RNA interference (RNAi) of 2-Cys Prx gene enhances PSII photoinhibition but does not affect PSI activity in tobacco leaves under high-temperature stress

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ABSTRACT
This study aimed to evaluate the effects of 2-Cys Prx gene inhibition on photochemical reaction and reactive oxygen species (ROS) metabolism under high temperature (35°C) with low light (HT + LL) or high temperature with high light (HT + HL) in tobacco. The results showed that HT significantly increased the production of O$_2^-$ and H$_2$O$_2$ compared with CK (25°C). Particularly, the oxidative damage of RNAi plants was significantly greater than that of wild type (WT) under HT + HL treatment, possibly due to the inhibition of superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities. HT treatment inhibited the photosystem II (PSII) activity, and the oxygen evolution complex (OEC) was the main injury site. Notably, the photosystem I (PSI) activity of WT and RNAi plants did not change significantly under HT + LL treatment compared with CK. Although the PSI activity of WT and RNAi plants decreased significantly under HT + HL treatment, there was no significant difference between WT and RNAi plants. Collectively, these findings indicate that high light increases the photoinhibition of PSI and PSI and oxidative damage under high-temperature stress. The results also revealed that 2-Cys Prxs plays a crucial role in alleviating oxidative damage and PSII photoinhibition under high-temperature stress in tobacco.

High temperature (HT) is one of the main factors limiting plant growth and development, including crop yield and quality (Lobell et al. 2005; Tubiello et al. 2007). HT stress affects plant growth and physiological functions through various metabolic processes, such as sugar and energy metabolism (Xu and Huang 2008; Liu et al. 2014), cell cycle (Yang et al. 2011, 2012) and signal transduction (Königshofer et al. 2008; Reddy et al. 2011). Photosynthesis is sensitive to HT stress (Essemble et al. 2011). HT stress affect carbon assimilation processes in plants, including stomatal closure or conductance (Rodrigues et al. 2018; Urban et al. 2018). HT also inhibits the activity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and RuBisCO activase (RCA) (Jagtap et al. 1998) and reduces the photosynthetic rate of plants (Haldimann and Feller 2005; Sharkey 2005). PSII and PSI reaction centers are the main sites vulnerable to HT stress (Allakhverdiev et al. 2008; Kalaji et al. 2011; Essemble et al. 2012). Light is the basis of plant photosynthesis. However, stress reduces plant’s ability to use light energy. Indeed, a high light intensity has been shown to damage photosynthetic apparatus (Ji et al. 2002). Several studies have confirmed that high light aggravates the damage to plant photosynthetic apparatus under stress (Chen et al. 2008; Zhao et al. 2011; Sun et al. 2013).

Plant photosynthesis is key in modulating reactive oxygen species (ROS) burst. Electron leakage in photosynthetic electron transport chain or excess excitation energy in chloroplast induces the accumulation of superoxide anion (O$_2^-$), H$_2$O$_2$ and singlet oxygen under stress. The excessive ROS inhibit the normal progress of photosynthesis, leading to further increase in ROS (Takahashi et al. 2004; Yamashita et al. 2008; Nishiyama et al. 2011; Bialasek et al. 2017). In chloroplast, superoxide dismutase (SOD) catalyzes O$_2^-$ to form H$_2$O$_2$, but there is no catalase (CAT) in chloroplast, thus H$_2$O$_2$ in chloroplast is mainly catalyzed by ascorbate peroxidase (APX) to H$_2$O and monodehydroascorbic acid (MDHA). Notably, APX in chloroplast is very sensitive to excessive ROS and is inactivated under high ROS content (Shafi et al. 2017; Zhang et al. 2017). Failure to remove excessive H$_2$O$_2$ generates highly active hydroxyl radicals (OH) through Haber–Weiss reaction, which damages plant photosynthetic apparatus (Xia et al. 2017).

Reducing ROS levels plays a vital role in improving plant resistance to stress. Thioredoxin-Thioredoxin peroxidase (Trx-Prx) pathway facilitates the scavenging of excess ROS to alleviate oxidative damage in plants (Zhang, Li, Guan et al. 2020; Zhang, Xu, Huo, et al. 2020). Prxs in Trx-Prx pathway can act as both antioxidant protein and enzyme and cooperate with ascorbic acid peroxidase (APX) in...
chloroplast AsA-GSH cycle to scavenge H$_2$O$_2$ (Nikkane and Rintamaki 2014; Vidigal et al. 2015). Stress induced the up-regulation of Prx protein expression in Arabidopsis thaliana (Ndinma et al. 2005), Cucumis sativus L. (Du et al. 2010), Physcomitrella patens (Wang et al. 2008), and Zea mays (Zörb et al. 2010). Prxs family members in higher plants mainly include 1-Cys Prx, 2-Cys Prx, Prx II and Prx Q. 2-Cys Prx is a class of chloroplast proteins encoded by nuclear genes located on the thylakoid membrane of chloroplast (Baier and Dietz 1999; Baier et al. 2000). They can convert H$_2$O$_2$ into water and alcohol in chloroplasts (Tripathi et al. 2009; Hong et al. 2017). When the redox potential is low, 2-Cys Prx can also polymerize with lipophilic molecules to reduce H$_2$O$_2$ (Konig et al. 2002; Dietz 2003).

2-Cys Prx plays a crucial role in scavenging ROS and improving plant stress resistance (Lee et al. 2015; Zhang, Xu, Teng, et al. 2019). Heterologous expression of rice 2-Cys Prx gene in Saccharomyces cerevisiae improved the redox balance in Saccharomyces cerevisiae cells (Kim et al. 2013). Kim et al. (2010) showed that 2-Cys Prx gene plays a role in protecting cells from oxidative damage in Festuca arundinacea. Overexpression of 2-Cys Prx gene has also been shown to alleviate the oxidative damage of Solanum tuberosum L. under high-temperature stress (Kim et al. 2011). 2-Cys Prx potentially modulates certain proteins in photosynthetic apparatus by changing the redox level of cells, thus affecting the photosynthetic electron transfer (Ivan et al. 2010; Leonor et al. 2015). Studies have mainly focused on elucidating the 2-Cys Prx function via its overexpression. Therefore, there is a need to explore the function of 2-Cys Prx by inhibiting its expression. RNA interference (RNAi) can specifically eliminate or shut down specific gene expression, thus being employed to study gene function. In this study, RNAi was used to inhibit the expression of 2-Cys Prx gene in tobacco plants. The effects of high temperature with low light (35°C + 200 μmol·m$^{-2}$·s$^{-1}$PFD, HT + LL) and high temperature with high light (35°C + 1500 μmol·m$^{-2}$·s$^{-1}$PFD, HT + HL) on ROS metabolism, PSII and PSI function of tobacco leaves were studied to determine the physiological function of 2-Cys Prx gene under high temperature and other stresses.

1. Materials and methods
1.1. Test materials
1.1.1. Construction of RNAi vector using gateway technology
This procedure was performed at the State Key Laboratory of forest genetics and breeding and Plant Photosynthetic Physiology Laboratory of Northeast Forestry University. 2-Cys Prx gene sequence was amplified from the leaves of tobacco plants (variety ‘Longjiang 911’) by RT-PCR in a previous study (Zhang et al. 2017). A 270 bp interference fragment was designed from the 3’ end of 2-Cys Prx gene. The primers of the interference fragment were as follows:

RNAi-S: TGCCCTGGGTCACAACCCGAAA,
RNAi-A: CAACACTTCATCCGGGTATCCTG.

PCR amplification of the 2-Cys Prx was performed using attB-RNAi-S and attB-RNAi-A primers containing a part of the B-adapter interference fragment. The primers were as follows:

attB-RNAi-S: AAAAAACGGCTTGCTGCGTGCTCAAAACCGAAA,
attB-RNAi-A: AGAAAGCTGGGTCAACACTTCATCCGGGTATCCTG.

The second PCR was performed with primers attB-adaptor-S and attB-adaptor-A, using the first PCR product as a template, and the B-adapter was added to the interference fragment. B-adapter sequences were as follows: attB-adaptor-S: GGGGACAGTTTGTAACAAAAAGCAGGCT,
attB-adaptor-A: GGGGACACCTTTGTACAAGAAAGCTGGGT.

LB medium containing 50 mg·L$^{-1}$ kanamycin and primers attB-RNAi-S and attB-RNAi-A were used for PCR identification, and the interference fragment was replaced with the intermediate vector (DONR). LB medium containing 100 mg·L$^{-1}$ spectinomycin and primers attB-RNAi-S and attB-RNAi-A were used for PCR identification. It was confirmed that the interference fragment was replaced from the donor vector to the inhibition expression vector pK7GWIG2D(II). One of the positive colonies was randomly selected and sequenced in BGI (Shenzhen, China).

1.1.2. Agrobacterium tumefaciens mediated transformation of tobacco
RNAi vector pK7GWIG2D(II) was transfected into competent cells of Agrobacterium tumefaciens (LBA4404) by electroporation. Subsequently, primers attB-RNAi-S and attB-RNAi-A were used for PCR amplification to identify positive clones with 270 bp specific band. Plasmids of the positive clones were extracted and further validated by PCR using primers attB-RNAi-S and attB-RNAi-A to obtain 270 bp specific RNAi positive clones of Agrobacterium tumefaciens.

Tobacco seeds were disinfected using 10% sodium hypochlorite (NaClO) and sown on Murashige and Skoog (MS) medium containing XXX. Tobacco leaves were harvested at 4–5 leaf stage and used as transformation materials. Agrobacterium tumefaciens (LBA4404) containing RNAi vector pK7GWIG2D(II) was cultured to OD$_{600}$ nm = 0.4, and then used to infect sterile tobacco leaf discs (about 1 cm×1 cm). Leaf discs from wild type tobacco (WT) plants were infected with Agrobacterium tumefaciens (LBA4404) without RNAi vector pK7GWIG2D(II) to serve as control. The Agro-infected tobacco leaf discs were cultured for three days in the dark, then transferred to a selective medium to induce callus formation. The callus was transferred to a differentiation medium to induce bud formation. When the putatively transformed buds elongated to about 1 cm, they were transferred to a rooting medium. After rooting, the plantlets were transferred to a well-mixed medium of peat soil and quartz sand (2:1 volume ratio) and cultivated in an artificial climate box at 23–25°C, light intensity of 200 μmol·m$^{-2}$·s$^{-1}$, and photoperiod of 12/12 h (light/dark).

1.1.3. PCR and semi-quantitative RT-PCR detection of tobacco plants containing RNAi 2-Cys Prx gene
Total DNA of WT and RNAi tobacco leaves was extracted using the CTAB method. The DNA of transgenic and WT plants was amplified by PCR using 35-S and 35-A primers. The primers of CaMV35S promoter gene were as follows: CaMV35S-S: 5’-AGATAGGCTTCTTCATTTGACTAAGGCTTGGGG, CaMV35S-A: 5’-CGAGGCTTCTTCATTTGACTAAGGCTTGGGG. The DNA from WT and RNAi tobacco
plants was subjected to semi-quantitative RT-PCR analysis to verify whether RNAi vector inhibited the expression of 2-Cys Prx gene in the RNAi tobacco plants.

1.2. Experimental treatment

When RNAi positive plants (RNAi) and wild-type plants (WT) had grown six-leaf stage, plants with relatively consistent growth were selected and put into incubators at 25°C (CK) and 35°C (Zobayed et al. 2005; Moon et al. 2007). In the high temperature treatment at 35°C, LED lamps provided 200 μmol·m⁻²·s⁻¹ or 1500 μmol·m⁻²·s⁻¹ light intensity. That is, high temperature low light (35°C+200 μmol·m⁻²·s⁻¹PFD, HT + LL) or high temperature strong light (35°C+1500 μmol·m⁻²·s⁻¹PFD, HT + HL). The physiological indexes and chlorophyll fluorescence parameters of the third fully developed leaf were determined after HT + LL or HT + HL treatment for 24 h.

1.3. Determination parameters

1.3.1. Determination of chlorophyll fluorescence kinetics (OJIP) curve and 820 nm reflection fluorescence (MR820) curve

After dark adaptation for 0.5 h, the OJIP curve and MR820 curve were measured using M-Pea continuous excitation fluorometer (Handy, UK). The OJIP curve was drawn using the average value of three repetitions, where O, J, I and P points correspond to 0.01, 2, 30 and 1000 ms time points, respectively. The initial fluorescence and maximum fluorescence at 0.01 and 1000 ms were expressed as F₀ and Fm, respectively. The OJIP curve was normalized according to the formula Vt=(Ft−F₀)/(Fm−F₀) to obtain the Vt curve, and the relative variable fluorescence V₅ and V₇ at 0.3 ms (K point) and 2 ms (J point) on the Vt curve were obtained. To further compare the variation range of relative variable fluorescence of each characteristic point, we obtained the ΔVt curve by determining the difference between the Vt curve of tobacco leaves under HT + LL and HT + HL treatment and that of tobacco leaves under 25°C. The parameters of chlorophyll fluorescence were obtained by JIP test analysis of OJIP curve (Strasser and Srivastava 1995): the maximum photochemical efficiency of PSII (Fv/Fm), photosynthetic index based on absorbed light energy (P1ABS), absorbed light energy per unit reaction center (ABS/RC), absorbed light energy per unit area (ABS/CSm) and the number of active reaction centers per unit area (RC/CSm) were measured. PSI activity is reflected by the relative drop in MR820 curve signal, i.e. ΔI/I₀, where I₀ is the maximum value of the reflected signal on the MR820 curve, and ΔI is the difference between the maximum and minimum values of the reflected signal on the MR820 curve (Oukarroum et al. 2013). The above chlorophyll fluorescence curves were measured for three biological repetitions.

1.3.2. Reactive oxygen species and antioxidant enzymes activity

The determination of each index here was done with three biological repetitions. The production rate of superoxide anion (O₂⁻·), the content of H₂O₂ and the activity of superoxide dismutase (SOD) were determined using the methods described by Zong and Wang (2010). Determination of the generation rate of O₂⁻·: 0.5 g leaves were weighed and added into 8 mL PBS solution for ice bath grinding, and centrifugate at 4000 r·min⁻¹ for 15 min. 1 mL of supernatant was taken, added with 1 mL of PBS and 2 mL of hydroxyamide hydrochloride, and the solution was stationary at 25°C for 20 min. Then 2 mL p-amino benzene sulfonic acid and 2 mL a-naphthylamine were added, and the absorbance at 530 nm was measured after standing at 25°C for 20 min. Determination of the content of H₂O₂: 0.5 g leaves were weighed and added into 8 mL aceton solution for ice bath grinding, centrifugated at 3000 r·min⁻¹ for 10 min, and the supernatant was used as sample extract. 1 mL of supernatant was absorbed, and 0.1 mL of 5% TiCl₄ solution and 0.2 mL of concentrated ammonia were added. After the mixture was precipitated, the mixture was centrifugated at 3000 r·min⁻¹ for 10 min, and the supernatant was discarded. Washed with acetone 3–5 times until the plant pigment is removed. Added 5 mL of 2 mol·L⁻¹ concentrated sulfuric acid to the precipitate, and added distilled water to the precipitate to 10 mL, and determined the absorbance value at 415 nm wavelength. SOD activity was determined using the nitro blue tetrazolium (NBT) method. The amount of enzyme required to phycologically reduce 50% of NBT in 1 mL of reaction solution in a 1-h unit of time (1 h) was considered one enzymatic activity unit (U), and the enzymatic activity units in each g of fresh samples were described as U·g⁻¹. The activity of ascorbate peroxidase (APX) was determined as described by Tang and Luo (2012). The enzyme activity units (again, U) were defined as the amount of enzyme used to catalyze the oxidation of 1 μmol of ascorbic acid in 1 min, and the enzyme activity unit in each g of fresh samples was described as U·g⁻¹.

1.4. Data analysis

Excel and SPSS (22.0) software were used for statistical analysis. Data in the figures are expressed as the mean ± standard deviation (SE) of three repetitions (n = 3). One-way analysis of variance (ANOVA) and least-significant difference (LSD) post hoc test was used to compare the differences among various treatments. P < 0.05 was considered to be statistically significant.

2. Results

2.1. Vector construction and generation of RNAi tobacco plants

A comparison of RNAi sequencing results with the full-length 2-Cys Prx gene sequence of tobacco revealed that the interference fragment was inserted into the inhibition expression vector pK7GWIWG2D(II), indicating that the RNAi vector was constructed successfully (Figure 1(A)). PCR performed on DNA samples from RNAi tobacco plants revealed a target band of about 800 bp, the same size as 35S promoter (5-22 bands in Figure 1(B)). No band was observed in samples from WT tobacco plants (1-4 bands in Figure 1(B)), indicating that the suppression expression vector containing 35S promoter was successfully transformed into the tobacco plants. The RNAi tobacco plants were randomly selected for further analysis with semi-quantitative RT-PCR to detect the 2-Cys Prx gene (Figure 1(C)). The expression level of 2-Cys Prx gene in the selected RNAi tobacco plants was significantly lower than that in WT.
plants, indicating that RNAi vector effectively inhibited the expression of 2-Cys Prx gene.

2.2. OJIP curve and PSII photochemical activity

The fluorescence intensities of O, J and I on OJIP curves of WT plants under HT + LL and HT + HL treatments were lower compared with CK, while the relative fluorescence intensity of F point \( (F_m) \) significantly decreased (Figure 2(A)). In addition to the significant decrease in \( F_m \), the relative fluorescence intensity of O point \( (F_o) \) was also significantly increased in RNAi plants compared with CK (Figure 2(B)). Compared with CK, the changes in \( F_o \) and \( F_m \) of WT plants under HT + LL and HT + HL treatments were not significant, but \( F_o \) of RNAi plants increased by 37.21% \( (P < 0.05) \) and 51.24% \( (P < 0.05) \), respectively, compared with CK (Figure 2(C)). Meanwhile, the decrease in \( F_m \) and PI\(_{ABS} \) of WT and RNAi plants showed a decreasing trend under HT + LL and HT + HL treatments. Moreover, the decrease in \( \Delta I/I_o \) under HT + HL treatment was higher than under HT + LL treatment. However, there was no significant difference in \( \Delta I/I_o \) between WT and RNAi plants under HT + LL or HT + HL treatments (Figure 4).
2.5. Correlation analysis of $V_K$ and $V_J$ with $F_v/F_m$ and $PI_{ABS}$

There was a significant negative correlation between $V_K$, $V_J$, and $F_v/F_m$, $PI_{ABS}$ in tobacco leaves (Figure 5). The correlation coefficients of $V_K$ and $V_J$ with $PI_{ABS}$ ($R^2 = 0.93$ and 0.78) were significantly higher than those with $F_v/F_m$ ($R^2 = 0.81$ and 0.73). Moreover, the correlation coefficients of $V_K$ with $F_v/F_m$ and $PI_{ABS}$ were significantly higher than those of $V_J$ with $F_v/F_m$ and $PI_{ABS}$.

2.6. Energy absorption per unit reaction center and unit area, number of active reaction centers per unit area

Compared with CK, the $ABS/RC$ of WT and RNAi plants increased under HT + LL and HT + HL treatments (Figure 6(A)), while the $ABS/CS_m$ decreased (Figure 6(B)). Changes in $ABS/RC$ and $ABS/CS_m$ under HT + HL treatment were greater than those under HT + LL treatment. Meanwhile, changes in $ABS/RC$ and $ABS/CS_m$ under RNAi plants were greater than those under WT. The $RC/CS_m$ of WT and RNAi plants decreased significantly under HT + LL and HT + HL treatments, especially under HT + HL treatment (Figure 6(C)).

2.7. ROS metabolism

There was no significant difference in SOD and APX activities between WT and RNAi plants at 25°C (Figure 7(A,B)). Compared with 25°C (CK), the SOD and APX activities of WT and RNAi plants treated with HT + LL increased slightly, but the differences were insignificant. The SOD activity of RNAi plants under HT + HL treatment was 18.38% ($P < 0.05$) lower than that of WT plants, and the SOD activity of RNAi plants was lower than that of HT + HL treatment 19.73% ($P < 0.05$) (Figure 7(A)). Although there was no significant difference in APX activity between WT and RNAi plants under HT + HL treatment, APX activity in RNAi plants was significantly lower than of WT plants under HT + LL treatment (Figure 7(B)). Compared with CK, the $O_2^-_2$ production rate of WT plants treated with HT + LL and HT + HL did not change significantly. However, the $O_2^-_2$ production rate of RNAi plants was higher than that of WT by 30.38% under HT + HL treatment ($P < 0.05$) (Figure 7(C)). Compared with CK, the $H_2O_2$ content of WT plants under HT + LL treatment did not increase significantly, but the content in RNAi plants increased by 43.41% ($P < 0.05$) (Figure 7(D)). Under HT + HL treatment, the contents of $H_2O_2$ in WT and RNAi plants were significantly higher than those under HT + LL treatment, and the RNAi plants had significantly higher $H_2O_2$ content than the WT.

3. Discussion

Photosystem II (PSII) is very sensitive to high temperature (HT) stress. Chlorophyll fluorescence analysis is an important technique used to assess plant photosynthetic function,
especially PSII photochemical activity, electron transfer ability and light energy utilization (Zhang, Li, Zhang, et al. 2018). In this study, $F_0$, $F_m$ and $F_v/F_m$ of WT plants did not change significantly under HT + LL or HT + HL treatments compared with CK (25°C). Although $PI_{ABS}$ decreased considerably, the difference was not significant ($p < 0.05$). In contrast, $F_o$ of RNAi plants increased significantly under HT + LL and HT + HL treatments. Also, $F_m$, $F_v/F_m$ and $PI_{ABS}$ of the RNAi plants decreased significantly under HT + HL treatment, indicating that 2-Cys Prx gene inhibition decreases the photochemical activity of PSII of tobacco leaves under HT + HL stress. The change of specific activity parameter of unit reaction center can not only reflect the absorption and utilization of light energy, but also reflect the activity of leaf reaction center (Strasser and Srivastava 1995). In this experiment, the $ABS/RC$ of WT and RNAi plants increased under HT + LL and HT + HL treatments, while $ABS/CS_m$ and $RC/CS_m$ decreased, compared with CK. Additionally, the changes in $ABS/RC$, $ABS/CS_m$ and $RC/CS_m$ of tobacco leaves under HT + HL treatment were greater than those under HT + LL treatment. These results suggest that HT stress potentially inactivated some reaction centers in tobacco leaves, decreasing light energy absorption per unit area of leaves. This could have accelerated the function of the remaining active reaction centers, increasing light energy absorption per unit area of tobacco leaves. Similar to $F_m$, $F_v/F_m$ and $PI_{ABS}$, the reduction in $RC/CS_m$ of RNAi plants was significantly higher than that by WT, implying that inhibition of 2-Cys Prx gene expression possibly reduced the activity and

Figure 3. Effects of RNAi 2-Cys Prx gene expression on standardized OJIP curve ($V_t$) in WT (A) and RNAi (B) plants, $\triangle V_t$ in WT (C) and RNAi (D) plants, $V_K$ (E) and $V_J$ (F) of tobacco leaves under high temperature with low light [HT + LL] or high temperature with high light [HT + HL] treatment. Note: The data in the figure are from three replicated experiments ($n = 3$), and represent means ± standard error (SE). Different lowercase letters show significant differences ($P < 0.05$).

Figure 4. Effects of RNAi 2-Cys Prx gene expression on 820 nm reflection fluorescence ($MR_{820}$) curve in WT (A) and RNAi (B) plants and $\triangle I/I_o$ (C) of tobacco leaves under high temperature with low light [HT + LL] or high temperature with high light [HT + HL] treatment. Note: The data in the figure are from three replicated experiments ($n = 3$), and represent means ± standard error (SE). Different lowercase letters show significant differences ($P < 0.05$).
number of PSII reaction centers in tobacco leaves under HT + HL treatment. The electron donor side of PSII is one of the sites prone to damage under HT stress (Barra et al. 2005; Chen et al. 2016; Ouakarroum et al. 2016). HT often inhibits the activity of oxygen-evolving complex (OEC) and down-regulates the expression of OEC or oxygen-evolving enhancer protein (OEE) (Han et al. 2009; Ahsan et al. 2010; Rocco et al. 2013). Some studies have found that 2-Cys Prx can affect the photosynthetic electron transport capacity of chloroplasts by changing their redox state (Ivan et al. 2010; Leonor et al. 2014). In the present study, V_K of WT and RNAi plants increased significantly under HT + LL and HT + HL treatments, especially under HT + HL treatment. An increase in V_K indicates damage to OEC activity of PSII electron donor side (Chen et al. 2017; Zhang, Shi, et al. 2019). Therefore, OEC activity on the electron donor side of PSII was significantly inhibited under HT stress. Furthermore, high light intensified the inhibition of OEC activity under HT stress. Although there was no significant difference in V_K between WT and RNAi plants under HT + LL treatment, the V_K of RNAi plants was significantly higher than that of WT under HT + HL treatment, indicating that 2-Cys Prx gene inhibition accelerates OEC damage in tobacco under HT + HL. In contrast, the V_I of RNAi plants increased significantly under HT + HL treatment. The increase in V_I indicates that the electron transfer from Q_A to Q_B in photosynthetic electron transport chain was blocked, and the accumulation of reduced Q_A increased gradually (Zhang et al. 2016; Zhang, Xu, Guo, et al. 2020). In this study, compared with CK, HT + LL treatment did not significantly change the value of V_I, indicating that HT + LL treatment did not significantly inhibit the transfer of Q_A to Q_B on PSII receptor side of tobacco leaves, possibly because the damage site of PSII by HT + LL treatment is mainly located on the donor side of PSII. The decrease of activity on the donor side weakened water photolysis on the donor side, thus reducing the electron pressure on the electron transfer receptor side. Under HT + HL treatment, the V_I increased significantly in RNAi plants, which is likely due to the weakening of the scavenging ability of H_2O_2 in tobacco leaves and the inhibition of electron transfer on the receptor side, following 2-Cys Prx gene inhibition (Wang, Guo, et al. 2022, Wang, Yu, et al. 2022). The correlation coefficients of V_K with F_v/F_m and P_lABS were significantly higher than that of V_I, further indicating that the damage of PSII donor side was greater than that of PSII receptor side under HT stress. The correlation coefficients of V_K and V_I with P_lABS were higher than F_v/F_m, indicating that P_lABS is a more accurate indicator of PSI function than F_v/F_m.

A decrease in PSI activity often inhibits the electron transfer from PSII to PSI, aggravating the damage of PSI (Bu et al. 2009; Zhang et al. 2009). The stability of PSI also affects the repair speed of damaged PSI (Yi et al. 2016, 2017). In the present study, the ΔI/II_o of WT and RNAi plants decreased by different degrees under HT + LL and HT + HL treatments, and the decrease was more significant under HT + HL treatment. PSI photoinhibition is related to ROS burst (Zhang et al. 2014), consistent with our results, O_2^- and H_2O_2 accumulation in WT and RNAi plants under HT + HL treatment were significantly higher than those under HT + LL treatment, thus the excessive ROS under HT + HL aggravated PSI damage. Although the accumulation of O_2^- and H_2O_2 was higher in RNAi plants than in WT under HT + LL and HT + HL treatments, the difference in ΔI/II_o between them was not significant, suggesting that the inhibition of 2-Cys Prx gene expression did not aggravate PSI damage in RNAi tobacco plants. Damage to PSI has been shown to occur only when the electron flow exceeds the capacity of the PSI acceptors (Tikkanen et al. 2014; Tikkanen and Aro 2016).

Figure 5. Correlation analysis of V_K and V_I with F_v/F_m and P_lABS.

Figure 6. Effects of RNAi 2-Cys Prx gene expression on ABS/RC (A), ABS/CS m (B) and RC/CS m (C) of tobacco leaves under high temperature with low light (HT + LL) or high temperature with high light (HT + HL) treatment. Note: The data in the figure are from three replicated experiments (n = 3), and represent means ± standard error (SE). Different lowercase letters show significant differences (P < 0.05).
PSI damage can be alleviated when the electron transfer from PSII to PSI is inhibited (Zhang et al. 2011; Huang et al. 2016). Therefore, although the PSII photochemical activity (Fv/Fm and PI_ABS) of RNAi plants was significantly lower than that of WT under HT + HL treatment, the electron transfer of PSII receptor side was blocked (Vj increased) in RNAi plants. This alleviated the electron pressure of PSI reaction center of RNAi plants, resulting in no significant difference in PSI activity between WT and RNAi plants. Plants can improve their resistance to stress by increasing respiratory intensity (Simons et al. 1999; Pascual et al. 2010). Therefore, the oxygen demand of plants increases under stress. An increase in intracellular free O2 induces the excess excitation energy in photosynthesis and the excess electrons in the photosynthetic electron transport chain to attack the free O2 in cells, leading ROS burst (Jaspers and Kangasjärvi 2010; Zhang, Xu, Li, et al. 2018). O2·− in the chloroplast is mainly produced in the thylakoid membrane as a result of the attack of excess electrons in photosynthetic electron transport chain on O2 (Takahashi and Asada 1988). Wang et al. (2020) found that overexpression of tamarix Th2-Cys Prx genes significantly affected the ROS content of tobacco leaves.

In the present study, O2·− production rate did not increase significantly in WT plants under HT + LL and HT + HL treatments. On the contrary, the O2·− production rate of RNAi plants was significantly higher than that of WT plants, especially under HT + HL treatment, possibly due to the increase in SOD activity in WT plants. SOD reduces most of the O2·− generated in the thylakoid membrane before it diffuses out (Foyer 2018); however, the SOD activity of RNAi plants decreased under HT + HL treatment, resulting in an increase in O2·− production rate. The accumulation of H2O2 in WT and RNAi plants increased by different degrees under HT + LL and HT + HL treatments. In particular, the increase in H2O2 accumulation under HT + HL treatment was greater than under HT + LL treatment. Although HT stress had little effect on PSII receptor side of tobacco leaves, it significantly reduced the activity of OEC on PSII donor side. Functional barrier of OEC can lead to incomplete cracking of water to produce H2O2 (Pospíšil 2012). Therefore, the increase in H2O2 in tobacco leaves under HT stress was directly related to the decrease in OEC activity and the increase in SOD disproportionation. The chloroplast of higher plants lacks CAT; therefore, H2O2 reduction in chloroplasts is mainly through the joint action of APX and ascorbic acid (AsA), which reduces H2O2 to H2O and MDHA (Aran et al. 2009). Alternatively, Prx can be used to convert H2O2 into H2O and alcohol to reduce the oxidative damage of H2O2 (Tripathi et al. 2009; Hong et al. 2017). Although the APX activity of WT and RNAi plants increased under HT + LL treatment compared with CK (25⍰), the APX activity of RNAi plants under HT + HL treatment decreased significantly, resulting in significantly higher H2O2 content in RNAi than WT plants under HT + HL treatment. These results indicate that 2-Cys Prx plays a crucial role in scavenging H2O2 under stress.

4. Conclusion

In this study, high light promoted the production of O2·− and H2O2 in tobacco leaves under HT stress and aggravated the damage degree of PSII and PSI activity in tobacco leaves under HT stress. The damage degree of PSII donor side was greater than that of PSII receptor side. RNA interference via 2-Cys Prx gene expression increased ROS production in tobacco leaves under HT stress, further inhibiting PSII activity. Particularly, the PSII donor side of RNAi plants was damaged, and the electron transfer of PSII receptor...
side was inhibited under HT + HL treatment. Affected by PSII function, RNAi 2-Cys Prx gene expression had little effect on the PSI activity of tobacco leaves under HT + LL or HT + HL treatments. This study provides a theoretical basis for reasonable utilization and popularization of tobacco in high temperature area, and it can provide a theoretical basis for genetic improvement of high temperature tolerance of tobacco.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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