Ectopic expression of a *Citrus kinokuni* β -carotene hydroxylase gene (*chyb*) promotes UV and oxidative stress resistance by metabolic engineering of zeaxanthin in tobacco

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Abstract

To investigate the biological function of zeaxanthin under UV light and oxidative stress we have increased its biosynthesis capacity in tobacco plants (*Nicotiana tabacum* cv. SR-1) by transformation with *Citrus kinokuni* β -carotene hydroxylase gene (*chyb*) under constitutive promoter control. The *chyb* transformants synthesized zeaxanthin about 30% more than the controls under UV irradiation. For revelation of direct effects, pigment composition, chlorophyll fluorescence, and photosynthesis were detected immediately after UV treatment. Pre-illuminated *chyb* transgenics showed higher photosynthesis (NPQ capacity and F_v/F_m ratio of *chyb* transformants about 50% more than the controls). In addition, the transgenic plants showed less lipid peroxidation (MDA level was reduced about 40%) and the SOD activity was about 1.5 times of the control plants. Furthermore, the methylviologen treatment assay (more than 60% of chlorophyll in the *chyb* transformants) suggested that the transgenic plants suffered less oxidative damage than the controls. Our results indicate that enhancing zeaxanthin amount in plant cell contributes to UV and oxidative stress protection.

Keywords Carotenoids · β -Carotene hydroxylase · Zeaxanthin · Tobacco · *Citrus kinokuni*

Abbreviations

<i>Chyb</i>	β -carotene hydroxylase gene
SOD	Superoxide dismutase
ROS	Reactive oxygen species
MS medium	Murashige–Skoog medium
PCR	Polymerase chain reaction
MV	Methylviologen
TAE	Tris-acetate-EDTA
qPCR	Quantitative real-time PCR
HPLC	High-performance liquid chromatography
MDA	Malondialdehyde
ANOVA	Analysis of variance.

Introduction

In nature, many unfavorable conditions, such as salinity, drought, and strong sunlight often influence plant growth and reproduction, even cause death (Chen and Murata 2002). These conditions, known as abiotic stresses, often produce reactive oxygen species (ROS) in plant cells and tissues (Niyogi 1999). During the long period of evolution, sessile plants have evolved a range of mechanisms to adapt to these abiotic stresses. Carotenoids, especially the xanthophyll cycle (the reversible conversion between two carotenoids, violaxanthin, and zeaxanthin), play key roles in protecting plant from ultraviolet and oxidative damage (Davison et al. 2002; Du et al. 2010; Nan et al. 2016).

Plant carotenoids are lipophilic pigments and widely found in nature with antioxidative and photoprotection properties (Abushita et al. 2000). Photoprotection function of carotenoids includes deactivation of ^3Chl . ^3Chl can lead to the formation of ROS which can be deactivated by carotenoids. And, carotenoids have been identified to be capable of the de-excitation of $^1\text{O}_2$ (Gotz et al. 2002; Niyogi 1999). In sessile plant, the xanthophyll cycle plays a key role in photoprotection (Jahns and Holzwarth 2012). Previous study had demonstrated that carotenoids can prevent lipid from

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peroxidation (Sarry et al. 1994). Some studies suggested that the structure and localization of carotenoids in plant cells determined the protective function of carotenoids (Britton 1995; Woodall et al. 1997a, b). When plant is encountered with abiotic stresses, zeaxanthin, a kind of important carotenoid taking part in the xanthophyll cycle, plays a key role in resistance to the stresses (Gotz et al. 2002). In plant, zeaxanthin participates in the process of non-photochemical quenching (NPQ) (Horton et al. 2005) and is a good kind of peroxy radicals inactivator (Woodall et al. 1997a, b).

In the natural environment, plants are encountered with combinations of various stresses. Among them are exposure to UV radiation and the oxidative damage produced by many stresses which can do harm to the plants in case the produced damage exceeds the tolerance of defence and repair mechanisms. UV radiation causes the formation of ROS in plant cells that are harmful to all cellular compartments (Jansen et al. 1998). Many studies demonstrate that UV stress can form hydroxyl and peroxy radicals, and then make cell membrane lipids peroxidized (Jansen et al. 1998). UV photoprotection of carotenoids *in vivo* has been reported for plants, fungi, and bacteria (Bonente et al. 2008; Havaux and Niyogi 1999; Steiger et al. 1999). Overexpression of the β -carotene hydroxylase (an enzyme catalyzing β -carotene to form zeaxanthin)-gene in *Arabidopsis thaliana* enhances its high-light stress tolerance by increasing the size of the xanthophyll cycle pool (Davison et al. 2002). These study results were related to the overexpression of the β -carotene hydroxylase gene which seemed to be the most important protective gene in the carotenoids biosynthesis pathway. However, the definite mechanism of the UV photoprotection by the xanthophyll cycle remains to be elucidated.

In contrast to fungi and bacteria, the dynamic protection mechanism of the xanthophyll cycle is specific to higher plants (Demmig et al. 1987). In the xanthophyll cycle, zeaxanthin is catalyzed by zeaxanthin epoxidase to produce violaxanthin, and the reverse reaction is catalyzed by violaxanthin deepoxidase to form the xanthophyll cycle (Johnson et al. 2008). The xanthophyll cycle enables the plant to resist to high-intensity white light by balancing energy quenching and transfer (Tan et al. 2007; Young 1991). Many studies have focused on the response of xanthophyll cycle to high-light stress (Niyogi 1999), but few research achievements of β -carotene hydroxylase against UV radiation are available.

In this present study, a transgenic approach was used to increase xanthophyll biosynthesis to control zeaxanthin formation in response to UV and oxidative stress and investigate protective function of zeaxanthin in higher plants. Here, the *Citrus kinokuni* β -carotene hydroxylase gene (*chyb*) (Genebank accession number: AM408552.1) was overexpressed in tobacco. Our results demonstrate that *Citrus kinokuni chyb* gene can increase the tolerance of UV and oxidative stress in higher plants. Furthermore, our results

provide supplemental evidence for the protective mechanism of UV and oxidative of carotenoids in higher plants, as well as a new idea for molecular breeding more promising crops.

Materials and methods

Plant material and tobacco transformation

The wild-type (WT) tobacco (*Nicotiana tabacum* cv. SR-1) was grown on solid Murashige–Skoog (MS) medium under controlled conditions (16 h light/8 h dark, 25 °C, 60% humidity). The sterile tobacco leaf-disc explants were used for genetic transformation according to previous study (Horsch et al. 1985). The successful transformants were confirmed by genomic PCR assay with primers specific for the *chyb* gene from *Citrus kinokuni*. The independent transgenic lines were grown in the greenhouse. After self-fertilization, seeds were germinated on MS medium containing 100 mg L⁻¹ kanamycin to generate homozygous plants overexpressing the *chyb* gene. The transformed tobacco plants with the empty plasmid (pCambia2300) were used as controls.

Plasmid and *Agrobacterium* strain

The *Agrobacterium tumefaciens* strain C58 harboring the plasmid pCambia2300-*Lbchyb* was employed in genetic transformation of tobacco. This plasmid containing the full-length cDNA sequence of a β -carotene hydroxylase gene (*chyb*) isolated from *Citrus kinokuni* was fused with 35S promoter of cauliflower mosaic virus and inserted into pCambia2300 binary vector with the neomycin phosphotransferase II (*nptII*) as selectable marker gene in plants (Fig. 1a).

Genomic DNA extraction, PCR, and Southern blotting analysis

Genomic DNA of tobacco was extracted using the DNeasy Plant Mini Kit (Qiagen, Germany). The specific primer sequences used for amplification of *chyb* gene were 5'-ATG GCTGCCGGAATTTTCAGGC-3' and 5'-TCACCTCTG TGGCTCTG-3', generating a 1000-bp product. The PCR products were electrophoresed on 0.75% TAE (Tris-acetate-EDTA)-agarose gels stained with ethidium bromide and visualized under UV light. For Southern blotting analysis, 80 μ g genomic DNA was digested with *Bam*HI at 37 °C for 16 h. Southern blotting was performed according to previous study (Chong 2001). The *chyb* probe was obtained by PCR amplification using the DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Switzerland) according to the manufacturer's instructions.

Fig. 1 Molecular analysis of *chyb* expression in the transgenic tobacco plants. **a** Construction of the plasmid pCambia2300-*Lbchyb* for expression of *Citrus kinokuni chyb* under the control of 35S promoter. 35S, 35S promoter of cauliflower mosaic virus, T35S terminator of cauliflower mosaic virus, Tnos NOS terminator, *nptII* kanamycin resistance gene, LB left border of T-DNA, RB right border of T-DNA. **b** Confirmation of *chyb* transgenic plants by genomic PCR. Lanes: M, DNA marker; 1, WT tobacco; 2, empty vector tobacco; 3, positive control; 4–9 and 10, kanamycin-resistant independent tobacco CL1–CL6 and CL8. **c** Southern blotting analysis of

transgenic tobacco plants. Three micrograms genomic DNA were digested with *Bam*HI, separated 0.75% agarose gel, transferred to a nylon membrane and probed with DIG-labeled *chyb* DNA fragment. Lanes: M, DNA marker; 1, positive control; 2 and 3, CL5 and CL8 T₂ transgenic lines; 4, WT tobacco. **d** Expression analysis of transgenic tobacco plants. Tobacco *Ubi* gene was used as the internal control gene. The X-fold changes of *chyb* gene transcripts relative to the control samples were used to calculate relative expression levels. Values are mean values ± SD ($n=3$)

Expression analyses by quantitative real-time PCR (qPCR)

Total RNA was isolated from 100 mg of frozen tobacco leaves using the RNeasy® Plant Mini Kit [QIAGEN China (Shanghai) Co., Ltd, China]. Total RNA was then quantified using SMA1000 UV Spectrophotometer (Merinton Technology Co., Beijing, People's Republic of China). Two µg of total RNA was used as a template for cDNA synthesis with PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Biotechnology (Dalian) Co., Ltd, China). Tobacco *Ubi* gene (GenBank accession no. X77456) (Zhao et al. 2014) was amplified along with *chyb* gene as an internal control to normalize the relative transcript levels. The specific primers of *chyb* (forward, 5'-CTCATCCCTGGACTCTGT-3'; reverse, 5'-GCTACCCTCCGAAAATAA-3') and tobacco *Ubi* (forward, 5'-GGCGAAAATCCAGGATAAAG-3'; reverse, 5'-CAACCTCTGCTGATGTTTGTGAC-3') were used to prepare probes, respectively. qPCR was performed on an optical 96-well plate in an Mx3000P QPCR Systems (StrataGene Mx3000, Agilent, USA) using SuperReal PreMix Plus

(SYBR Green) (TIANGEN, China) following the manufacturer's protocol. The qPCR amplification reactions were performed under the following thermal cycling conditions: 95 °C for 3 min, 40 cycles at 95 °C for 20 s, 60 °C for 15 s and 72 °C for 20 s. The comparative Ct method was employed to analyze the data of qPCR (Liu et al. 2017a, b).

Stress treatments

For UV radiation, the 8-week old tobacco plants were treated with UV-B (15 W m⁻²) for 2 h as described by Zhao et al. (Zhao et al. 2014). For oxidative stress, the 8-week old tobacco leaves were sprayed with 30 µmol L⁻¹ methylviologen (MV), and the leaves were stained with diaminobenzidine for detecting H₂O₂ level after two days stress as described by Du et al. (Du et al. 2010).

Pigment extraction and composition analysis

The protocol of total pigments extraction and composition analysis was performed as described previously (Zhao et al.

2014). The quantification of carotenoids was determined according to Zhao et al. (2014). For pigment composition, the pigments were separated on a reverse-phase nucleosil 100-3 C18, 250×4.6 mm (MN, Germany) column with an acetonitrile/methanol/2-propanol (85:10:5, v/v/v)-based isocratic mobile phase. Zeaxanthin and lutein were separated on a C30 YMC column as described by Wu et al. (2012).

Measurement of net photosynthesis rate and chlorophyll fluorescence

Photosynthesis rate and chlorophyll fluorescence were measured using the LI-6400 Portable Photosynthesis System (LI-COR Biosciences, USA) as in the previous study (Zhao et al. 2014).

Determination of superoxide dismutase (SOD) activity, malondialdehyde (MDA), and chlorophyll content

The activity of SOD (superoxide dismutase, EC1.15.1.1) was measured using Superoxide Dismutase (SOD) Detection Kit (Nanjing Jiancheng Bioengineering Institute, China). The contents of MDA for the estimation of lipid peroxidation were measured using thiobarbituric acid-malondialdehyde assay (TBA-MDA) as described (Hodges et al. 1999). The total chlorophyll content was extracted using acetone (80%) after salt stress treatment and calculated according to Arnon (Arnon 1949) as follows: total chlorophyll (g L^{-1}) = $20.21 \times (\text{A}_{645}) + 8.02 \times (\text{A}_{663})$. A₆₄₅ and A₆₆₃ are the absorbance values at 645 and 663 nm wavelengths, respectively.

Statistical analysis

Expression analysis of transgenic tobacco plants were means of three biological replicates and other data were means of five biological replicates. Student's *t* test and ANOVA were applied to analyze the significant difference between the wild-type and transgenic lines.

Results

Tobacco transformation and expression of *chyb* in transgenic tobacco

Leaf disc explants of tobacco explants were transformed with the plasmid pCAMBIA2300-*Lbchyb*, which contains a *chyb* cDNA isolated from *Citrus kinokuni* under control of the CaMV 35S promoter (Fig. 1a). Empty vector pCAMBIA2300 was also introduced into tobacco plants as control. The target gene was detected by genomic PCR analysis in the kanamycin-resistant independent tobacco plants

(Fig. 1b). Integration and expression of the *chyb* transgene was confirmed by southern blot and qPCR analysis using T₂ generation transgenic tobacco plants. Southern blot results revealed that only one hybridization signal was observed for CL5 and CL8 (Fig. 1c). The other transgenic tobacco plants analyzed have likewise displayed only one hybridization signal (data not shown). qPCR results showed that all the analyzed plants had expressed *chyb* except the WT and control plants (Fig. 1d). The transgenic lines CL5 and CL8 showed higher levels of *chyb* transcripts.

Carotenoids accumulation profiles in wild-type and *chyb* transformants

Carotenoids and chlorophyll were extracted from leaves of the selected lines (T₂) and the control plants (WT and transformed with empty plasmid). Under artificial light conditions (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), the amount of total carotenoids was similar in the transgenic lines and the control plants (Table 1). Determination of carotenoid distribution by HPLC demonstrated that under artificial light conditions the amount of β -carotene decreased in the *chyb* transformants as compared to the control plants (86% of the controls). However, zeaxanthin level was not detectable under these light conditions.

Upon transfer of the plants grown under artificial light conditions to high-light conditions (1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) zeaxanthin was dramatically formed in the *chyb* lines (Table 1). After a high-light treatment of 2 h, zeaxanthin content significantly increased in the *chyb* lines as compared to the control plants (wild-type about 5.3 $\mu\text{g g}^{-1}$ FW, *chyb* transgenics about 9.3 $\mu\text{g g}^{-1}$ FW, 5 independent determinations). This suggested that overexpression of *chyb* manipulated the metabolic flux of carotenoids to excessive accumulation of zeaxanthin.

To further elucidate the influence of UV irradiation on pigment composition in the *chyb* lines and control plants, the plants, pre-illuminated with high-light of 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, were exposed to UV light for 2 h. As we expected, the *chyb* tobaccos displayed more tolerance to UV irradiation (Fig. 2a). After exposure to UV radiation for 2 h, zeaxanthin content was 30% higher in the transformants than the controls (Table 1). In addition, the violaxanthin and antheraxanthin content remained higher, too.

Direct effects of UV radiation: photosynthetic efficiency, NPQ capacity, MDA content, and SOD activity

After UV stress, the *chyb* lines displayed less leaf necrosis, which suggested that *chyb* lines suffered less impact of UV radiation (Fig. 2a).

Table 1 Content and composition of carotenoids of *chyb* transgenic lines (CL5 and CL8, T₂ progeny) and tobacco control plants (A) in artificial light conditions (200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), (B) after transfer to high-light (1000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) for 2 h and (C) exposed to UV radiation for 2 h after pre-illuminated with high-light of 1000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$

Carotenoid ($\mu\text{g g}^{-1} \text{ FW}$)	WT	EP (empty plasmid)	CL5	CL8
A				
Total carotenoids	205.84 $\pm$ 15.38	221.85 $\pm$ 9.57	285.68 $\pm$ 11.59*	273.54 $\pm$ 12.58*
Violaxanthin	3.85 $\pm$ 0.41	4.01 $\pm$ 0.47	15.84 $\pm$ 1.25*	13.68 $\pm$ 1.06*
Neoxanthin	1.79 $\pm$ 0.25	2.01 $\pm$ 0.34	3.15 $\pm$ 0.29*	3.08 $\pm$ 0.33*
Lutein	45.23 $\pm$ 1.85	43.57 $\pm$ 1.74	48.94 $\pm$ 2.04	44.36 $\pm$ 1.59
β -Carotene	148.6 $\pm$ 10.69	142.39 $\pm$ 9.29	129.28 $\pm$ 13.21*	121.85 $\pm$ 11.69*
Zeaxanthin	Not detected	Not detected	Not detected	Not detected
B				
Total carotenoids	218.68 $\pm$ 12.58	220.14 $\pm$ 11.95	306.29 $\pm$ 16.98*	298.34 $\pm$ 15.04*
Violaxanthin	5.79 $\pm$ 1.02	6.57 $\pm$ 1.14	7.69 $\pm$ 1.24	7.81 $\pm$ 1.63
Neoxanthin	2.61 $\pm$ 0.95	2.72 $\pm$ 0.88	2.97 $\pm$ 0.96	3.08 $\pm$ 1.31
Lutein	92.28 $\pm$ 5.87	94.06 $\pm$ 6.34	98.69 $\pm$ 7.25	103.01 $\pm$ 8.94
β -Carotene	68.32 $\pm$ 4.65	65.28 $\pm$ 5.04	10.92 $\pm$ 2.58*	12.25 $\pm$ 2.04*
Zeaxanthin	5.32 $\pm$ 1.15	5.23 $\pm$ 1.07	9.36 $\pm$ 2.31*	8.91 $\pm$ 1.85*
C				
Total carotenoids	195.36 $\pm$ 11.71	190.42 $\pm$ 10.89	298.67 $\pm$ 17.23*	284.51 $\pm$ 16.39*
Violaxanthin	4.41 $\pm$ 0.76	4.81 $\pm$ 0.82	10.92 $\pm$ 1.76*	10.92 $\pm$ 2.27*
Neoxanthin	2.81 $\pm$ 1.05	2.71 $\pm$ 1.14	2.89 $\pm$ 1.29	2.73 $\pm$ 1.38
Lutein	89.36 $\pm$ 5.26	90.36 $\pm$ 5.86	92.19 $\pm$ 6.23	95.24 $\pm$ 7.29
β -Carotene	58.94 $\pm$ 5.25	60.24 $\pm$ 5.97	9.84 $\pm$ 3.09*	10.39 $\pm$ 3.15*
Zeaxanthin	4.85 $\pm$ 1.28	4.61 $\pm$ 1.28	6.53 $\pm$ 3.81*	5.94 $\pm$ 3.73*

Values shown are mean values $\pm$ SD of five T₂ transgenic tobacco plants and corresponding control plants

* $P < 0.05$. The unit is $\mu\text{g g}^{-1} \text{ FW}$

Without the photoprotective function of xanthophylls, plants often suffered from photooxidation damage (Niyogi 1999; Tian et al. 2003). To determine the effect of zeaxanthin on F_v / F_m and NPQ, chlorophyll fluorescence was measured in *chyb* transgenics and control plants. The control tobacco plants showed significantly lower amplitude of NPQ compared with the *chyb* lines (Fig. 2b). The control tobacco plants also had significant lower F_v / F_m ratios than the *chyb* lines (Fig. 2c). These results suggested that increasing the size of xanthophyll cycle pool had significantly elevated the photosynthetic efficiency of PSII reaction center, which was consistent with previous study (Du et al. 2010).

An increase in the xanthophyll cycle can lead to decreased vulnerability of *Arabidopsis thaliana* to oxidative damages (Davison et al. 2002). We detected a significantly lower level of MDA, which is an important end product of lipid peroxidation (Wu et al. 2010), in *chyb* tobacco plants than in the control plants (Fig. 2d). In addition, we also measured SOD activity in the soluble proteins fractions extracted from the *chyb* overexpression lines and the control plants before and after UV stress. Compared with the control plants, the SOD activity in the transgenic plants was similar before the stress but was significantly increased after the stress (Fig. 2e). All these results above suggested that overexpression of *chyb* in tobacco enhances UV stress resistance in plants.

Increased resistance of *chyb* transgenic tobacco plants to oxidative stress

UV radiation is a kind of oxidative stress via the formation of ROS in plant cells (Jansen et al. 1998). Subsequently, we measured the response of the *chyb* transgenic lines and control plants to oxidative stress using methylviologen (MV), a well-known kind of oxidative stress inducer in chloroplasts under light (Cheng 2006). The leaves of controls and *chyb* lines were treated with 30 μM MV for two days. After two days treatment, necrosis fraction was observed on the leaves of all tested tobacco plants, whereas the MV damage of *chyb* lines was a little weaker than that of control plants (Fig. 3a). Chlorophyll content was also significantly reduced in the control plants (Fig. 3c). We measured the accumulation of H_2O_2 further on in the tested leaves by diaminobenzidine staining. After MV treatment for 2 d, a darker staining was observed in the wild type than in the *chyb* lines, which suggested that the content of H_2O_2 was accumulated more in the wild type (Fig. 3b). These results indicated that the enhanced resistance of the *chyb* transgenic plants to oxidative stress may result from an increased ROS-scavenging ability. From these results, the importance of *chyb* in oxidative stress resistance was further established.

Fig. 2 Response of *chyb*-expressing transgenic tobacco plants to 2 h UV radiation. **a** Phenotype of tobacco plants before and after UV radiation; **b** NPQ of chlorophyll fluorescence ($\mu\text{mol photons m}^{-2}$

s^{-1}), **c** chlorophyll fluorescence of tobacco plants; **d** MDA content of tobacco plants before and after UV treatment; **e** SOD activity in the tobacco leaves. Values are mean values $\pm$ SD ($n=5$). * $P<0.05$

Discussion

Sessile plants have developed many mechanisms to adapt to the ever-changing environment. Carotenoids, especially zeaxanthin, play important roles in plants to oxidative stress (Davison et al. 2002; Johnson et al. 2008; Liu et al. 2017a, b). These findings prompted us to investigate the protective ability of the novel β -carotene hydroxylase gene, *Citrus kinokuni chyb* gene, to against UV and oxidative stresses in higher plants. Compared with many researches on

Arabidopsis thaliana β -carotene hydroxylase gene, the *Citrus kinokuni chyb* gene has not been reported. To our knowledge, for the first time, we report that the *Citrus kinokuni chyb* gene plays a key role in UV and oxidative resistance.

Characterization of the tobacco transformants by genomic PCR, southern blotting, and qPCR analysis proved that the *Citrus kinokuni chyb* gene is expressed (Fig. 1). Previous study demonstrated that zeaxanthin could not accumulate in higher plant for its rapid conversion to violaxanthin under low-light conditions (Gotz et al. 2002). Our results, high

Fig. 3 Response of *chyb*-expressing transgenic tobacco plants to MV treatment. **a** Leaf phenotype under 30 μ M MV stress for 2 days; **b** diaminobenzidine staining for H_2O_2 level; **c** chlorophyll content before and after MV treatment. Values are mean values $\pm$ SD ($n=5$). * $P<0.05$

violaxanthin levels in the *chyb* transgenic plants under low-light conditions (Table 1), were consistent with this finding. Whereas under high-light conditions, violaxanthin might not be able to bind to the light-harvesting complex of photosystem II (Caffarri et al. 2001). Subsequently, zeaxanthin could replace the violaxanthin and bind to the site. Our results also suggested that the *chyb* lines accumulated high levels of zeaxanthin under high-light conditions (Table 1). However, the other carotenoid components had relatively small changes, which may be caused by a tight control of carotenoid synthesis pathway (Misawa et al. 1993).

As a member of xanthophyll cycle, zeaxanthin has been demonstrated to play an important role in plant photoprotection under high-light stress (Demmig et al. 1987; Niyogi 1999; Young 1991). As compared to the *Arabidopsis* mutants with decrease of xanthophyll cycle activity (Havaux and Niyogi 1999; Hurry et al. 1997), our *chyb* transgenics possess higher capacity of catalyzing β -carotene to form zeaxanthin and consequently promote the function of xanthophyll cycle.

UV-B radiation has many effects on plants (Rozema et al. 1997). The UV stress often influences the growth and culture regime of high plants (Allen et al. 1998). Although our experiments did not test the biomass of UV-treated tobacco, the results showed that the leaves of transgenic tobacco were damaged more slightly than that of the controls after UV treatment (Fig. 2a). As parameters of the xanthophyll cycle

(Du et al. 2010), F_v/F_m , NPQ and SOD were tested after UV treatment (Fig. 2). The results showed that these parameters were significantly higher in the *chyb* lines than in the controls under UV stress conditions as expected, further supporting the role of *chyb* gene in enhancing tobacco resistance to UV stress through metabolic engineering of zeaxanthin. Furthermore, the lower MDA amount in the *chyb* lines demonstrated that enhanced zeaxanthin content alleviated lipid peroxidation. The enhanced UV resistance is likely connected with the higher *Citrus kinokuni* β -carotene hydroxylase capacity for zeaxanthin formation. This assumption is established on the significantly higher zeaxanthin content in the UV-tolerant *chyb* plants. It cannot be excluded, however, that the other increased carotenoids in the transgenics and the lutein, a kind of zeaxanthin stereoisomer, also plays a role in the increased resistance to UV radiation.

UV radiation often produces excessive ROS by transporting photosynthetic electron in the thylakoid membrane, and excessive ROS can cause plant cell damage (Bouvier et al. 1998). However, plant endogenous carotenoids can scavenge the excessive ROS (Lokstein et al. 2002). To examine the antioxidant properties of zeaxanthin, MV treatment was applied in this study. MV could generate H_2O_2 in chloroplasts by inhibiting electron transport during photosynthesis (Cheng 2006). Meanwhile, the decrease of chlorophyll content in the *chyb* lines was less than that in the controls. The enhanced antioxidant protection in *chyb* lines is probably

related to the increased zeaxanthin content. Previous study demonstrates that zeaxanthin incorporates in the membrane and does help to its rigidity (Havaux and Niyogi 1999). This may be the reason for higher chlorophyll content in the *chyb* lines. Therefore, these results indicate that the enhanced antioxidant activity in the *chyb* transgenics might be achieved by elevating zeaxanthin contents in plant cell.

Conclusions

In conclusion, our study reports the antioxidant characterization of *Citrus kinokuni* β -carotene hydroxylase (*chyb*) gene in tobacco. This gene produced an increased xanthophyll cycle pool, which conferred UV and oxidative resistance to transgenic tobacco plants. Considering our results, forming additional zeaxanthin via β -carotene can give plants a distinct advantage to resist UV light and oxidative stress. Our study indicates that the *Citrus kinokuni chyb* gene may be a promising candidate gene for increasing UV and oxidative resistance in plants, especially in crops.

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Author contributions Jiang Wu conceived and designed the study. Jiang Wu, Xuedong Ji, Shenhong Tian, Shaoxia Wang and Huarong Liu performed the experiments. Jiang Wu wrote the paper. Jiang Wu reviewed and edited the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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