression levels of 7 single genes were predicted according to the FPKM value, and the expression levels of the selected 7 genes were verified by RT-qPCR (quantitative real-time PCR).

Supplementary data

Fig. S1 Chlorophyll synthesis pathway diagram.

Fig. S2 Principal Component Analysis Plot.

Fig. S3 Inter-sample correlation analysis.

Fig. S4 Zeatin content in leaves of different colors.

Table S1 Agronomic trait data for yellow leaf material and normal green plants.

Table S2 Phtotsynthetic pigment content.

Table S3 Relative content of chlorophyll synthesis precursor.

Table S4 Transcriptome quality control data statistics.

Table S5 Differentially expressed genes in the three materials.

Table S6 Gene expression levels related to chloroplast development.

Table S7 Chlorophyll-related gene expression.

Table S8 Genes and primers analyzed by RT-qPCR.

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