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Full Length Article

Genetic, Physiological and Biochemical Analysis of the Formation of Yellow-green Leaf Color of Burley Tobacco (*Nicotiana tabacum*)

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Abstract

To investigate the formation of the yellow-green leaf color of burley tobacco, the inheritance of leaf color, the contents of plastid pigments and the characteristics of chlorophyll metabolism in burley tobacco leaves were determined with burley tobacco cv. Burley21 and normal green Maryland tobacco cv. Maryland609 as the experimental plants. Field genetic populations of the reciprocal F1 generation, inbred F2 generation and the backcrossed BC1 generation of Burley21 and Maryland609 were constructed and the statistical analysis revealed that the inheritance of the yellow-green leaf color of burley tobacco was controlled by a double recessive gene. Chlorophyll content and carotenoid content were measured using spectrophotometric method and the results found that chlorophyll contents in the leaves of Burley21 were always lower than in that of Maryland609 during the whole growth period; the content ratios of chlorophyll to carotenoid showed a decreasing trend in Burley21, while in normal green Maryland609, it maintained at about 5. Further researches on the characteristics of chlorophyll metabolism indicated that the precursors of δ- aminolevulinic acid (ALA), porphobilinogen (PBG), uroporphyrinogen III (Urogen III), coproporphyrinogen III (Coprogen III), protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (Mg-Proto IX) and protochlorophyllide (Pchlide) in chlorophyll biosynthesis in Burley21 were lower than in Maryland609 at vigorous growing period; the activity of δ-aminolevulinate dehydratase (ALAD) in Burley21 was 0.43% as compared to Maryland609, but the activity of chlorophyllase in Burley21 was 2.04 times as high as in that of Maryland609. In conclusion, the yellow-green leaf color of burley tobacco was caused by low chlorophyll content, which might due to the combination of inhibited biosynthesis and accelerated degradation of chlorophyll regulated by key enzymes of ALAD and chlorophyllase, respectively.

Keywords: Burley tobacco; Yellow-green leaf color; Inheritance; Low chlorophyll content; Synthetic precursor; Key enzyme

Introduction

Leaf color of higher plants is a comprehensive effect of chlorophyll, carotenoid and anthocyanin. Leaf color mutation would occur as the kinds, distributions or contents of these plastid pigments varied. Of all the leaf color mutants, green-deficient is the most common one (He *et al*., 2006; Pan *et al*., 2006). Chlorophyll content is one of the determining factors of plant leaf color, which kept a dynamic balance under the continuous biosynthesis and degradation in normal green plants, the leaf color of green-deficient or stay-green would occur as this balance was destroyed (Reinbothe and Reinbothe, 1996; Terry and Kendrick, 1999; Shi *et al*., 2009).

Chlorophyll biosynthesis in higher plants was carried and accomplished by sequential reactions, δ-aminolevulinic acid (ALA), porphobilinogen (PBG), uroporphyrinogen III (Urogen III), coproporphyrinogen III (Coprogen III), protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (Mg-Proto IX) and protochlorophyllide (Pchlide) were the major synthetic precursors during these sequential reactions (Ilag *et al*., 1994; Nagata *et al*., 2005; Shi *et al*., 2009). If any step of chlorophyll synthesis was blocked, it can be determined by the contents of those synthetic precursors. The blocked chlorophyll synthesis would result in low chlorophyll content and the corresponding green-deficient leaf color in higher plants (Koski and Smith, 1951; Reinbothe and Reinbothe, 1996; Rebeiz, 2014). In Su *et al*. (1990), studied the chlorophyll biosynthesis of green-deficient leaf color mutant of wheat and found that both the contents of ALA and Pchlide were lower in the leaves of mutant than in control plants (normal green wheat cv. Aibian-1). In recent years, the similar researches have been reported in green-deficient mutant in rice (Xu *et al*., 2006; Wu *et al*., 2007), wheat and barley(Falbel *et al*., 1994; Cao *et al*., 2010), tomato (Terry and Kendrick, 1999) and oilseed rape (Sun *et al*., 2007), etc.

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Plant leaf color mutation has the cytological and genetic basis, which maybe the perpetual mutant character due to gene mutation, or the temporary mutant character of the environmental impact (Whelan and Chubey, 1973; Hosticka and Hanson, 1984; Ilag *et al*., 1994). Usually, the perpetual mutant character is the results of varied bases, number or structure of chromosome of some catalytic enzymes, and these would lead to changes of the activities of the enzyme that catalyze reaction related to plastid pigment metabolism (Terry and Kendrick, 1999; Beale, 2005; Sun *et al*., 2011; Braumann *et al*., 2014). δ-aminolevulinate dehydratase (ALAD), porphobilinogen deaminase (PBGD) and magnesium chelatase (Mg-chelatase) were the key enzymes reported in the chlorophyll biosynthesis in higher plants. ALAD catalyzes the production of PBG from ALA, PBGD catalyzes the biosynthesis of hydroxymethylbilane from PBG and Mg-chelatase catalyzes the production of Mg-Proto IX (McMahan *et al*., 1990; Ilag *et al*., 1994; Cornah *et al*., 2003; Santos, 2004; Chen *et al*., 2012). On the other hand, the synthesized chlorophyll was accompanied with degradation spontaneously in plants. The chlorophyllase that catalyzes the production of chlorophyllide from chlorophyll at the first step of chlorophyll degradation is also the key enzyme that affects the chlorophyll contents in higher plants (Fernandez-Lopez *et al*., 1992; Tsuchiya *et al*., 1999; Harpaz-Saad *et al*., 2007).

Burley tobacco (*Nicotiana tabacum* L.) is the green-deficient leaf color tobacco type mutated from normal green Maryland tobacco under natural conditions, it has the typical character of yellow-green leaf color (Bill and Mann, 1960; Tong, 1997), but the cause of its leaf color formation remained unclear and the researches on the characteristics of chlorophyll metabolism in its leaves has not been reported. In view of this, the contents of chlorophyll and carotenoid, the contents of the major synthetic precursors in chlorophyll biosynthesis and the activities of key enzymes that catalyze the biosynthesis and degradation of chlorophyll in the leaves of burley tobacco were determined in this study. Also, the inheritance of the yellow-green leaf color of burley tobacco was discussed. This study was conducted to evaluate the formation of the yellow-green leaf color character of burley tobacco on genetic, physiological and biochemical basis.

Materials and Methods

Analysis of the Inheritance of the Yellow-green Leaf Color Trait in Burley Tobacco

The experiment was performed in the experimental field of Biological and Agricultural Engineering College, Weifang University, Weifang, China during 2009-2011. Burley tobacco cv. Burley21 and Maryland tobacco cv. Maryland609 were used as the parental line, their reciprocal F1 progeny (Burley21 × Maryland609 and Maryland609 × Burley21, respectively) were bagging inbred and backcrossed to get seed of F2 and BC1 hybrid, respectively. Seeds of F1, F2 and BC1 hybrid were cultivated and the seedlings were randomly selected to transplant in experimental field for 100 plants to get the genetic population of F1 progeny, 300 plants to get the genetic population of F2 progeny and 200 plants to get the genetic population of BC1 progeny, respectively. The number of tobacco plants with yellow-green or normal green leaf color were investigated and statistically analyzed.

Physiological and Biochemical Analysis of the Chlorophyll Metabolism in Burley Tobacco leaves

Plant Materials

Burley tobacco cv. Burley21 was used in the experiments with normal green Maryland tobacco cv. Maryland609 as the control. Tobacco seedlings were conventionally cultivated and transplanted in experimental field of Biological and Agricultural Engineering College, Weifang University, Weifang, China. Each tobacco variety was transplanted for three planting rows with twenty seedlings in each row. Unified managements of watering, fertilization, pest and disease controls were used during the whole growth period.

Sample Preparation

One middle leaf per plant was picked from three randomly selected tobacco plants in each planting row, washed with water to remove the dirt, cut into pieces and mixed. The mixed leaf pieces were used for determining the contents of chlorophyll and carotenoid, contents of synthetic precursors in chlorophyll biosynthesis and the activity of the key enzymes. Tobacco leaves picked from each planting row were set as a replicate and three replicates were performed for each experiment.

Analysis of the Contents of Chlorophyll and Carotenoid at Different Growth Periods

Leaf pieces (0.1 g) were immersed in 10 mL mixture of 95% ethanol and 80% acetone (the volume ratio is 1:1) in conical flask, sealed with parafilm and kept in darkness at the temperature of 30℃ for 24 h to extract the chlorophyll and carotenoid. The absorbance values (A_{663}, A_{646} and A_{470}) of the extracted solution were measured using UV-visible spectrophotometer at the wavelength of 663nm, 646nm and 470nm, the concentration (mg L^{-1}) of chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (Cx.c) of the extracted solution and the corresponding contents of each kinds of pigment (mg g^{-1}) were calculated according to the equation of Li (2000). Leaves of Burley21 and Maryland609 plants at transplanting period, rosette period, vigorous growing period, flower budding period and mature period were analyzed, respectively.

Analysis of the Contents of Major Synthetic Precursors in Chlorophyll Biosynthesis

Leaves of Burley21 and Maryland609 plants at vigorous growing period were used and leaf pieces were prepared as described in sample preparation section. Contents of ALA, PBG, Urogen III and Coprogen III in the leaves of Burley21 and Maryland609 plants were analyzed and calculated according to the methods described by Dei (1985) and Sun *et al*. (2007) with UV-visible spectrophotometer; Proto IX, Mg-Proto IX and Pchlide were analyzed and calculated according to the methods described by Rebeiz *et al*. (1975) with fluorescence spectrophotometer.

Determination of the Activities of Key Enzymes in Chlorophyll Metabolism

Leaves of Burley21 and Maryland609 plants at vigorous growing period were used to determine the activities of the key enzyme of δ- aminolevulinate dehydratase (ALAD) and chlorophyllase. Leaf pieces were prepared as described in sample preparation section.

In determining the activity of ALAD, the substrate of ALA was prepared as the method adopted by Dei (1985), extraction of enzyme solution (ALAD-containing solution), the reaction system and the activity of ALAD were performed and calculated according the methods described by Scarponi *et al*. (1985); in determining the activity of chlorophyllase, the substrate of chlorophyll a was purchased from Sigma Chemical Company, the enzyme solution (chlorophyllase-containing solution) was prepared according to the methods described by Mosequera-Minguez *et al*. (1994), the reaction system and the activity of ALAD were performed and calculated according the methods described by Fernandez-Lopez *et al*. (1992) and Fang *et al*. (1998). Activity of the enzyme was evaluated by reaction product produced per hour (μmol g^{-1} h^{-1}).

Statistical Analysis

The data of chlorophyll contents and carotenoid contents were expressed as mean ± standard deviation, fig. were made with Excel 2003. The SAS system (Version 8, SAS Institute, Inc, Cary, NC) was used to analyze the significant difference of data of plastid pigments contents, activities of key regulatory enzymes and statistical test of burley tobacco leaf color inheritance in our experiments.

Results

Inheritance of Burley Tobacco Leaf Color Trait

The segregation of tobacco plants with yellow-green or normal green leaf color in the reciprocal F1 progeny, backcrossed BC1 progeny and inbred F2 progeny were showed in Table 1. The results indicated that all tobacco plants of F1 progeny were normal green leaf color; in 299 tobacco plants of F2 progeny, 22 plants were yellow-green leaf color, 277 were normal green leaf color, the segregation ratio of normal green tobacco plants to yellow-green tobacco plants was 12.5:1; in 190 tobacco plants of BC1 progeny, 32 plants showed yellow-green leaf color, other 158 plants, the normal green, the segregation ratio of normal green tobacco plants to yellow-green tobacco plants was 4.9: 1. Statistical analysis found that the probability value (*P*) of F2 and BC1 progeny were 0.401 and 0.066, respectively, both were larger than 0.05, this estimated that the inheritance of yellow-green leaf color of burley tobacco was consistent with the genetic law of double recessive trait controlled by nuclear gene.

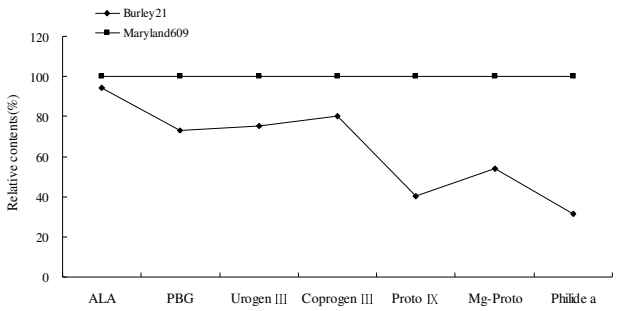

Fig. 1: Relative contents of the seven kinds of precursors in the biosynthesis of chlorophyll in the leaves of Burley21 and Maryland609 plants at vigorous growing period

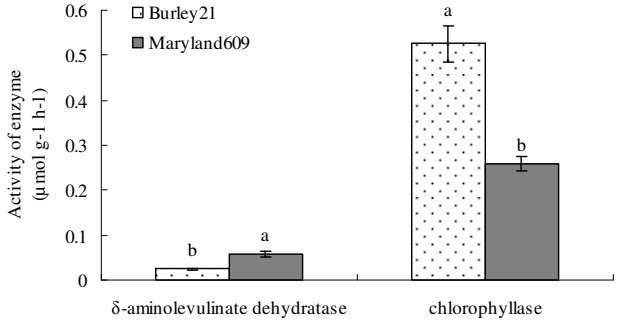

Fig. 2: Activities of the key enzymes in the leaves of Burley21 and Maryland609 plants at vigorous growing period. The value of the bars with the same small letter mean non-significantly in activity of the same key enzyme between Burley21 and Maryland609 plant leaves at the level of P=0.05

Contents of Chlorophyll and Carotenoid

Contents and content ratios of the chlorophyll and carotenoid in the leaves of Burley21 and Maryland609 plants at different growth periods were showed in Table 2. The results indicated that the contents of the same pigment (chlorophyll and carotenoid, respectively) in Burley21 were always lower than in that of Maryland609 at the same

Table 1: Segregation of leaf color trait in the hybrid progenies of Burley21 and Maryland609

Hybrid progeny	Hybrid combination	Number of different leaf color plants		Theory value	Practical value	Chi square value (χ^2)	Probability value (*P*)
		Normal green	Yellow-green				
F1	Burley21×Maryland609	48	0	1: 0	1: 0		
	Maryland609×Burley21	50	0	1: 0	1: 0		
F2	Burley21×Maryland609	277	22	15: 1	12.5: 1	0.706	0.401
BC1	(Burley×Maryland609)×Burley21	158	32	3:1	4.9:1	3.371	0.066

Table 2: Contents and content ratios of chlorophyll and carotenoid in the leaves of Burley21and Maryland609 plants at different growth periods

Growth period	Tobacco varieties	Contents of total chlorophyll (Ca+b, mg g^{-1})	Contents of carotenoid (Cx, mg g^{-1})	Ratio of chlorophyll a to b (Ca/Cb)	Ratio of total chlorophyll to carotenoid (Ca+b/Cx)
Transplanting period	Burley21	1.66±0.11 a	0.29±0.15 a	3.42	5.70
	Maryland609	1.73±0.06 a	0.32±0.01 a	3.71	5.40
Rosette period	Burley21	1.91±0.06 a	0.35±0.13 a	3.55	5.49
	Maryland609	2.07±0.05 a	0.39±0.02 a	3.53	5.26
Vigorous growing period	Burley21	1.07±0.05 B	0.26±0.01 b	3.82	4.15
	Maryland609	1.89±0.11 A	0.36±0.07 a	3.99	5.20
Flower budding period	Burley21	0.75±0.05 B	0.18±0.02 b	3.35	3.92
	Maryland609	1.06±0.05 A	0.20±0.01 a	3.27	5.41
Mature period	Burley21	0.24±0.02 B	0.08±0.07 b	3.48	3.24
	Maryland609	0.71±0.14 A	0.13±0.11 a	3.03	5.57

Mean ± standard deviation. Value with the same small and capital letter mean non-significantly of the same plastid pigment contents between Burley21 and Maryland609 plant leaves at the same growth period at the level of P=0.05 and P=0.01, respectively

growth period; from vigorous growing period, this difference became significant, but the difference magnitude of carotenoid contents were smaller (P=0.05) than chlorophyll contents (P=0.01). At mature period, total chlorophyll content in Burley21 plants was 0.24 mg g^{-1}, but it was 0.71 mg g^{-1} in Maryland609 plants, the latter was 2.95 times as high as the former.

The content ratio of chlorophyll a to chlorophyll b (Ca/Cb) at the same growth period maintained at about 3 both in Burley21 and Maryland609 from transplanting period to mature period; the content ratio of total chlorophyll to carotenoid (Ca + b/Cx) in Maryland609 also maintained at about 5, but in Burley21, it showed a downward trend (Table 2).

Contents of the Major Synthetic Precursors in Chlorophyll Biosynthesis

Contents of the seven kinds of major synthetic precursors in chlorophyll biosynthesis in the two kinds of experimental tobacco plants at vigorous growing period were determined by relative quantitative method described by Xu *et al*. (2006), Sun *et al*. (2007) and Chen *et al*. (2013). That is, the contents of the precursors measured in the leaves of normal green Maryland609 plants were set as 100% and in Burley21, it was expressed as the percentage compared to Maryland609 at the corresponding growth period. The quantitative results were showed in Fig. 1.

We can see from Fig. 1 that contents of the all seven synthetic precursors of ALA, PBG, Urogen III, Coprogen III, Proto IX, Mg-Proto IX and Pchlide in Burley21 plants were lower than in that of Maryland609 plants and the difference became obvious from PBG.

Activities of the Key Enzymes in Chlorophyll Metabolism

The activities of ALAD in the leaves of Burely21 and Maryland609 plants at vigorous growing period were 0.025 μmol g^{-1} h^{-1} and 0.056 μmol·g^{-1}·h^{-1}, respectively, it was 0.43% in Burley21 as compared to Maryland609; the activity of chlorophyllase was 0.53 μmol g^{-1} h^{-1} in Burley21, while in Maryland609, it was only 0.26 μmol g^{-1} h^{-1}; the activity of chlorophyllase in burley21 was 2.04 times as high as in Maryland609 plants at vigorous growing period. Statistical analysis indicated that the difference of the activities of the same enzyme (ALAD and chlorophyllase, respectively) between the two kinds of experimental tobacco varieties were significant (P=0.05, Fig. 2).

Discussion

In higher plants, the chlorophyll a present a blue-green color, chlorophyll b, yellow-green and carotenoid, yellow. The normal content ratio of chlorophyll a to chlorophyll b is about 3: 1 (Jeffrey, 1961; He *et al*., 2006; Pan *et al*., 2006). The normal green leaf color of higher plants can be affected as the content and content ratio of chlorophyll a to chlorophyll b or total chlorophyll to carotenoid varied (Zhao *et al*., 2001; Pan *et al*., 2006). Our research results showed that from transplanting period to mature period, the contents of the total chlorophyll in the leaves of Burley21 plants were always lower than in that of Maryland609 at the same growth period and the content ratio of total chlorophyll to carotenoid performed a decreasing trend in Burley21, but the content ratio of chlorophyll a to chlorophyll b remained at about 3 both in Burley21 and Maryland609 plants

throughout the five different growth and development periods (Table 2). These results indicated that the yellow-green leaf color of burley tobacco was not the result of imbalance in content ratio between chlorophyll a and chlorophyll b, but the abnormal lower content of total chlorophyll in its leaves.

Chlorophyll-deficient mutant has been reported in rice (Jung *et al*., 2003; Zong *et al*., 2013), wheat (Cao *et al*., 2006), maize (Lonosky *et al*., 2004), soybean (Stockinger and Walling, 1994) and *Arabidopsis thaliana* (Carol *et al*., 1999). Some research results on rice and *Arabidopsis thaliana* found that the formation of chlorophyll-deficient mutant was the block of chlorophyll biosynthesis regulated by enzymes of δ-aminolevulinate dehydratase (ALAD), porphobilinogen deaminase (PBGD) and magnesium chelatase (Mg-chelatase) (Rissler *et al*., 2002; Xu *et al*., 2006; Wu *et al*., 2007; Zong *et al*., 2013). Results of our study also showed that the contents of the seven major precursors and the activity of key enzyme of ALAD in chlorophyll biosynthesis were lower in the leaves of Burley21 plants as compared to that of normal green Maryland609 at vigorous period (Fig. 1 and Fig. 2). However, the activity of chlorophyllase, a key enzyme in the degradation of chlorophyll, was high in Burley21 as compared to Maryland609 (Fig. 2). These results indicated that the formation of the yellow-green leaf color of burley tobacco might due to the combination of inhibited chlorophyll biosynthesis and accelerated chlorophyll degradation regulated by the activities of key enzymes of ALAD and chlorophyllase from the point of view of physiology and biochemistry.

In our research, content differences of chlorophyll at the same growth period between the two kinds of experimental tobacco varieties of Burley21 and Maryland609 became significant from vigorous growing period (Table 2), so further researches of the contents of synthetic precursors and activities of key enzymes in chlorophyll metabolism were analyzed only at the vigorous growing period. δ-aminolevulinate dehydratase (ALAD) as well as other catalytic enzymes (such as porphobilinogen deaminase and magnesium chelatase) were viewed as key enzyme in chlorophyll biosynthesis (McMahan *et al*., 1990; Ilag *et al*., 1994; Cornah *et al*., 2003; Santos, 2004; Chen *et al*., 2012), but in our research, the dramatic content difference of the seven synthetic precursors in chlorophyll biosynthesis between Burley21 and Maryland609 was began from PBG (Fig. 1), so only the activity of ALAD were determine.

Some research results reported that the yellow-green leaf color of burley tobacco was controlled by a double recessive gene (yb_1 yb_2) (Henika, 1932; Chaplin, 1969), but others, however, approved that it was the combination results of recessive gene and minor gene (Wang and Si, 1986). In our research, the segregation of leaf color trait in field genetic population and the statistical analysis (Table 1) estimated that the inheritance of yellow-green leaf color trait of burley tobacco might be controlled by a double recessive gene.

Burley tobacco is the green-deficient leaf color tobacco type mutated from normal green Maryland tobacco under natural conditions (Bill and Mann, 1960; Chaplin, 1969). So in our study, all research results of burley tobacco (Burley21) were analyzed by the methods of parallel determination and relative quantitative comparison with normal green Maryland tobacco (Maryland609) as the control.

To conclude, the yellow-green leaf color of burley tobacco, which might be controlled by a double recessive gene, was caused by low chlorophyll content in its leaves. From the point of view of physiological and biochemical, the low chlorophyll content was due to the combination of inhibited biosynthesis and accelerated degradation of chlorophyll regulated by key enzymes of δ-aminolevulinate dehydratase (ALAD) and chlorophyllase, respectively. However, Green-deficient leaf color, a useful trait in plant research, has been studied extensively in *Arabidopsis thaliana* and other higher plants and the mutated genes related in chlorophyll metabolism have been reported (Tsuchiya *et al*., 1999; Rissler *et al*., 2002; Nagata *et al*., 2005; Peter and Grimm, 2009). Thus, the related genes involved in chlorophyll metabolism in burley tobacco need further study.

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