Journal of Agricultural Science and Technology B 10 (2020) 201-217
doi: 10.17265/2161-6264/2020.04.001

Interference of Quinolinate Phosphoribosyltransferase Gene *QPT* Affects Agronomic Traits and Leaf Quality in *Nicotiana tabacum* L.

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Abstract: Quinolinate phosphoribosyltransferase (QPRTase), a key enzyme in ensuring nicotinic acid is available for the synthesis of defensive pyridine alkaloids in *Nicotiana* species, also plays an important role in nicotinamide adenine dinucleotide (NAD) biosynthesis. In this study, the morphological traits, the quality characteristics and photosynthetic parameters in *QPT*-overexpressing/interfering tobacco plants were investigated, respectively. Results showed that the interference of *QPT* gene not only reduced significantly the morphological traits including plant height, stem girth, leaf number and leaf length, etc. at 20 days after transplanting (DAT), but the flowering period was delayed 10-15 d in interfered tobaccos compared with the overexpressed, control and wild-type counterparts. However, at 40 DAT and 60 DAT, only three indexes (plant height, stem girth and leaf number) in *QPT*-interfering plants appeared significant difference in comparison with other three types of tobacco lines. Meanwhile, the determination results from nicotine, sugar, K^+ and Cl^- content showed the nicotine content in interfered plants was always significantly lower than that in overexpressed plants, control and the wild-type ones respectively whatever toppling or not. At the same time, the toppling treatment also caused the increasement of K^+ content among the four different tobacco lines, but the maximum increase amplitude of K^+ content was found in *QPT*-overexpressing tobaccos while the minimum appeared in *QPT*-interfering plants. Finally, *QPT*-interference in transgenic tobaccos likewise affected the photosynthesis by reducing net photosynthetic rate (Pn), stomata conductance (Gs) and transpiration rate (Tr), while there was no significant difference between *QPT*-overexpressing plants and the controls and the wild-types.

Key words: *Nicotiana tabacum*, *QPT* gene, physiological characteristics, tobacco quality.

1. Introduction

The plant kingdom is a rich source of biologically active secondary metabolites with the largest and most diverse group being the alkaloids [1]. Alkaloids represent an extensive group of nitrogen-containing secondary metabolites [2], which have been proved that such metabolites in plants play important roles in growing development, ecological regulation, and defense of disease and insect [3, 4]. In the ~75 *Nicotiana* species, the pyridine alkaloids such as anabasine, anatabine, nicotine and its demethylated derivative nornicotine are characteristically found in leaves and/or roots [5, 6]. It is generally accepted that these pyridine alkaloids serve as defensive compounds against herbivores, both within the context of native plant habitats [7-9]. Among these alkaloids, nicotine is the most predominant alkaloid found in a majority of tobacco varieties, typically accounting for 90% to 95% of the total alkaloid pool while another alkaloid is a relatively minor alkaloid, comprising

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about 2% to 5% in the mature leaf [10]. Besides, nicotine induces antifeed toxicity and hinders protein digestion in insects [9] and is used as insecticides in agricultural applications [11]. The research indicates that nicotine synthesis requires the concerted regulation of the metabolic pathways leading to both the production of nicotinic acid and to N-methylpyrrolinium [12, 13].

In the methylpyrroline pathway, biosynthesis can be initiated either directly through the decarboxylation of ornithine by ornithine decarboxylase (ODC) to form putrescine [14], or indirectly through the arginine decarboxylase (ADC)-mediated decarboxylation of arginine to agmatine, which can be converted to putrescine via a N-carbamoyl putrescine intermediate [15, 16]. It is the methylation of putrescine to N-methylputrescine that commits this ubiquitous plant metabolite toward alkaloid production. Thus it is not surprising that putrescine methyltransferase (PMT), the enzyme catalyzing this reaction, has been shown to be a key regulatory enzyme in nicotine production [17, 18]. The pyridine-nucleotide cyclic pathway originates with L-aspartic acid, which is oxidized by L-aspartate oxidase to form α-iminosuccinic acid (Fig. 1) [19]. In the following step, glyceraldehyde 3-phosphate condenses with the α-iminosuccinate to form quinolinic acid, catalyzed by quinolinate synthetase [20]. Subsequently, quinolinic acid is converted to nicotinate mononucleotide (NaMN) by quinolinate phosphoribosyltransferase (QPT), serving as the entry point into the pyridine nucleotide cycle that leads to the production of nicotinic acid.

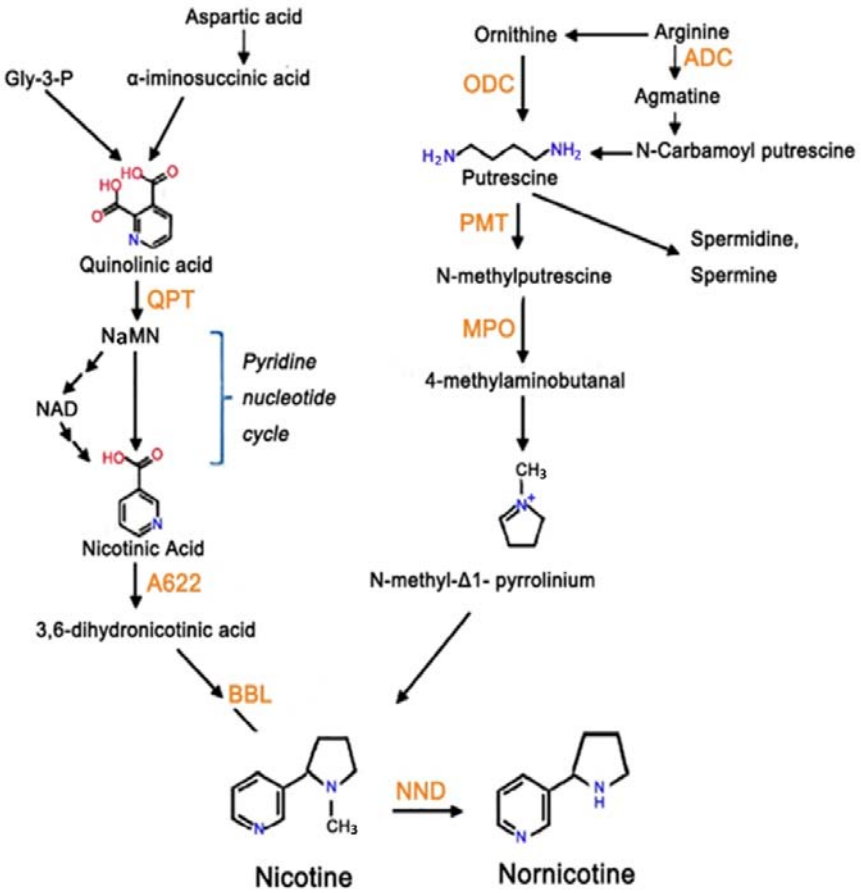

Fig. 1 Schematic diagram of alkaloid biosynthesis in *Nicotiana tabacum* [19].

A622: isoflavone reductase-like protein; ADC: arginine decarboxylase; BBL: berberine bridge enzyme-like; MPO: N-methylputrescine oxidase; NND: nicotine N-demethylase; ODC: ornithine decarboxylase; PMT: putrescine methyltransferase; QPT: quinolinate phosphoribosyltransferase.

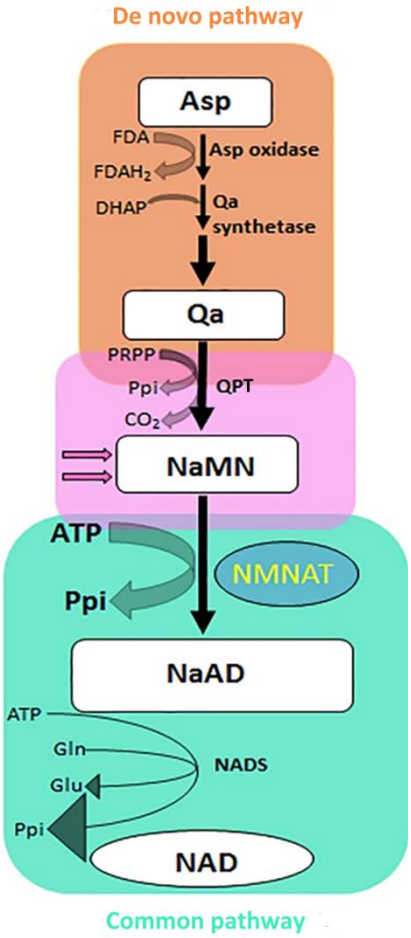

Fig. 2 Schematic diagram illustrating four possible pathways for nicotinamide adenine dinucleotide (NAD) biosynthesis [21].

Asp: aspartate; FAD: flavin adenine dinucleotide; DHAP: dihydroxyacetone phosphate; Qa: quinolinate; PRPP: 50-phosphoribosyl-10-pyrophosphate; Ppi: pyrophosphate; NaMN: nicotinate mononucleotide; NaAD: nicotinate adenine dinucleotide; Gln: glutamine; Glu: glutamate; QPT: quinolinate phosphoribosyltransferase; NMNAT: nicotinate (and/or nicotinamide) mononucleotide adenylyltransferase; NADS: NAD synthetase; NAD ppase: NAD pyrophosphatase; NADase: NAD glycohydrolase (NAD nucleosidase).

Similar to the pivotal role that PMT has shown to play in the methylpyrroline pathway, most of the early studies pointed to QPT as the rate-limiting step in the pyridine-nucleotide branch of nicotine biosynthesis [22, 23].

In addition to the pivotal role in nicotine biosynthesis, quinolinate phosphoribosyltransferase (QPT) is also a key enzyme in de novo nicotinamide adenine dinucleotide (NAD) synthesis (Fig. 2) [24]. And the latter and its derivative nicotinamide adenine dinucleotide phosphate (NADP) are traditionally known as the central metabolites orchestrating plant cellular redox homeostasis. These nucleotides also play vital roles in signaling via the generation and scavenging of reactive oxygen species (ROS) [25] and in systems controlling adaptation to environmental stresses such as ultraviolet (UV) irradiation, salinity, heat shock and drought [26-28]. Recent evidence suggests new roles for NAD and its derivatives in regulating complex cellular processes, including transcriptional regulation and microtubule metabolism via NAD-dependent deacetylation and/or mono/poly (ADP-ribos) ylation, and intracellular Ca^{2+} signalling via NAD-derived cyclic ADP-ribose [29-31]. These cellular events are accompanied by a decrease in cellular NAD level independently of redox reactions. To avoid cell death due to NAD depletion, plant cells possess a mechanism for maintaining cellular NAD levels, i.e., NAD is immediately re-synthesized when the level is decreased. Recent advances in the characterization of the *Arabidopsis* NAD biosynthetic genes have shed light on the significance of NAD biosynthesis in various developmental processes, including seed germination and pollen tube growth [32, 33].

In this review, the two tobacco *QPT*-contained plant expression vectors, namely p35S-QPT and p35S-QPTi, were constructed to investigate the biological function of *QPT* gene on morphological characteristics, photosynthetic parameters and tobacco leaf quality in tobacco plants. Special attention will be focused on influence of overexpression and interference of *QPT* gene on photosynthetic indexes and tobacco leaf quality.

2. Materials and Methods

2.1 Plant Materials, Reagents and Instruments

Plant materials are preserved and provided by the

Institute of Agricultural Bioengineering of Guizhou University. The cultured *Nicotiana tabacum* "Xanthi" plants were grown in a greenhouse. Other reagents included Plant DNA Extraction Kit (Tiangen Biotech, China), MultiScribe™ Reverse Transcriptase Kit (Applied Biosystems, USA) and Power SYBR® Green PCR Master Mix Kit (Applied Biosystems, USA); the specific upstream/downstream primers (CYP-f//CYP-r and CYPi-f/CYPi-r) for detecting transgenic plants and primers for real-time PCR were artificially synthesized by Shanghai Xuguan Biological Technology Development Co., Ltd. Polyethoxyllaurin (brij-35) was purchased from Beijing Baierdi Biological Co., Ltd.; Disodium hydrogen phosphate ($Na_2HPO_4{\cdot}12H_2O$) (analytically pure) was purchased from Xilong Chemical Co., Ltd.; Citric acid ($C_6H_8O_7$, analytically pure) was purchased from Chongqing Chuandong Chemical (Group) Co., Ltd.; P-aminobenzene sulfonic acid ($NH_2C_6H_4SO_3H$) was purchased from Tianjin Kermel Chemical Reagent Co., Ltd.; Sodium dichloroisocyanurate ($C_3Cl_2N_3NaO_3$) was purchased from Afaisha (Tianjin) Chemical Co. Ltd.; Nicotine (C_5NH_4-$C_4H_7NCH_3$) was provided by British BDH Laboratory; Flue-cured tobacco standard sample was provided by Qingzhou Tobacco Research Institute of National Tobacco Corporation; P-hydroxybenzoyl hydrazide ($HOC_6H_4CONHNH_2$) (analytically pure) was purchased from Xilong Chemical Co., Ltd.

C1000 Touch™ Thermal Cycler, PowerPac Basic™ gel and ChemiDoc™ XRS$^+$ were purchased from BIO-RAD, USA; 7500 fluorescence PCR was purchased from Applied Biosystems, USA; Thermo Scientific Varioskan Flash was purchased from American Thermo Company, USA; LI-6400 Photosynthetic Analyzer was purchased from Beijing Ligaotai Technology Co., Ltd., China.

2.2 Plasmid Constructions

All DNA manipulations were performed essentially as described by Green and Sambrook [34]. Binary plasmid pSH737 (Fig. 3a) contained a replication of wide-range host origin, one kanamycin resistance gene, neomycin phosphotransferase I (NPT I, from Tn903) for bacterial selection, and one chimeric gene bearing β-glucuronidase (GUS) reporter gene (from *Escherichia coli*) with an NPT II (from Tn5) selectable gene for plant expression and selection, respectively. The chimeric gene *GUS::NPT II* was driven by the cauliflower mosaic virus 35S (CaMV 35S) promoter. Plasmid pSH737 was provided by the Institute of Agro-Bioengineering (Guizhou University). Binary plasmids pSH737-QPT (Fig. 3b) and pSH737-QPTi (Fig. 3c) had the same origin of replication. The former contained integrated *N. tabacum QPT* gene driven by CaMV 35S promoter, while the latter possessed the interference fragment of *QPT* gene. Plasmids pSH737, pSH737-QPT and pSH737-QPTi were sequentially transformed into *Agrobacterium tumefaciens* strain LBA4404 [35]. Colonies resistant to kanamycin were selected to ensure the presence of all plasmids by enzyme digestion and PCR amplification.

The whole-length CDS sequence of *QPT* gene (*NtQPT*; Gen Bank: AB038494.1) with *Bam*H I and *Eco*R I at 5' and 3' ends respectively was artificially synthesized by Shanghai Xuguan Biological Technology Development Co., Ltd. And the *QPT*-containing interference fragment (including 240 bp conserved sequence of *NtQPT* sense strand, 224 bp intron sequence of *Solanum lycopersicum* pyruvate phosphate dikinase gene *PDK* and 240 bp conserved sequence of *NtQPT* antisense strand) with the same restriction enzyme sites at 5' and 3' ends respectively as the former was also artificially synthesized by the company. The pSH737 and the two artificially synthesized fragments were digested by *Bam*H I/*Eco*R I enzymes synchronously. The pSH737-QPT and pSH737-QPTi were severally constructed by T_4 DNA ligase-mediated ligation reaction.

2.3 Plant Co-transformation and Identification

Tobacco leaf explants were co-cultivated with genetic engineering strain LBA4404 containing two binary

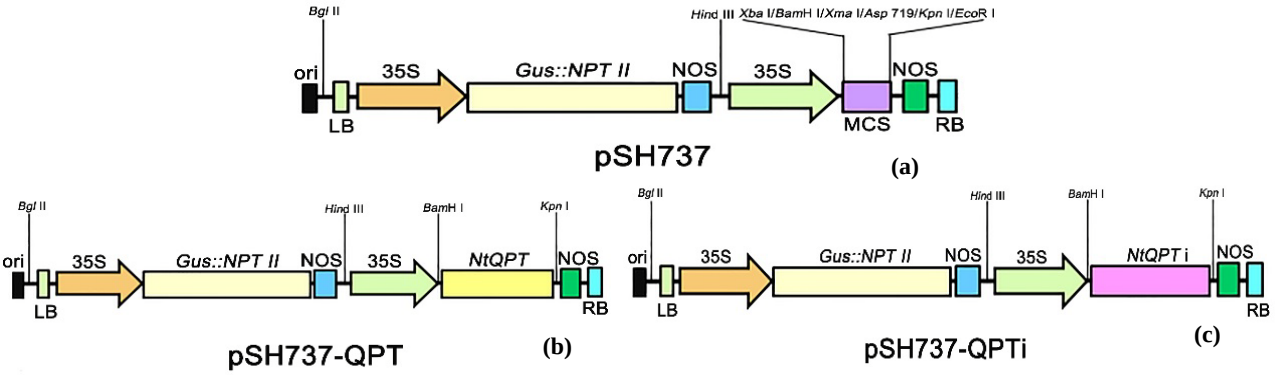

Fig. 3 Linear maps of the T-DNA regions of the plant expression vectors.

(a) The T-DNA region of the vector pSH737 which harboured 35S-*GUS::NPT II* gene expression cassette. The expression of chimeric gene *GUS::NPT II* was necessary for the selection of transformants.

(b) The T-DNA region of the vector pSH737-QPT which contained 35S-*QPT* expression cassette.

(c) The T-DNA region of the vector pSH737-QPTi which contained 35S-*QPTi* expression cassette.

35S, cauliflower mosaic virus 35S (CaMV 35S) promoter; *GUS::NPT II*, β-glucuronidase gene fused with neomycin phosphotransferase II (NPT II); NOS: nopaline synthase gene polyadenylation signal; LB: left T-DNA border; RB: right T-DNA border; Ori: origin of replication.

plasmids (pSH737-QPT and pSH737-QPTi, pSH737 as the control group) respectively (OD_{600} = 0.8), using a leaf disk transformation procedure [36]. Plants were regenerated on media (Phyto Technology Laboratories, USA) containing 100 mg/L kanamycin-sulfate (Sigma-Aldrich, St Louis, MO, USA), as described by Radke *et al.* [37]. The kanamycin-resistance plantlets were transferred to soil and grown for four weeks before identification. T_0 transformants were confirmed by the histochemical GUS assay and the assay working solution contained 0.5 mmol/L potassium ferrocyanide (Sigma-Aldrich, Shanghai, China), 0.1% (*v*/*v*) of Triton X-100 (Sigma-Aldrich, Shanghai, China), and 1 mg/mL of 5-bromo-4-chloro- 3-indolyl-D-glucuronide (X-gluc) (Sigma-Aldrich, Shanghai, China) in 50 mmol·/L of phosphate buffer with a final pH of 7.0 [38]. Genomic DNA of GUS-staining positive plants was isolated from frozen leaf tissues using the protocol of the Plant DNA Kit (Tiangen Biotech, Beijing, China). Tobacco genomic DNA was analyzed by PCR amplification with specific primers, resulting in fragments of 751 bp for QPT transformants and 500 bp for QPTi ones. Primer sequences were 5'-CGCACAATCCCACTATCCTT-3' and 5'-TTAGAGCTTTGCCGACACCT-3' for QPT fragments (84 bp of 35S promoter sequence and 667 bp of *QPT* sequence) or with QPTi-specific primers (84 bp of 35S promoter sequence and 426 bp of *QPTi* sequence). The PCR parameters included 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The 6 mL of total PCR product in each reaction was detected and electrophoresed in 1.2% agrose at 90 V. And the results were observed under the gel imaging system (Universal Hood II Gel Doc, Hercules, CA, USA).

2.4 Measure and Observation of Agronomic Characters

Morphological indexes of transgenic tobacco plants, including plant height, number of leaves, stem circumference, length and width of waist leaves, leaf area and florescence, were observed and recorded after transplantation at resettling stage, long-term stage and flower stage, respectively. The investigation method of morphological indexes of tobacco in different periods referred to the standard (YC/T 142-2010) issued by State Tobacco Monopoly Bureau of China in 2010. Straightedge, tapeline and digital vernier caliper (Deli Group, Ningbo, China)

were used to measure stem circumference, length and width of waist leaves of tobacco plant. The anthers of fully flowering tobacco plants were cut off; their pollens were removed with surgical tweezers, stained and sliced with iodine tincture, and then observed and photographed under optical microscope. The iodine tincture dye (Weber Liyang Chemical Group, China) was used for pollen activity analysis. Pollen diameter was measured with a scale in the eyepiece, and 30 pollen diameters were measured for each genotype.

2.5 Plant Sample Pretreatment

Tobacco leaves in the upper and middle parts of tobacco plants 14 d before and after topping were treated according to the tobacco industry standard of the People's Republic of China (YC/T 31-1996). After removing the dust on the tobacco leaves, they are firstly placed in an electric oven at 110 °C for 10 min, and then in an oven at 80 °C for 2 d, followed by the grinding and pulverization until the tobacco leaves were completely dried. The tobacco leaf powder is sealed for use after 40-mesh screening. Accurately weighed 0.25 g (with the accuracy of 0.0001 g) of dried tobacco powder sample in 50 mL triangular flask with rubber stopper and added 25 mL distilled water. After that, the triangular flask was placed in a shaker at 150-200 rpm for extracting tobacco components under room temperature. The tobacco powder water extract was filtered with a qualitative filter paper, first 2-3 mL was discarded and the subsequent filtrate was collected.

2.6 Determination of Total Nicotine and Total Nitrogen

The water-extracted sample is subjected to the determination of total alkaloids of tobacco and tobacco products according to the continuous flow potassium thiocyanate method in the tobacco industry standard of the People's Republic of China (YC/T 468-2013). The reaction product is colorimetric at a wavelength of 460 nm, and the total plant alkali (as measured by nicotine) is calculated by Eq. (1). Determination of total alkaloids of tobacco and tobacco products is carried out according to the continuous flow determination of total nitrogen in the tobacco industry standard of the People's Republic of China (YC/T 161-2002). And the reaction product was determined at 660 nm and the total nitrogen content was calculated using Eq. (2).

$$\alpha = \frac{c \times V}{m \times (1 - w) \times 1{,}000} \times 100 \quad (1)$$

where, c represents the instrumental observation of total alkaloids in the extract in milligrams per milliliter (mg/mL); V represents the volume of the extract in milliliters (mL); m represents the mass of the sample in grams (g); w represents the mass fraction of the sample moisture.

$$\text{Total nitrogen}\ (\%) = \frac{c}{m \times (1 - w) \times 1{,}000} \times 100 \quad (2)$$

where, c represents the instrumental observation of total nitrogen in the sample liquid in milligrams (mg); m represents the mass of the sample in milligrams (mg); w represents the moisture content of the sample.

2.7 Determination of Total Sugar and Reducing Sugar Content

The water-extracted sample is subjected to the determination of total nitrogen content in tobacco and tobacco products water soluble sugars in the tobacco industry standard of the People's Republic of China (YC/T 159-2002). The reaction product is colorimetric at a wavelength of 410 nm, and water-soluble sugar content is calculated by Eq. (3).

$$\text{Total (reduced)sugar}\ (\%) = \frac{c \times V}{m \times (1 - w)} \times 100 \quad (3)$$

where, c represents the instrumental observation of total (reduced) sugar in the sample solution in milligrams per milliliter (mg/mL); V represents the volume of the extract in milliliter (mL); m represents the mass of the sample in milligrams (mg); w represents the moisture content of the sample.

2.8 Determination of Potassium Ion and Chloride Ion Content

The water-extracted sample is subjected to the determination of potassium content of tobacco and tobacco products according to the continuous flow determination of potassium method in the tobacco industry standard of the People's Republic of China (YC/T 217-2007), and the potassium content is calculated according to Eq. (4). The pretreated sample is subjected to the determination of chlorine content of tobacco and tobacco products according to the continuous flow determination of chlorine method in the tobacco industry standard of the People's Republic of China (YC/T 162-2011). The reaction product is colorimetric at a wavelength of 460 nm, and potassium content is calculated by Eq. (5).

$$C = \frac{X \times V}{(m1 - m2) \times (1 - w) \times 1{,}000} \times 100 \quad (4)$$

where, C represents the content on a dry basis, the value is expressed in %; X represents the instrumental observation of potassium in the sample solution in milligrams per milliliter (mg/mL); V represents the volumetric volume of the sample solution in milliliters (mL); $m1$ represents the sum of the mass of the measuring bottle and the mass of the sample (g); $m2$, represents the mass of the bottle (g); w represents the moisture content of the sample.

$$\alpha = \frac{c \times V}{m \times (1 - w)} \times 100 \quad (5)$$

where, c represents the instrumental observation of extraction of chlorine in milligrams per milliliter (mg/mL); V represents the volume of the extract in milliliters (mL); m represents the mass of the sample in milligrams (mg); w represents the mass fraction of moisture in the sample in %.

2.9 Assignment and Evaluation of Tobacco Leaf Quality Coordination

After calculating coordination indexes, including the sugar-nitrogen ratio, the ratio of sugar to alkali, the ratio of nitrogen to alkali and the ratio of potassium to chlorine based on these data of above-mentioned experimental steps, the chemical composition indicators and coordination indicator of transgenic tobacco leaves are assigned according to the standard of the "Tobacco Planting Division of China". Meanwhile, the quality evaluation weights of nicotine, total nitrogen, reducing sugar, potassium, sugar-alcohol ratio, nitrogen-base ratio, potassium-chloride ratio were receptively set as 0.18, 0.10, 0.15, 0.09, 0.27, 0.12, 0.10 based on the standard (Table 1).

Table 1 Assignment reference table of tobacco chemical composition.

Score	100	100-90	90-80	80-70	70-60	< 60
N	2.20-2.80	2.20-2.00	2.00-1.80	1.80-1.70	1.70-1.60	< 1.60
		2.80-2.90	2.90-3.00	3.00-3.10	3.10-3.20	> 3.20
TN	2.00-2.50	2.00-1.90	1.90-1.80	1.80-1.70	1.70-1.60	< 1.60
		2.50-2.60	2.60-2.70	2.70-2.80	2.80-2.90	> 2.90
RS	18.00-22.00	18.00-16.00	16.00-14.00	14.00-13.00	13.00-12.00	< 12.00
		22.00-24.00	24.00-26.00	26.00-27.00	27.00-28.00	> 28.00
K	≥ 2.50	2.50-2.00	2.00-1.50	1.50-1.20	1.20-1.00	< 1.00
S/A	8.50-9.50	8.50-7.00	7.00-6.00	6.00-5.50	5.50-5.00	< 5.00
		9.50-12.00	12.00-13.00	13.00-14.00	14.00-15.00	> 15.00
N/A	0.95-1.05	0.95-0.80	0.80-0.70	0.70-0.65	0.65-0.60	< 0.60
		1.05-1.20	1.20-1.30	1.30-1.35	1.35-1.40	> 1.40
K/Cl	≥ 8.00	8.00-6.00	6.00-5.00	5.00-4.50	4.50-4.00	< 4.00

N: nicotine; TN: total nitrogen; RS: reducing sugar; K: potassium; S/A: the ratio of sugar to alkali; N/A: nitrogen/alkali; K/Cl: the ratio of potassium to chlorine.

2.10 Determination of Photosynthetic Parameters

Photosynthetic physiological parameters including the net photosynthetic rate (Pn), transpiration rate (Tr), stomata conductance (Gs), water use efficiency (WUE), and intercellular carbon dioxide (CO_2) concentration (Ci) of the inverted fourth leaves of genetically modified and wild-type tobacco were measured using the LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA) before and after drought treatment. Measurements were taken from 09:00 AM to 17:00 PM (avoiding 12:00 AM-14:00 PM) with an artificial light intensity varying from 0 to 2,000 mmol/m^2/s [39]. Each test was repeated three times.

3. Results

3.1 Identification of Transgenic Plants

The Xanthi leaf blocks were infected with *Agrobacterium tumefaciens* (contained pSH737-QPT and pSH737-QPTi, respectively), co-cultured for 2 d (Fig. 4Aa), and then transferred into a screening medium (Fig. 4Ab). After green calluses were found on the leaf margins (Fig. 4Ac), the contents were transferred into a screening and differentiation medium, which induced the growth of resistant sprouts (Fig. 4Ad). The resistant sprouts were cut into 1 cm pieces and incubated in a rooting medium for induction of rooting (Fig. 4Ae). The seedlings that grew to 3-5 cm were transplanted into nutrient-containing soil (Fig. 4Af). The growing situation of transgenic and wild-type tobacco plants at rosette stage in field was shown in Fig. 4B. The successfully transplanted resistant seedlings were selected and used for GUS histochemical staining. The transgenic plants were GUS-positive, as evidenced from the blue staining, while the wild-type plants were GUS-negative, as evidenced from the light yellow staining (Fig. 4C). The GUS-positive plants were further used for extraction of the total genomic DNA, followed by amplification of the 35S-QPT-specific fragment (778 bp) and 35S-QPTi-specific fragment (545 bp), respectively (Fig. 4D). Finally, 56 plants containing this target fragment were acquired. Then, sequencing of the PCR amplification products of the PCR-positive plants (data not shown) and southern blotting (Fig. 4E) were conducted. The results showed that the introduced elements were integrated into the tobacco genome. The transgenic plants contained one to three copies of the foreign gene.

3.2 Effect of QPT-Overexpression/Interference on Major Agronomic Traits of Tobaccos

Morphological characters such as plant height, stem circumference, leaf number, leaf length, leaf width and leaf area were determined for transgenic and wild-type tobaccos bred in the same batch at three stages after transplanting: resettling stage (20 days after transplanting (DAT)), long-term stage (40 DAT) and mature stage (60 DAT), respectively. The results showed that there was no significant difference in the morphological and morphological indexes between *QPT* tobacco, *CK* tobacco and wild-type tobacco at different growth stages. However, at 20 DAT, the plant height, stem circumference, leaf number and leaf area were 14.26 cm, 17.76 mm, 13 blades and 57.57 m^2, which were significantly lower than the wild-type plants. At 40 DAT, the plant height, stem circumference and leaf number of the interfering plants were 31.77%, 27.51% and 30.47% lower than those of the wild-type ones, respectively, and the differences were all extremely significant. At 60 DAT, the plant height, stem circumference and leaf number of the interfering plants were 71.69%, 84.50% and 78.63% of the wild-type plants, all significantly lower than that of the wild-type plants (Table 2). At the same time, the flowering time of the interfered tobacco plants was delayed, and the flowering time was 10-15 d later than the *QPT*-overexpressing, control and wild-type tobacco. It was speculated that the interference of *QPT* gene would affect the growth and development of tobacco and the delay of the flowering time.

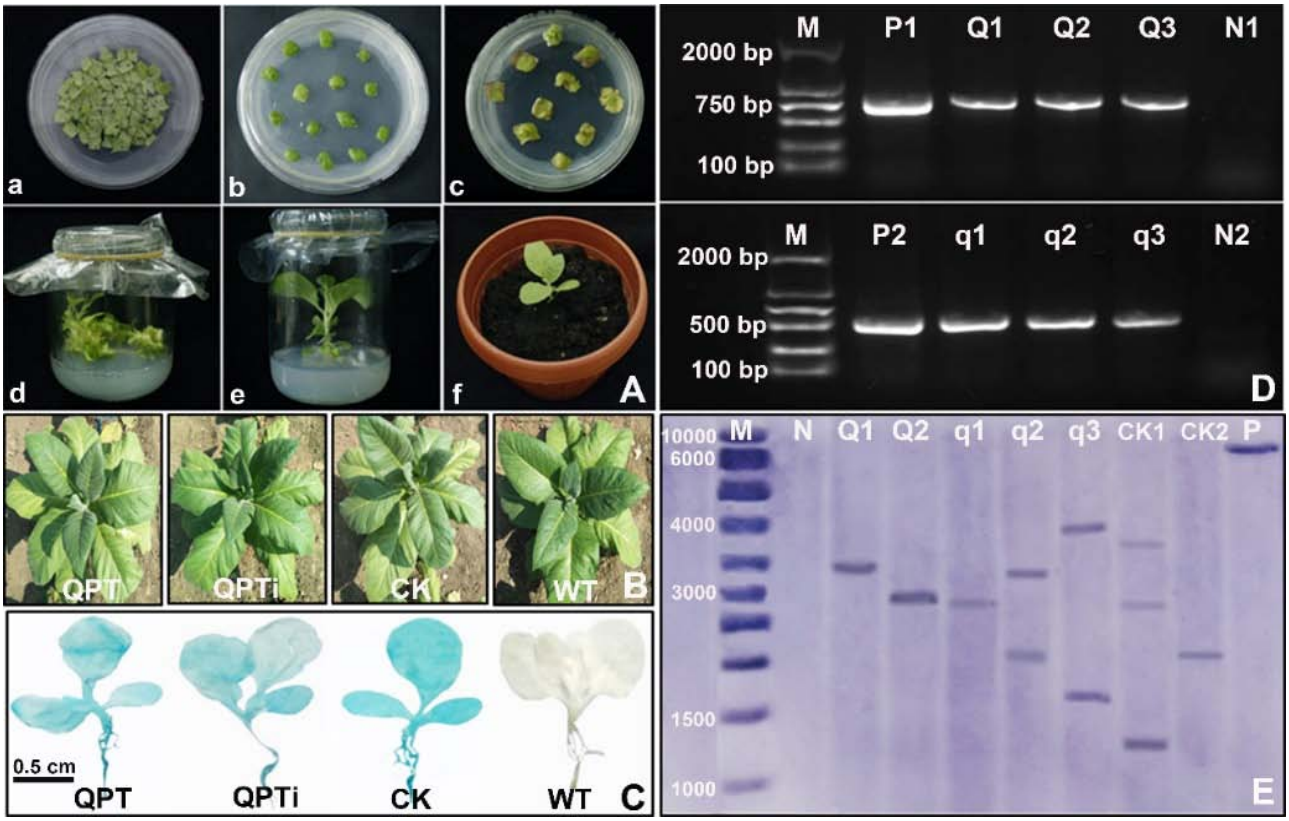

Fig. 4 Genetic transformation of tobacco and selection of transgenic tobacco plants.

(A) Genetic transformation of tobacco leaf: (a) co-culture; (b) screening culture; (c) induction culture; (d) differentiation culture; (e) rooting culture; (f) plant transplanting. (B) Comparison of growth state between transgenic tobaccos and wild-type ones. (C) histochemical staining by GUS for kanamycin-resistant tobacco plantlets. QPT, QPTi, CK and WT represent for *QPT*-overexpressing, *QPT*-interfered, empty carrier-transformed and wild-type tobaccos. (D) The PCR amplification of GUS-staining positive plants: M, DL 2,000 DNA Maker; Q1~Q3, different *QPT*-overexpressing lines; q1~q3, different *QPT*-interfered lines; N1/N2, negative control; P1/P2, positive control. (E) Southern blotting analysis for PCR-positive plants: M, DL 10,000 DNA Maker; Q1~Q2, q1~q3, CK1~CK2, different transgenic lines; N, negative control; P, positive control.

Table 2 Comparison of major agronomic traits of transgenic and wild-type tobaccos at 20, 40 and 60 d after transplantation, respectively.

Transplanting days (d)	Genotype	Plant height (cm)	Stem girth (mm)	Leaf number	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)
20	*WT*	26.08 ± 2.23	25.28 ± 3.52	16.88 ± 0.88	16.79 ± 1.37	11.83 ± 0.97	118.87 ± 10.55
	QPT	25.64 ± 1.86	27.36 ± 2.59	17.12 ± 1.21	17.02 ± 1.22	11.32 ± 0.79	124.37 ± 18.94
	QPTi	15.28 ± 2.12**	16.84 ± 1.77**	12.93 ± 1.63**	12.07 ± 0.86**	6.96 ± 0.66**	48.69 ± 9.97**
	CK	25.27 ± 1.86	26.24 ± 2.87	16.25 ± 1.44	17.12 ± 2.24	10.39 ± 0.82	115.29 ± 8.54
40	*WT*	68.21 ± 5.83	39.02 ± 2.06	26.12 ± 2.83	19.88 ± 1.05	12.93 ± 0.82	197.42 ± 20.11
	QPT	71.02 ± 6.82	41.87 ± 3.63	25.87 ± 1.40	21.11 ± 1.84	13.24 ± 0.97	217.83 ± 23.52
	QPTi	45.61 ± 4.94**	25.55 ± 3.09**	16.92 ± 0.96**	20.27 ± 1.34	12.81 ± 1.15	202.55 ± 18.90
	CK	66.68 ± 4.77	40.26 ± 1.96	27.06 ± 1.72	20.12 ± 0.96	11.37 ± 1.12	199.34 ± 19.45
60	*WT*	80.12 ± 7.24	40.12 ± 1.75	27.08 ± 2.02	22.78 ± 0.79	13.87 ± 1.28	202.84 ± 24.17
	QPT	79.83 ± 6.62	42.07 ± 3.82	25.97 ± 1.52	22.57 ± 2.20	15.22 ± 0.86	228.63 ± 21.32
	QPTi	52.85 ± 4.37**	28.70 ± 1.69**	20.42 ± 0.87**	22.27 ± 0.86	14.72 ± 0.82	211.36 ± 19.85
	CK	81.52 ± 9.26	39.47 ± 2.68	27.45 ± 3.16	21.62 ± 1.88	14.44 ± 2.17	206.38 ± 19.84

$N = 30$; ** represents $p < 0.01$.

3.3 Comparison of Major Chemical Composition in Leaves of Different Tobaccos

The transgenic (*QPT*, *QPTi* and *CK*) and wild-type tobacco (*WT*) leaves before and after toppling were sampled and treated, and their chemical components were determined by continuous flow meter. The results showed that the nicotine content of the interfered plants was 48.23% and 50% lower than that of the over-expressed plants and the wild-type ones respectively before toppling while the nicotine content was also significantly lower than the over-expressed plants (53.48%) and the wild-type ones (42.16%) after toppling (Fig. 5a). The mostly increasing amplitude of nicotine content appeared in *QPT*-overexpressing plants (63.12%), flowed by *QPT*-interfering tobaccos (46.58%), which implied the toppling could significantly affect the nicotine content of transgenic plants whatever *QPT*-overexpression or not. Additionally, the results also showed that the interference of *QPT* gene not only affected the nicotine content of tobacco plant, but also affected the sugar, nitrogen and chloride content of tobacco plant. Compared with wild-type tobacco, after toppling, the content of total sugar and reducing sugar in *QPT*-interfering tobaccos both showed a significant decrement, respectively, 32.06% and 53.39% (Fig. 5b and 5c). The effect of toppling treatment on the total nitrogen content in interfered plants was less while there was a minor change of nitrogen content before or after tobacco toppling. However, toppling treatment could cause significant reduction of nitrogen content between wild-type and *QPT*-overexpressing tobacco plants. There was no significant difference of tobacco total nitrogen content in transgenic and wild-type plants after toppling (Fig. 5d). The toppling treatment also caused the content of potassium ion in four different genotypes decreased slightly by removing the shoot apex. The potassium ion content of tobacco of the four genotypes before toppling was *QPT* > *QPTi* > *WT* > *CK*, and the sequence after topping was *QPT* > *WT* > *CK* > *QPTi* (Fig. 5e). At the same time, the content of chlorine ion in the *QPT*-overexpressed plants before topping was higher, reaching 0.96% while the content decreased obviously with the topping treatment (Fig. 5f).

3.4 Coordination Evaluation of Chemical Components in Genetically Modified Organism (GMO) and Its Wild-Type Tobacco

Considering the quality of tobacco leaves is usually the result of the combination of various chemical components, the study also comprehensively evaluates the quality of tobacco leaves based on the measured values of the above components. The results showed that although the ratio values of chemical components in *QPT*-interfering plants was significantly lower than that of the wild-type ones, the chemical composition coordination was superior to the other tobacco plants. These ratios of sugar to nitrogen, sugar to alkali, nitrogen to alkali and potassium to chloride of the *QPT*-interfering plants before the topping treatment were significantly higher than those in *QPT*-overexpressing, wild-type and control tobaccos (Figs. 6a-6d). Among them, the ratio of sugar to nitrogen and the ratio of sugar to nicotine in the *QPT*-interfering plants were 28.37% and 89.67% higher than those in the wild-type ones, respectively, and the difference reached a significant level (Figs. 6a and 6b). After topping treatment, with the increase of nicotine content in the *QPT*-interfering plants and the significant decrease of total sugar and reducing sugar content, the chemical composition coordination also showed significant changes, and the coordination difference with wild-type and over-expressed tobacco becomes smaller. However, except for the ratio of sugar to nitrogen, the other three values are still higher than wild-type tobacco.

3.5 Changes of Photosynthetic Efficiency in QPT-Overexpressing/Interfering Tobaccos

The mean values of Pn between transgenic tobacco and its wild-type plants at nine different points in

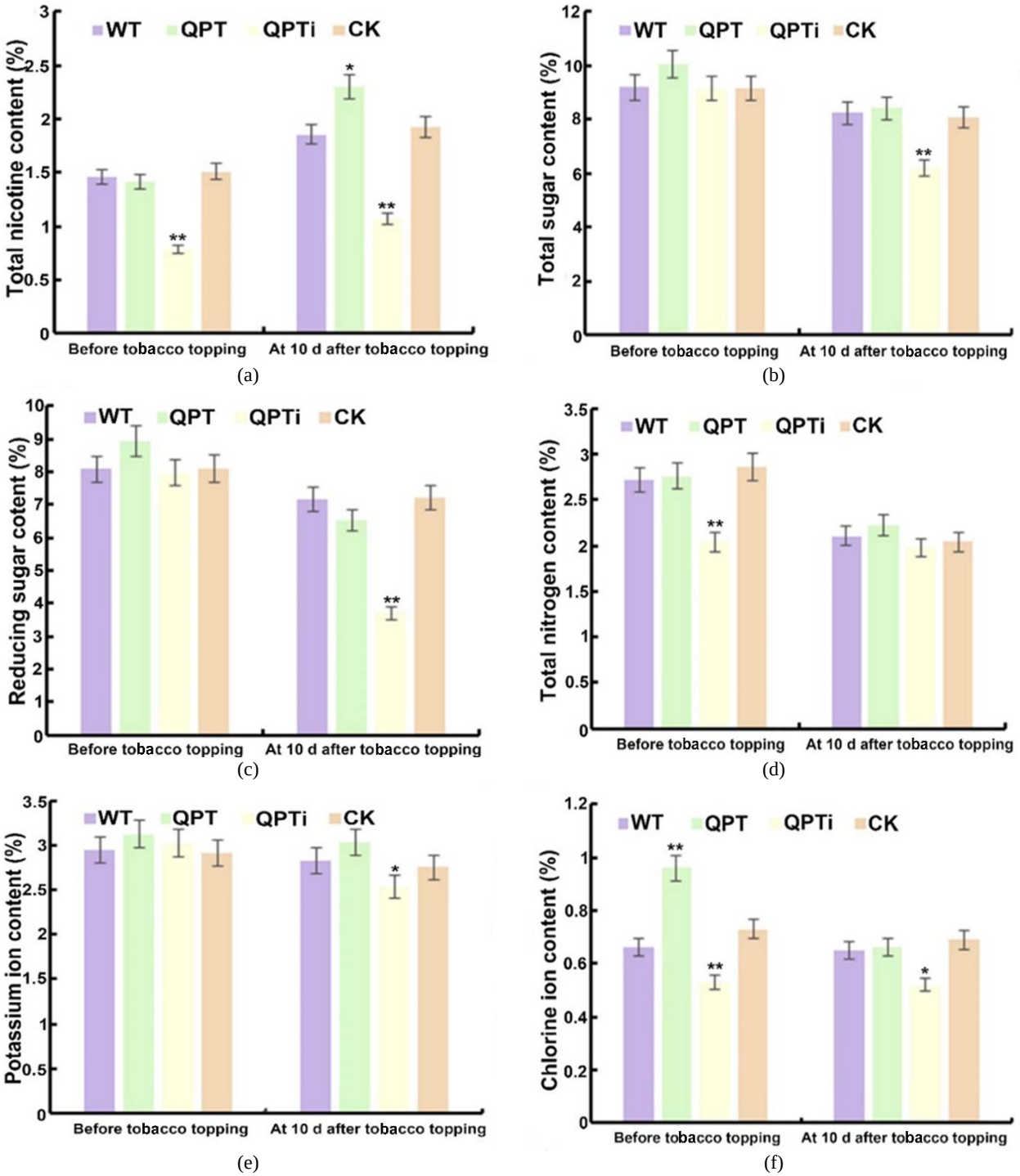

Fig. 5 Determination of major chemical composition of tobacco leaves: (a) total nicotine content; (b) total sugar content; (c) reducing sugar content; (d) total nitrogen content; (e) potassium ion content; (f) chlorine ion content.

WT: wild-type tobacco plants; QPT: *QPT*-overexpressing tobacco plants; QPTi: *QPT*-interfering tobacco plants; CK: empty vector-transformed tobacco plants. All values represent the average of the three repeats (** $p < 0.01$; * $p < 0.05$).

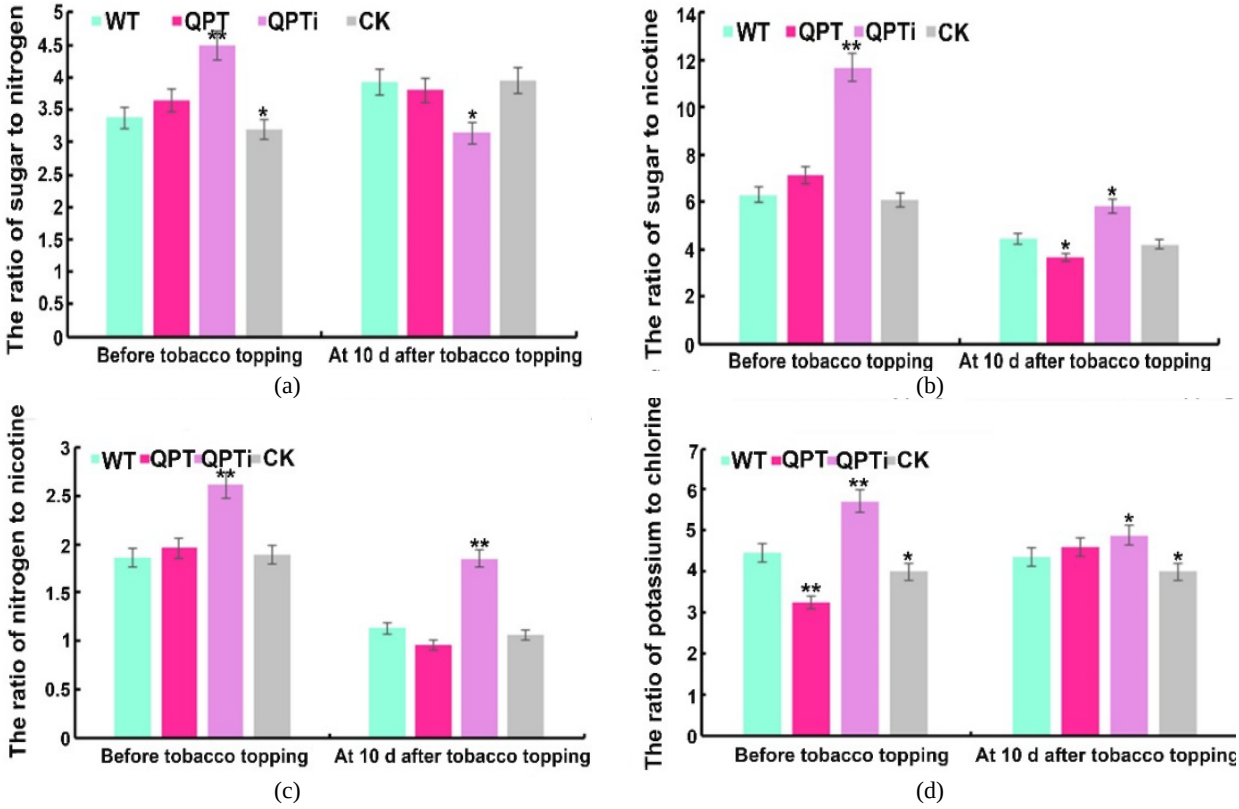

Fig. 6 Coordination of chemical composition in tobaccos before/after topping: (a) the ratio of sugar to nitrogen; (b) the ratio of sugar to nicotine; (c) the ratio of nitrogen to nicotine; (d) the ratio of potassium to chlorine.

WT: wild-type tobacco plants; QPT: *QPT*-overexpressing tobacco plants; QPTi: *QPT*-interfering tobacco plants; CK: empty vector-transformed tobacco plants. All values represent the average of the three repeats (** $p < 0.01$; * $p < 0.05$).

a day were analyzed, respectively. The results showed that there was no significant difference in Pn, Gs, Ci, Tr between the *QPT*-overexpressing tobacco and the wild-type tobacco, while the mean values of Pn and Gs in *QPT*-interfering tobacco were 10.62 μmol/m^2/s and 0.29 μmol/m^2/s, respectively, which were significantly lower than the wild-type one. The diurnal variation of Pn in both overexpression plants and wild-type plants showed a bimodal curve, but its peak size and occurrence time were different. The two maximum peaks of Pn in the former appeared at 12:00 PM (19.92 μmol/m^2/s) and 14:00 PM (17.45 μmol/m^2/s), respectively, while the other maximum peaks in the latter were 18.59 μmol/m^2/s at 12:00 PM and 17.21 μmol/m^2/s at 15:00 PM, which implied the difference in times of photosynthetic lunch break between the two types of tobacco plants (Fig. 7a). However, the daily change of Pn in interfering plants showed a single peak curve, and its Pn showed a decreasing trend after reaching a peak value of 12.15 μmol/m^2/s at 13:00 PM. The Gs in both *QPT*-overexpressed and wild-type tobaccos showed different degrees of decline from 12:00 PM, which may be related to leaf water loss and stomata closure caused by strong light and high temperature at noon, but the decreasing amplitude of Gs in overexpressed plants at noon was lower than that of wild-type tobacco (Fig. 7b), whereas, the Gs change of *QPT*-interfered tobacco also showed a single peak change similar to Pn, and its peak appeared at about 13:00 PM and 14:00 PM, and then the overall trend of decline. Meanwhile, in the diurnal variation curve of Tr, the variation trend of the three different genotypes of tobacco was relatively consistent, showing an upward trend from 8:00 AM, and gradually decreasing after reaching the peak at 14:00 PM. There was no significant difference in Ci

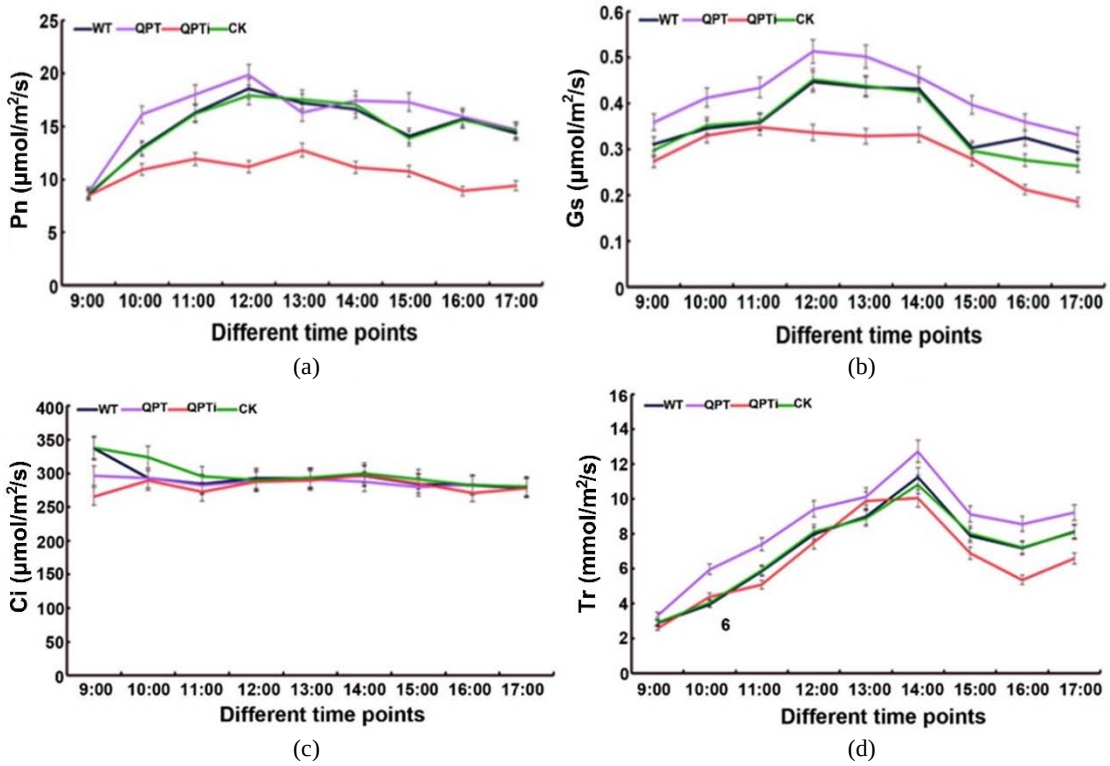

Fig. 7 Diurnal variations of net photosynthetic rate (Pn), stomata conductance (Gs), intercellular carbon dioxide (CO_2) concentration (Ci), and transpiration rate (Tr) in transgenic and wild-type tobaccos: (a) Change of Pn; (b) change of Gs; (c) change of Ci; (d) change of Tr.

WT: wild-type tobacco plants; QPT: *QPT*-overexpressing tobacco plants; QPTi: *QPT*-interfering tobacco plants; CK: empty vector-transformed tobacco plants.

value among tobacco plants after 12:00 PM (Fig. 7c). The maximum Tr value of the three genotypes was ranked as QPT > WT > QPTi (Fig. 7d).

4. Discussion

Recent studies have shown that *QPT* is not only involved in the biosynthesis and regulation of nicotine, but also a key enzyme in the de novo synthesis of NAD [40]. NAD, as the electron receptor of energy metabolism and respiration metabolism in organisms, is involved in a variety of redox reactions in cells [41]. Therefore, QPT plays an important role in both primary and secondary metabolism of plants [42]. Meanwhile, multiple important derivatives produced by QPT through pyridine nucleotide cycle, such as NADP, reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), NAD and reduced form of nicotinamide-adenine dinucleotid (NADH), are critical for the normal growth and development of plants [43] Although there was little straightforward evidence demonstrating the interrelation between QPT and plant phenotype, some studies indicated that the decrease of pyridine nucleotide derivatives in tobacco plants and *Arabidopsis* could result in abnormal phenotypes such as dwarf plants and internode retardation. Hashida *et al.* [21] reported another vitally pyridine nucleotide-synthesized gene *NMNAT* (At5g55810) in *Arabidopsis thaliana* which mediated to catalyze the synthesis of nicotinate adenine dinucleotide (NaAD) from NaMN in the Preiss-Handler-dependent pathway, and of NAD from nicotiamide mononucleotide (NMN) in the Preiss-Handler-independent pathway. Moreover, the research results showed that *AtNMNAT* expression was not only spatio-temporally regulated during microspore development and pollen tube growth, but

its expression was obviously detected in the male gametophyte. Further, an *AtNMNAT* gene mutant (*atnmnat*) was characterized by a decrease in NAD content in pollen and cytological examinations which revealed that the *atnmnat* mutant was gametophytically impaired in *in vivo* and *in vitro* pollen tube growth. In this study, the agronomic traits, including plant height, stem circumference, leaf number and leaf area were investigated in *QPT*-overexpressing and *QPT*-interfering tobacco plants. The results showed that the silence of *QPT* gene significantly affected the plant height, stem circumference and leaf number. At 20 DAT, the morphological indexes of plant height, stem circumference, leaf number and leaf area in *QPT*-interfering tobaccos were significantly ($p < 0.05$) lower than these in *QPT*-overexpressing, wild-type and control plants, while all of these were extremely significantly ($p < 0.01$) lower than the last three types of tobacco at 40 DAT or 60 DAT, which implied interference of *QPT* gene could significantly affect the morphological development of transgenic tobacco plants. Moreover, flowering time was also delayed 10-15 d in the interfering tobaccos compared with control, overexpressing and wild-type ones. It was speculated that the interference of *QPT* gene would affect the growth and development of tobacco and the delay of the flowering time and the relationship between the morphological abnormality and the content of pyridine nucleotide will be further studied. Except for its ubiquitous role in enabling NAD(P)(H) synthesis, the QPT as one of vital enzymes plays a key role in facilitating the availability of precursors for alkaloid synthesis [43]. Tobacco (*N. tabacum* L.) is one of pyridine alkaloids-containing economic crops. These pyridine alkaloids include anabasine, anatabine, nicotine and its de-methylated derivative nornicotine and they are characteristically found in leaves and/or roots of the ~75 *Nicotiana* species that are naturally distributed in North and South America, mainland Australia, a few Pacific Islands and Southern Africa [5, 6, 44, 45]. Among these pyridine alkaloids, the nicotine is the most predominant alkaloid found in cultivated tobacco varieties, typically accounting for 90%-95% of the total alkaloid pool [10].

Many studies have confirmed quinolinate phosphoribosyl-transferase (EC 2.4.2.19) (QPT) functions as the anapleurotic enzyme of the pyridine nucleotide cycle and thus a key component of primary metabolism [46, 47] and also secondary metabolism in pyridine alkaloid producing species [48, 23]. In this study, the major chemical composition in different types of tobaccos was also investigated, including the content of nicotine, sugar, reducing sugar, K^+ and Cl^-. The results showed that the nicotine content in interfered plants was 48.23% and 50% lower than that of the overexpressed and the wild-type ones, respectively, before toppling while the nicotine content was also significantly lower than the overexpressed (53.48%) and the wild-type tobaccos (42.16%) after toppling, which indicated the toppling treatment could significantly increase the nicotine-accumulation in *QPT*-overexpressing plants but not in *QPT*-interfering ones. Additionally, the interference of *QPT* gene could also affect the content of sugar, nitrogen and chloride in different degree. The content of total sugar and reducing sugar in *QPT*-interfering tobaccos both showed a significant decrement, respectively 32.06% and 53.39% after toppling compared with wild-type tobaccos. In addition, the toppling treatment also caused the content of K^+ in four different genotypes decreased slightly by toppling treatment. The highest K^+ content was found in *QPT*-overexpressing tobacco plants, followed by *QPT*-interfering ones before toppling, which implied the interference of *QPT* gene had little effect on potassium accumulation. In addition, photosynthesis is one of the important indicators of plant growth and metabolism. Hence, the photosynthetic capacity was also investigated in this study simultaneously.

The results showed that photosynthesis in the interfered plants was significantly suppressed compared with the other three types of tobacco lines.

Khan *et al*. [49] also reported the *QPT*-RNAi plants reduced content of nicotine and anabasine. Also, silencing of *QPT* led to a decrease in chlorophyll content, maximum quantum efficiency of PSII, net CO_2 assimilation and starch content. The results clearly demonstrated that *QPT* was not only involved in the biosynthetic pathway of the alkaloids but also affected plant growth and development, and these results provided information to be considered when trying to engineer the secondary metabolite quality and quantity.

5. Conclusions

This study is a preliminary evaluation of the differences of agronomic traits and tobacco leaf quality in *QPT*-overexpressing and *QPT*-interfering tobacco plants. The interference of *QPT* gene not only reduced significantly the morphological traits including plant height, stem girth, leaf number and leaf length, etc., at 20 DAT, but the flowering period was delayed 10-15 d in interfered tobaccos compared with the overexpressed ones. Synchronously, determination of tobacco leaf quality showed the content of nicotine, sugar, K^+ and Cl^- in interfered plants was always significantly lower than that in overexpressed plants, control and the wild-type ones, respectively, whatever toppling or not. Further, *QPT*-interference in transgenic tobaccos likewise influence the photosynthesis by reducing Pn, Gs and Tr.

Acknowledgments

This study was supported by the Major Special Projects for the Cultivation of New National Genetically Modified Varieties (Contract No. 2016ZX08010013-002), the Guizhou Science and Technology Plan Project (Contract No. [2016] 7449) and the Talent Training Program of Guizhou University (Contract No. [2018] 5781).

Funding

This study was funded by Guizhou Science and Technology Plan Project (2016-7449) and Talent Training Program of Guizhou University (2018-5781).

Conflict of Interest

All the authors declare that there is no conflict of interest.

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