Front. Agr. Sci. Eng. 2021, 8(2): 262–279 https://doi.org/10.15302/J-FASE-2021383

RESEARCH ARTICLE

PROTECTIVE ROLES OF D1 PROTEIN TURNOVER AND THE XANTHOPHYLL CYCLE IN TOMATO (*SOLANUM LYCOPERSICUM*) UNDER SUB-HIGH TEMPERATURE AND HIGH LIGHT STRESS

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KEYWORDS

D1 turnover, photoinhibition, photoprotection, photosynthesis, tomato, xanthophyll cycle

HIGHLIGHTS

- D1 turnover plays a more important role than xanthophyll cycle in photoprotection under sub-high temperature and high light (HH) conditions in tomato.
- D1 protein turnover and xanthophyll cycle avoided the accumulation of excess energy and photooxidative damage of photosystem through maintaining photosynthetic pigments content and inducing changes in NPQ core components.
- D1 protein turnover and xanthophyll cycle can also avoided accumulation of ROS and membrane lipid peroxidation by maintaining the activity of the ROS scavenging system.

Received August 2, 2020;
Accepted December 14, 2020.

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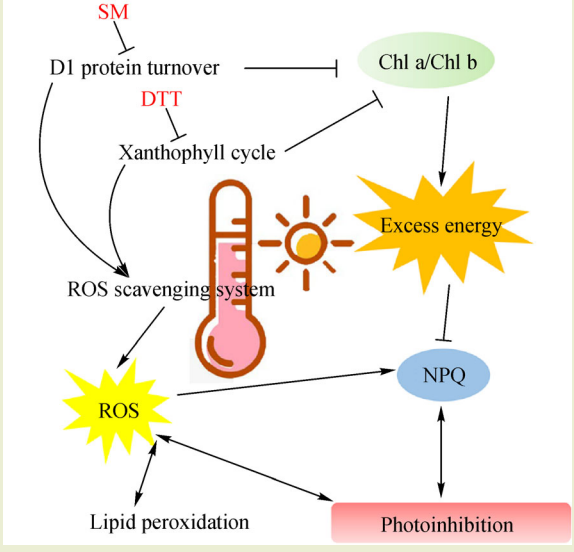
GRAPHICAL ABSTRACT



ABSTRACT

D1 protein turnover and the xanthophyll cycle (XC) are important photoprotective mechanisms in plants that operate under adverse conditions. Here, streptomycin sulfate (SM) and dithiothreitol (DTT) were used in tomato plants as inhibitors of D1 protein turnover and XC to elucidate their photoprotective impacts under sub-high temperature and high light conditions (HH, 35°C, 1000 μmol·m^{-2}·s^{-1}). SM and DTT treatments significantly reduced the net photosynthetic rate, apparent quantum efficiency, maximum photochemical efficiency, and potential activity of photosystem II, leading to photoinhibition

and a decline in plant biomass under HH. The increase in reactive oxygen species levels resulted in thylakoid membrane lipid peroxidation. In addition, there were increased non-photochemical quenching and decreased chlorophyll pigments in SM and DTT application, causing an inhibition of D1 protein production at both transcriptional and translational levels. Overall, inhibition of D1 turnover caused greater photoinhibition than XC inhibition. Additionally, the recovery levels of most photosynthesis indicators in DTT-treated plants were higher than in SM-treated plants. These findings support the view that D1 turnover has a more important role than XC in photoprotection in tomato under HH conditions.

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1 INTRODUCTION

A severe consequence of climate change is global warming with heat waves usually accompanied by high light intensity occurring globally, including many important agricultural production areas. High temperature and high light stress are major environmental stresses that can limit plant growth and development. Photosynthetic activity is sensitive to adverse environmental conditions. When the solar energy absorbed by plants exceeds their capacity, generation of excess energy disturbs several physiologic processes in the cells, including the destruction of antenna systems, oxygen evolving complex and reaction centers, as well as the denaturation of plant proteins. These result in photoinhibition or photodamage of photosystems[1]. Previous studies have shown that moderately high temperatures and strong light can enhance the tolerance of plants to photoinhibition, but high-intensity high temperatures as well as strong light can restrict the ability of plants to assimilate photosynthetic carbon by stomatal or non-stomatal factors[2]. It is well-established that photosystem II (PSII) is the primary site of photosynthetic activity, which can be destroyed by strong light and high temperatures[3]. Despite the conserved nature of PSII vulnerability, the actual photodamage mechanism varies from one crop to another, with the main discrepancy being whether photodamage is caused by the donor or acceptor side of the PSII reaction center[4].

Plants employ a series of photoprotection and photodefense mechanisms to maintain physiologic functions of PSII, including degradation and restoration of damaged D1 protein, rapid synthesis of new D1 protein, and thermal dissipation of excess energy by the xanthophyll cycle (XC)[5]. One of the major subunits of the PSII protein complex, the D1 protein, is encoded by *psbA*, a chloroplast localized gene that is the cofactor binding site and the most vulnerable part of the PSII reaction center[4,6]. D1 protein maintains the stable structure of the PSII reaction center and is related to the separation and transmission of the primary charge. Therefore, the timely degradation of damaged D1 protein is a key factor in accelerating PSII recovery and improving photosynthetic efficiency[7]. Likewise, XC has an important role in environmental stress defense. Violaxanthin is de-epoxidated to antheraxanthin by violaxanthin de-epoxidase (VDE) under excess energy. A is then further de-epoxidated to zeaxanthin, which enables the transition of PSII antenna complexes to a conformation that dissipates excess excitation energy[8]. Additionally, some studies show that XC has a protective effect on D1 protein by effectively preventing D1 protein degradation[9]. However, whether D1 protein turnover can protect XC is poorly studied. In cyanobacteria, specific details of the inactivation of D1 protein synthesis by reactive oxygen (ROS) reveals that *psbA* transcription and translation are specifically inactivated by hydrogen peroxide and singlet oxygen (H_2O_2 and $^1O_2$)[10]. ROS can be depleted by xanthophyll epoxidation reactions to ensure D1 protein synthesis and PSII renewal, but the specific mechanisms of XC during these processes remain unclear.

In addition to the above mechanisms, plants have another important strategy to dissipate excess light energy, non-photochemical quenching (NPQ), which prevents the electron transport chain from being over-reduced through thermal dissipation[11]. The induction and relaxation of NPQ is facilitated by three components: qI, qE and qT. qI is associated with zeaxanthin accumulation. Its relaxation is slow (hours or longer) and its effects are similar to some strong forms of photoinhibition (e.g., lowered F_v/F_m). In addition, D1 protein damage leads to a constant low quantum yield of PSII (i.e., photoinhibition), which can be easily measured. Therefore, D1 protein damage also has similar traits to qI[12]. qE has a rapid transformation (seconds to minutes) and depends upon the proton gradient across the thylakoid membrane (ΔpH). There are two well-characterized components that regulate qE. One is the accumulation of XC carotenoid zeaxanthin, which is associated with the development of qE[13]. The other is the PsbS protein of PSII, whose role in qE was found by screening *Arabidopsis* mutant populations for altered

chlorophyll fluorescence quenching[11,12]. Lastly, qT has recently been shown to have an induction and reversion profile on a timescale of tens of minutes. In addition to its correlation with zeaxanthin synthesis and removal, qT may also be associated with chloroplast pigment antenna[14]. Rapid relaxation of qE is usually the primary mechanism of NPQ under conditions of rapid plant growth, although situations exist when qT and qI become more prominent[15]. Changes in NPQ components are often an indication that light intensity is no longer ideal for plant growth.

Streptomycin sulfate (SM) suppresses the synthesis of chloroplast proteins and can be used as an inhibitor of D1 protein synthesis and turnover[16]. Dithiothreitol (DTT) is a specific inhibitor for VDE that inhibits the conversion of violaxanthin to zeaxanthin, and can be used as an inhibitor of XC[17]. Tomato (*Solanum lycopersicum*) is commonly cultivated in northern China and always faces high temperature and high light intensity stresses hindering its growth and development in long season of cultivation over the summer. Here, SM and DTT were used to compare the effects of D1 protein turnover and XC to PSII function in tomato leaves under sub-high temperature and high light stress (HH). Our study reveals the possible functions of D1 turnover and XC in the prevention of photoinhibition of tomato seedlings under HH. This work has implications for high production cultivation and the creation of stress-tolerant tomato cultivars.

2 MATERIALS AND METHODS

2.1 Plant material and inhibitor treatment

Tomato cv. Liaoyuanduoli (a popular cultivar in North-eastern China) was cultivated in pots (12 cm × 12 cm) in a greenhouse without supplementary illumination and with regular cultivation management. Tomato seedlings at the six-leaf stage were separated into four groups of 40 pots each. The first and second groups were sprayed with an equivalent volume of distilled water and the second group treated in HH (35°C, 1000 μmol·m^{-2}·s^{-1}). The third and fourth groups were sprayed with 3 mmol·L^{-1} SM and 10 mmol·L^{-1} DTT under HH stress. Each seedling received about 35 mL. The control plants were cultivated under normal conditions (25°C, 500 μmol·m^{-2}·s^{-1}). Treated plants were returned to control conditions after 5 d of stress treatment.

The fourth fully expanded functional leaves were collected for physiologic and biochemical analysis 0, 1, 3 and 5 d after treatment as well as 5 and 10 d after recovery. Six replicates were selected for each treatment, with at least three plants included in each sample.

2.2 Measurement of chlorophyll fluorescence and photosynthesis

Chlorophyll fluorescence, P700 redox state and gas exchange were synchronously measured by DUAL-PAM-100 and GFS-3000 (Heinz Walz, Effeltrich, Germany), as described by Yamori et al.[18]. To obtain photosynthetic light-response curves, the net photosynthetic rate (Pn) under different photosynthetic photon flux densities (PPFDs) was recorded after light adap-tation of 3 min. PPFDs varied from 13 to 1287 μmol·m^{-2}·s^{-1}. Linear regression of the Pn-photoresponse curves during low light stage were generated and the slopes were identified as the apparent quantum yield (AQY). The seedlings were dark-adapted for 20 min before measurement and the determinations were made at 400±10 μmol·m^{-2}·s^{-1} CO_2 concentration.

2.3 Microscopic morphology of tomato leaves

To observe microstructures of the tomato leaves, the leaf epidermis was sliced and removed from the back of the leaf blade. The slice was placed upside down on an Axio Observer A1 inverted fluorescence microscope (Zeiss International, Hallbergmoos, Germany) for observation and photography. Enumeration and measurement of stomatal parameters were conducted at 20 and 40 times magnification using Zen 2012 software blue edition (Zeiss International).

2.4 Determination of non-photochemical quenching components

NPQ and its three components, qE, qT, and qI, were measured according to Horton and Hague[5]. First, F_m was measured in dark-adapted plants as A. Then, the actinic light with continuous and appropriate supersaturated light (~ 1200 μmol·m^{-2}·s^{-1}) was turned on and F_m' was measured by giving a saturation pulse light every 2 min. F_m' was measured six times before the actinic light was turned off and the stationary state of F_m' was used as B. Finally, under dark conditions, the recovery value of F_m' was measured six times while giving a saturation pulse of light every 2 min. The 6th min of F_m' was used as C and the final F_m' was used as D. NPQ components were calculated as: qE = C − B, qT = D − C, and qI = A − D.

2.5 Protein extraction, electrophoresis and western blot analysis

Total protein in tomato leaves was extracted according to the methods of Wittenberg et al.[19] and Lu et al.[3]. Extraction was done with 80% (v/v) buffered acetone to measure the total chlorophyll concentration using the formula C (mg·L^{-1}) = 7.12 ×

A_{660} + 16.8 × $A_{642.5}$. The membrane suspension containing 100 μg chlorophyll was then centrifuged for 5 min at 3000 × *g*. The pellet was homogenized in protein sample buffer [2% SDS (v/v), 100 mmol·L^{-1} DTT, 5 mmol·L^{-1} Tris-Cl, pH 6.8, 5% glycerin (v/v), and 0.05% bromophenol blue (w/v)] to obtain a 0.5 mg·L^{-1} chlorophyll concentration. Before loading onto the gel, the samples were incubated at 95°C for 5 min. SDS-PAGE (4% stacking gels and 15% resolving gels with 10% SDS) was used to separate the protein samples. When electrophoresis was complete, PVDF membrane (Millipore, Molsheim, France) and 5% skimmed milk were used to transfer and block the proteins, respectively. The membrane was then incubated with anti-D1 protein antibody (Agrisera, Vännäs, Sweden). With the help of an immunoblot imaging system (Azure Diosystems c600, Dublin, CA), the signals were probed using a western blotting detection kit (Tiangen, Beijing, China). D1 protein quantification was made with a laboratory imaging system (Bio-Rad Laboratories, Hercules, CA).

2.6 Total RNA extraction and RT-qPCR analysis

A commercial RNA extraction kit (Tiangen, Beijing, China) was used to extract total RNA from tomato leaves. The RNA samples were reverse transcribed into cDNAs which were then used as a template for real-time fluorescence quantitative PCR amplification using a SYBR Premix Ex Taq kit (TaKaRa, Tokyo, Japan). The ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) and Software 7500 V2.0.6 were used in RT-PCR and gene expression analysis. Primer Express 5.0 was used to design primers (Table 1). RT-PCR was run on three replicates.

2.7 Determination of chlorophyll content and xanthophyll cycle pigments

Total chlorophyll content is proportional to antenna size, while the ratio of Chl a/Chl b indicates the composition of reaction centers and antenna proteins. Measurement of chlorophyll content was adapted from a previous protocol with some modifications[20]. A UV-visible spectrophotometer (Beckman, Brea, CA) was used to measure the absorbance at 663 and 645 nm. Chlorophyll concentrations were calculated as Ca = 12.21 × A_{663} − 2.81 × A_{645} and Cb = 20.13 × A_{645} − 5.03 × A_{663}. Six replicates of each treatment were analyzed.

The HPLC analysis of XC compositions was done as previously described[11]. Solvent A (acetonitrile:ethyl acetate 85:15) was used for sample elution for 14.5 min, followed by a linear gradient to solvent B (methanol:ethylacetate 68:32) for 2 min, which continued isocratically until separation at 30 min. The C18 column (5 μm particle size, 4.6 mm × 250 mm; Waters Corporation, Milford, MA) was re-equilibrated for 10 min in solvent A before injection. The flow rate and column temperature were set at 5 mL·min^{-1} and 30°C, respectively. The pigments were detected at 445 nm of their absorbance and analyzed using a Waters 2695 (Alliance HPLC System, Waters Corporation). Peak areas were calculated with Empower 3.

2.8 Measurement of malondialdehyde and H_2O_2

We detected the content of malondialdehyde (MDA) with an MDA assay kit (Jiancheng, Nanjing, China). H_2O_2 estimation in leaves was conducted according to the instructions of the H_2O_2

Table 1 Response gene accession numbers and primer sequences of the genes described here

Category	Accession	Primer sequence (5'–3')
$_{(Cu/Zn)}$SOD	AF034411.1	F 5'-ACCAGCACTACCAATTCTTTCT-3' R 5'-GGGGTTTAGGGGTAGTGACA-3'
$_{(Mn)}$SOD	M37151.1	F 5'-GGCACCTACCTCTTCACTCA-3' R 5'-GGATTGTAATGTGGTCCTGTTGA-3'
APX	AF413573.1	F 5'-ATGACGCGGGGACTTACAA-3' R 5'-GGCTGGAGAAGTTTCAGTGC-3'
PsbA	AY568719.1	F 5'-TGCTCATAACTTCCCTCTAGACC-3' R 5'-AGCACCCTCTTGACAGAACA-3'
PsbS	U04336.1	F 5'-TGTTCCTACCTTCTCTTCCTTTG-3' R 5'-ATTGAAACAGAGCGAGAGAGT-3'
Actin	Q96483	F 5'-TGTCCCTATTTACGAGGGTTATGC-3' R 5'-AGTTAA ATCACGACCAGCAAGAT-3'

assay kit. The supernatant was read at 405 nm against a blank ELISA plate using an Infinite M200 Pro spectrophotometer (Tecan, Männedorf, Switzerland).

2.9 Histochemical staining and *in situ* localization of H_2O_2 and O_2^-

3,3'-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) were used for the histochemical staining of H_2O_2 and O_2^-, respectively, as described in Gong et al.[21]. For the *in situ* localization of H_2O_2, 2',7'-dichlorofluorescin diacetate (DCFH-DA), a highly sensitive and a cell-permeable probe, was used as reported by Capone et al.[22]. In the case of O_2^-, dihydroethidium (DHE) was used as superoxide free radical as described by Liu et al.[23]. An Axio Observer A1 system (Zeiss International) was used to capture images with standard filters and collection modalities for DCFH-DA green fluorescence (emission 525 nm and excitation 488 nm) and for DHE yellow fluorescence (emission 525 nm and excitation 515 nm).

2.10 Antioxidant enzyme activities

The activities of total antioxidant (T-AOC), total superoxide dismutase (T-SOD), and catalase (CAT) were measured using applicable kits (Solarbio, Beijing, China). The supernatants were read at 520 nm, 550 nm, 405 nm and 420 nm against a blank ELISA plate using an Infinite M200 Pro spectrophotometer (Tecan, Männedorf, Switzerland).

2.11 Data analysis and visualization

Quantitative assessment was conducted on randomly selected samples. The data are the mean±standard deviation of five replicates. The data were statistically analyzed using SPSS 20 Software (IBM SPSS STATISTICS, IBM Armonk, NY, USA) by analysis of variance. Statistically significant difference was set at a probability level of 0.05. The figures were drawn with Origin 9.0 Software (Origin Laboratory, Northampton, MA).

3 RESULTS

3.1 Photosynthetic rate and apparent quantum efficiency in response to SM and DTT under HH

The Pn-light-response curves of HH treatments increased more slowly than those of the control group. As the illumination gradient increased, the values of the curves peaked at significantly lower values, especially under moderate and high light gradients. Moreover, the AQY decreased markedly with DTT and SM treatments (Fig. 1(a)). After recovering under normal conditions, the AQY of H_2O, DTT, and SM treatments were 74%, 54% and 50%, respectively. These are similar to the recovery trends of Pn-light-response curves (Fig. 1(b)). DTT and SM treatments significantly intensified the reduction of Pn. Additionally, the Pn recovery rates of DTT and SM were 53% and 40%, respectively, and were significantly lower than that of the control (Fig. 1(c)). Since the large decline in Pn was followed by AQY, we hypothesized that this was caused by photoinhibition. DTT and SM impaired the resistance of the plant to HH and the ability to use low light in tomato leaves. Moreover, once D1 turnover or XC was disturbed the adverse effect was persistent and the pigment protein complex, which absorbs and converts light energy, could not recover well.

3.2 Changes in guard cells and stomata in response to SM and DTT under HH

H_2O-treated leaves under HH were 7.2% shorter and 10.6% narrower, and the guard cells 16.2% smaller in area. Stomata were 21.1% narrower and 13.9% smaller in area, but 8.7% longer (Table 2). Compared with H_2O, DTT and SM greatly intensified the changes in the above parameters. Moreover, SM and DTT treatment lead to a significant decrease in stoma quantity. During the recovery process, most parameters returned to normal, with the exception of stoma and guard cell area in SM- and DTT-treated plants. These results indicate that HH reduced the area of stoma primarily by decreasing stomatal openings.

3.3 Changes in photosystem quantum yield, photochemical activity and chlorophyll fluorescence in response to SM and DTT under HH

We studied the photosynthetic properties of tomato to elucidate the possible protective effects of D1 turnover and XC on PSII. F_v/F_o was recorded at different times in order to determine F_v/F_m (Fig. 1(a,b)). On day 0, the values of F_v/F_m were close to 0.82 but decreased upon light exposure, which is consistent with previous studies[24]. F_v/F_m and F_v/F_o both decreased faster and reached lower values in SM group than DTT- and H_2O-treated plants. In addition, the decrease in F_v/F_m and F_v/F_o was mostly due to an increase in F_o and a decrease in F_m (Fig. 1(c)). Although Y(NPQ) and Y(NO) increased significantly under HH compared with H_2O-treated plants, Y(NPQ) and Y(NO) of SM group were significantly higher, but no significant differences in Y(NO) were found between H_2O- and DTT-treated plants. Chlorophyll content changes were similar to F_m and the percentage of Chl a increased significantly with DTT and SM (Fig. 3(a)). Likewise, Y (II) and qP of the HH treatment groups were significantly lower than the control, especially SM and DTT treatments (Fig. 2(c)

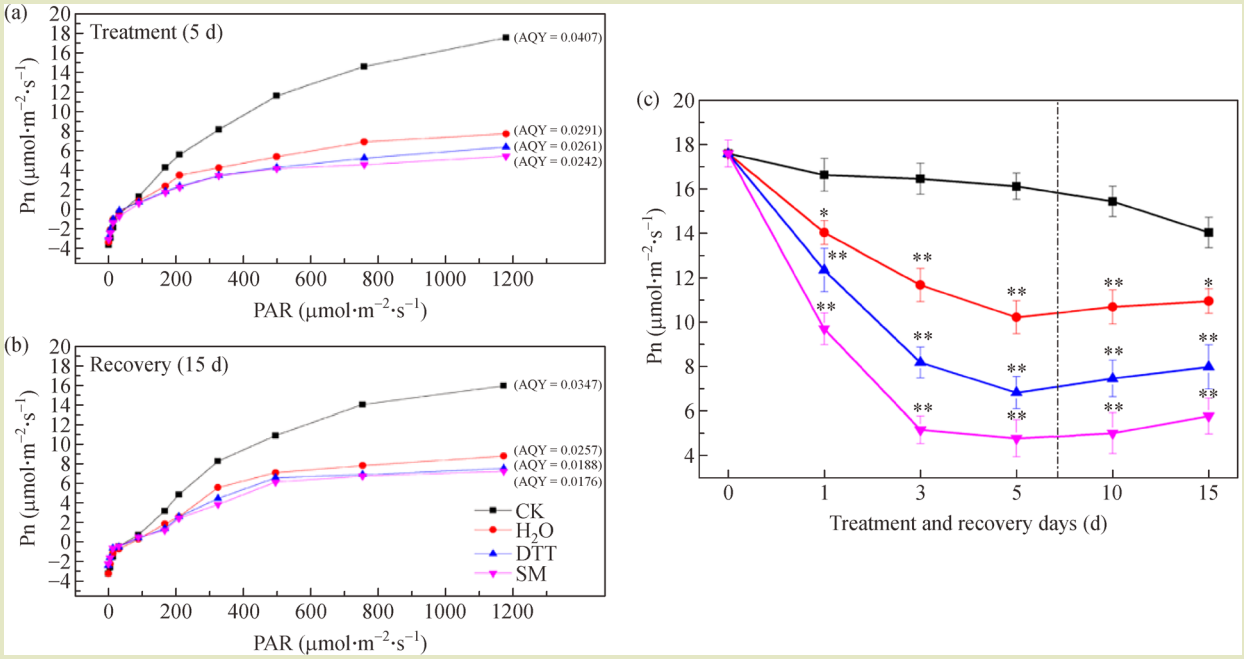

Fig. 1 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment (a) and recovery (b) on the photosynthetic rate (Pn) and apparent quantum efficiency (AQY) of tomato seedlings under sub-high temperature and high light (HH) stress (c). CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. The left side of the dotted line shows different treatments and the other side shows the treatments of recovery. Data are mean values of five replicates with standard errors shown as vertical bars. *, significant difference ($P \leqslant 0.05$); and **, highly significant difference ($P \leqslant 0.01$).

Table 2 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on guard cells and stomata in tomato leaves under sub-high temperature and high light stress.

Time (d)	Treatment	Guard cell				Stoma					
		Length (μm)	Width (μm)	Area (μm²)	L/W	Length (μm)	Width (μm)	Area (μm²)	L/W	A_S/A_G	Quantity
0	Control	24.80	18.28	357.10	1.36	10.78	3.40	28.80	3.19	0.083	36
1	SM	22.04^{cB}	13.36^{bB}	231.34^{bB}	1.65^{bA}	11.73^{aAB}	3.06^{aA}	28.31^{aA}	4.16^{aA}	0.123^{bA}	20^{cB}
	DTT	23.73^{abAB}	15.16^{bAB}	284.45^{bB}	1.59^{abA}	13.09^{bB}	3.05^{aA}	31.38^{aA}	4.42^{aA}	0.112^{abA}	27^{bB}
	H_2O	23.12^{bcAB}	14.94^{bAB}	271.05^{bB}	1.56^{abA}	12.78^{bB}	3.25^{aA}	32.79^{aA}	3.94^{aA}	0.121^{bA}	26^{bB}
	CK	24.85^{aA}	18.62^{aA}	362.22^{aA}	1.36^{aA}	11.01^{aA}	3.43^{aA}	29.71^{aA}	3.22^{aA}	0.083^{aA}	33^{aA}
3	SM	21.98^{bB}	16.95^{abA}	291.96^{bAB}	1.33^{aA}	12.81^{bB}	2.93^{aA}	29.62^{aA}	4.46^{aA}	0.104^{aA}	21^{bA}
	DTT	22.24^{bB}	15.23^{bA}	266.22^{bB}	1.47^{aA}	11.79^{abAB}	2.87^{aA}	26.69^{aA}	4.20^{aA}	0.102^{aA}	24^{abA}
	H_2O	23.13^{bAB}	16.58^{abA}	301.36^{bAB}	1.40^{aA}	11.45^{abAB}	3.14^{aA}	28.04^{aA}	3.80^{aA}	0.093^{aA}	26^{abA}
	CK	24.54^{aA}	18.59^{aA}	356.75^{aA}	1.35^{aA}	10.82^{aA}	3.43^{aA}	28.89^{aA}	3.80^{aA}	0.083^{aA}	28^{aA}
5	SM	21.01^{aA}	16.99^{aA}	280.45^{bA}	1.24^{aA}	11.55^{bA}	2.64^{bB}	24.50^{bA}	4.48^{cC}	0.088^{aA}	21^{bB}
	DTT	22.52^{aA}	16.75^{aA}	298.90^{abA}	1.35^{aA}	11.97^{abA}	2.62^{bB}	24.92^{bA}	3.83^{cC}	0.084^{aA}	21^{bB}
	H_2O	22.68^{aA}	16.52^{aA}	294.36^{abA}	1.38^{aA}	11.35^{abA}	3.06^{bB}	27.35^{abA}	3.72^{bB}	0.094^{aA}	28^{aAB}
	CK	24.43^{aA}	18.47^{aA}	350.51^{aA}	1.36^{aA}	10.44^{aA}	3.88^{aA}	31.77^{aA}	3.22^{aA}	0.093^{aA}	26^{aA}
10	SM Recovery	23.41^{bB}	16.77^{bA}	309.20^{bB}	1.40^{aA}	12.69^{bA}	2.95^{aA}	29.27^{aA}	4.38^{abA}	0.096^{aA}	21^{aA}
	DTT Recovery	24.47^{bAB}	17.85^{abA}	342.09^{bAB}	1.38^{aA}	13.04^{abA}	2.91^{aA}	29.73^{aA}	5.08^{bA}	0.088^{aA}	19^{aA}

(*Continued*)

Time (d)	Treatment	Guard cell				Stoma					
		Length (μm)	Width (μm)	Area (μm^2)	L/W	Length (μm)	Width (μm)	Area (μm^2)	L/W	A_S/A_G	Quantity
	H_2O Recovery	24.90^{abAB}	16.74^{bA}	327.40^{bA}	1.49^{aA}	12.58^{abA}	3.66^{aA}	36.09^{aA}	3.48^{abA}	0.111^{aA}	22^{aA}
	CK Recovery	26.40^{aA}	18.80^{aA}	390.16^{aA}	1.45^{aA}	11.38^{aA}	3.73^{aA}	33.32^{aA}	3.05^{aA}	0.086^{aA}	21^{aA}
15	SM Recovery	23.34^{aA}	17.29^{aA}	316.62^{bA}	1.35^{aA}	12.29^{aA}	3.56^{bB}	34.36^{bA}	3.46^{aAB}	0.109^{aA}	13^{bB}
	DTT Recovery	23.42^{aA}	17.91^{aA}	328.77^{bA}	1.31^{aA}	12.64^{aA}	3.04^{cC}	30.09^{bA}	4.18^{bB}	0.092^{aA}	13^{bB}
	H_2O Recovery	23.43^{aA}	17.91^{aA}	330.86^{bA}	1.32^{aA}	12.09^{aA}	3.64^{bAB}	34.68^{bA}	3.32^{aA}	0.107^{aA}	24^{aA}
	CK Recovery	25.89^{aA}	18.65^{aA}	379.52^{aA}	1.39^{aA}	11.56^{aA}	4.00^{aA}	36.43^{aA}	2.89^{aA}	0.097^{aA}	21^{aA}

Note: Lowercase letters indicate the significance of differences between different treatments on the same day ($P \leqslant 0.05$); capital letters indicates highly significant differences ($P \leqslant 0.01$).

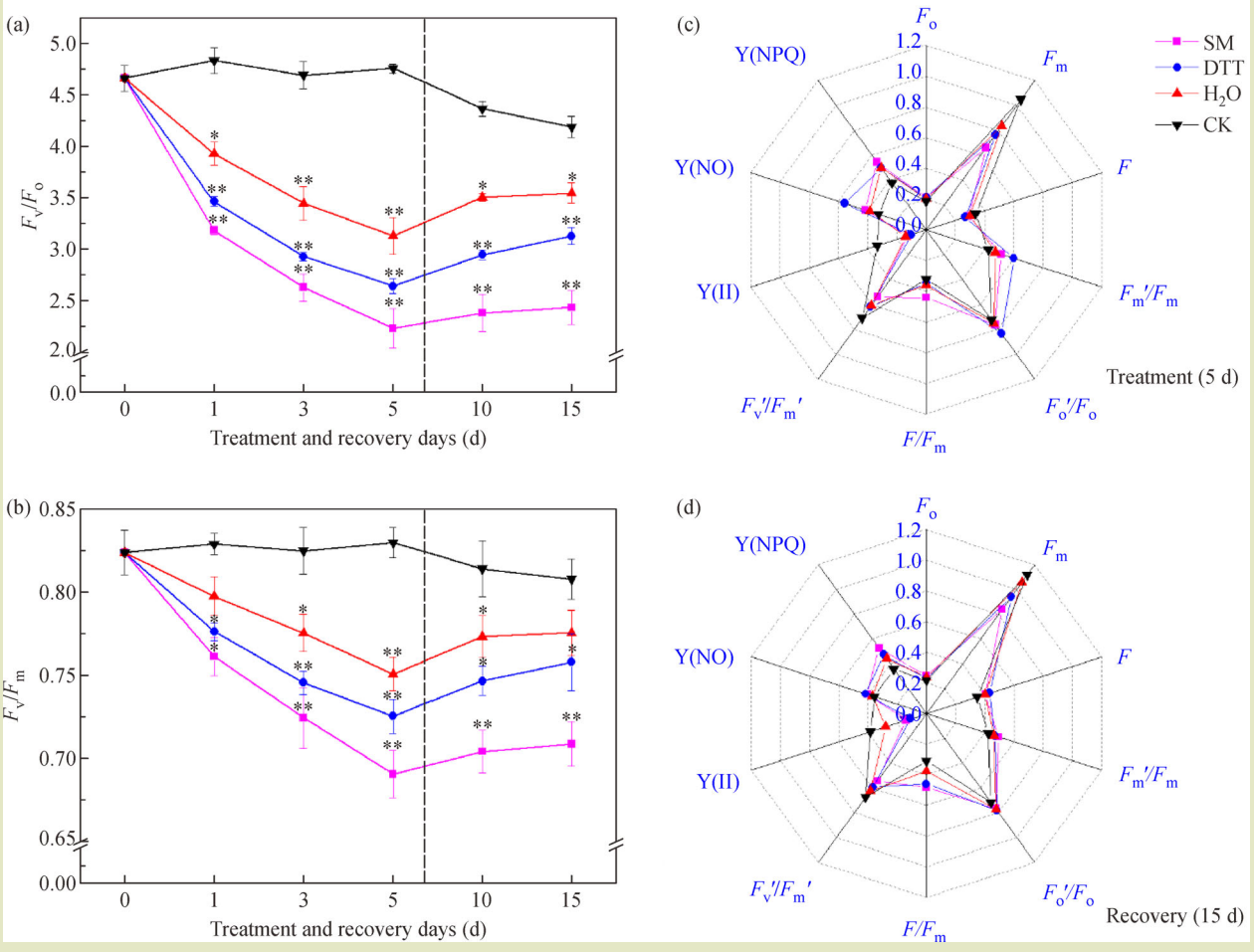

Fig. 2 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on maximum photochemical efficiency (F_v/F_m) (a), potential activities of PS II (F_v/F_o) (b), chlorophyll fluorescence parameters [F_o, the initial fluorescence; F_m, the maximal fluorescence; F, the steady-state fluorescence; F_v'/F_m', excitation efficiency capture by open PS II centers; Y(II), efficient quantum yield of PS II;Y(NPQ), yield of unregulated energy dissipation of PS II; Y(NO), yield of regulated energy dissipation of PS II; F_m'/F_m, ratio of F_m' and F_m; F_o'/F_o, ratio of F_o' and F_o; F/F_m, ratio of F and F_m] (c,d) of tomato leaves under HH. CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. The left side of the dotted line shows different treatments and the other side shows the treatments of recovery. Data are mean values of five replicates with standard errors shown as vertical bars. *, significant difference ($P \leqslant 0.05$); and **, highly significant difference ($P \leqslant 0.01$).

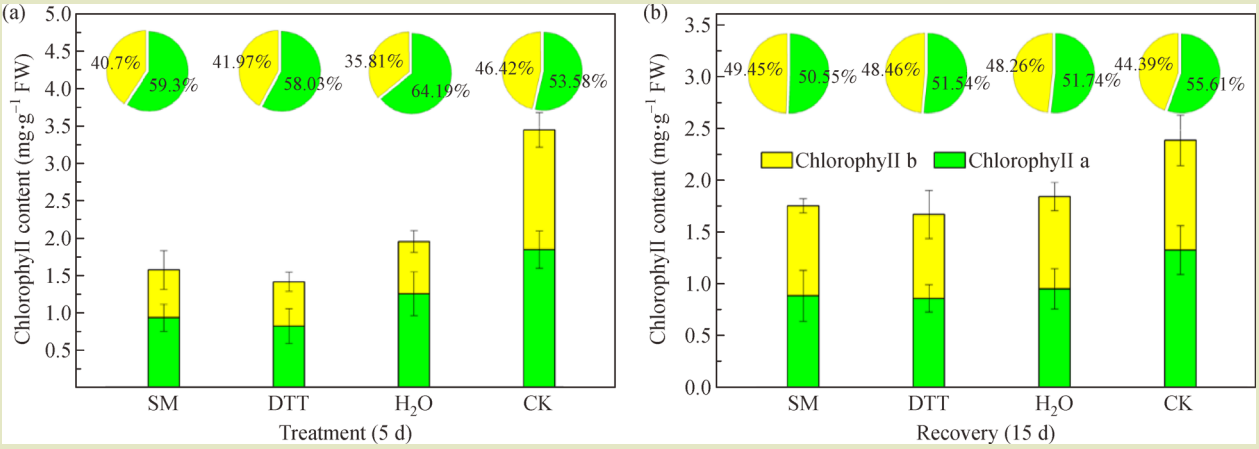

Fig. 3 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on chlorophyll content and proportions of chlorophyll a and chlorophyll b in tomato leaves under sub-high temperature and high light stress. CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. Data are mean values of five replicates with standard errors shown as vertical bars.

Table 3 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on the photochemical quenching coefficient (qP) and non-photochemical quenching coefficient (qN) in tomato seedlings under sub-high temperature and high light stress.

Time (d)	Treatment	qP	qN
0	Control	0.54±0.02	0.62±0.04
1	SM	0.42 ± 0.11^{aA}	0.54 ± 0.03^{bB}
	DTT	0.49 ± 0.04^{aA}	0.54 ± 0.09^{bB}
	H_2O	0.50 ± 0.03^{aA}	0.61 ± 0.01^{aA}
	CK	0.52 ± 0.04^{aA}	0.64 ± 0.07^{aA}
3	SM	0.38 ± 0.11^{bA}	0.52 ± 0.11^{bB}
	DTT	0.39 ± 0.05^{bA}	0.55 ± 0.09^{bB}
	H_2O	0.48 ± 0.09^{abA}	0.60 ± 0.03^{aA}
	CK	0.57 ± 0.03^{aA}	0.62 ± 0.07^{aA}
5	SM	0.33 ± 0.06^{bA}	0.50 ± 0.04^{bB}
	DTT	0.32 ± 0.07^{bA}	0.53 ± 0.05^{bB}
	H_2O	0.36 ± 0.05^{bA}	0.58 ± 0.02^{aA}
	CK	0.58 ± 0.11^{aA}	0.62 ± 0.04^{aA}
10	SM Recovery	0.32 ± 0.11^{bA}	0.58 ± 0.04^{aA}
	DTT Recovery	0.31 ± 0.05^{bA}	0.57 ± 0.06^{aA}
	H_2O Recovery	0.31 ± 0.17^{bA}	0.63 ± 0.01^{aA}
	CK Recovery	0.59 ± 0.07^{aA}	0.62 ± 0.03^{aA}
15	SM Recovery	0.28 ± 0.08^{bAB}	0.60 ± 0.04^{bA}
	DTT Recovery	0.19 ± 0.03^{bB}	0.60 ± 0.05^{abA}
	H_2O Recovery	0.29 ± 0.08^{bAB}	0.63 ± 0.03^{aA}
	CK Recovery	0.57 ± 0.16^{aA}	0.62 ± 0.03^{aA}

Note: Lowercase letters indicate the significance of differences between different treatments on the same day ($P \leqslant 0.05$); capital letters indicate highly significant differences ($P \leqslant 0.01$).

and Table 3). During the recovery phase, significant recoveries of F_v/F_m and F_v/F_o were observed in H_2O-treated plants, whereas the recovery was lower in both SM- and DTT-treated plants (Fig. 2(a,b)). In addition, Y(II) and qP barely recovered in SM- and DTT-treated plants, respectively (Fig. 2(d) and Table 3). As Y(II) is the product of F_v'/F_m' multiplied by qP, our findings indicate that the significant changes in Y(II) for SM and DTT were mainly due to the changes in qP. D1 protein turnover and XC can help tomato seedlings recover from photodamage, but once they are inhibited, irreversible photodamage of PSII leads to permanent loss of the PSII reaction center.

3.4 Chlorophyll content, components of xanthophyll cycle, non-photochemical quenching, and D1 protein turnover in response to SM and DTT under HH

Under HH stress, compared with the control, SM and DTT treatments reduced the total chlorophyll of tomato leaves by 54% and 57%, respectively; while Chl a/Chl b increased by 27% and 20%. After 15 d of recovery the total chlorophyll content of the plants in the SM and DTT treatment groups increased slightly, and Chl a/Chl b was close to unity (Fig. 3).

NPQ includes the dissipation of heat from excess energy absorption, which surpasses the capacity of downstream metabolic reactions. Under HH stress conditions, NPQ values were higher than the control, and qI, qE and qT changed significantly. In addition, SM and DTT treatment intensified the increase in qI portion of NPQ (Fig. 4(a)). Likewise, D1 protein was significantly inhibited at both transcriptional and translational levels and tended to decrease with qI (Fig. 5(a) and Fig. 6). D1 protein damage led to a reduction in PSII quantum yield (i.e., photoinhibition) and thus exhibited similar characteristics to the qI type of NPQ. Our results indicate that the obvious changes in qI were mainly due to the changes in D1 protein turnover.

qE in SM- and DTT-treated plants was significantly lower than the control (Fig. 4(a)). In addition, SM caused a significant increase in the accumulation of antheraxanthin and zeaxanthin but inhibited the generation of violaxanthin (Fig. 7). However, as DTT suppressed the activity of VDE, the contents of violaxanthin and antheraxanthin markedly increased, and the synthesis of zeaxanthin was turned off. Likewise, the gene expression of PSII protein PsbS was significantly decreased by SM and DTT under HH (Fig. 5(b)).

qT is correlated with the synthesis and removal of zeaxanthin, and may also be associated with the chloroplast pigment antenna[25]. qT values were significantly decreased under HH. SM and DTT caused a loss of 74% and 72%, respectively (Fig. 4(a)), while chlorophyll levels decreased in a similar manner to qT (Fig. 3(a)). Thus, by inhibiting the synthesis of chloroplast pigment antenna and the accumulation of zeaxanthin, SM led to a reduction in qT. DTT inhibited qT by impeding both of the above mechanisms.

3.5 Thylakoid membrane, lipid peroxidation and reactive oxygen species in response to SM and DTT under HH

The histochemical observations of H_2O_2 and O_2^- detection in

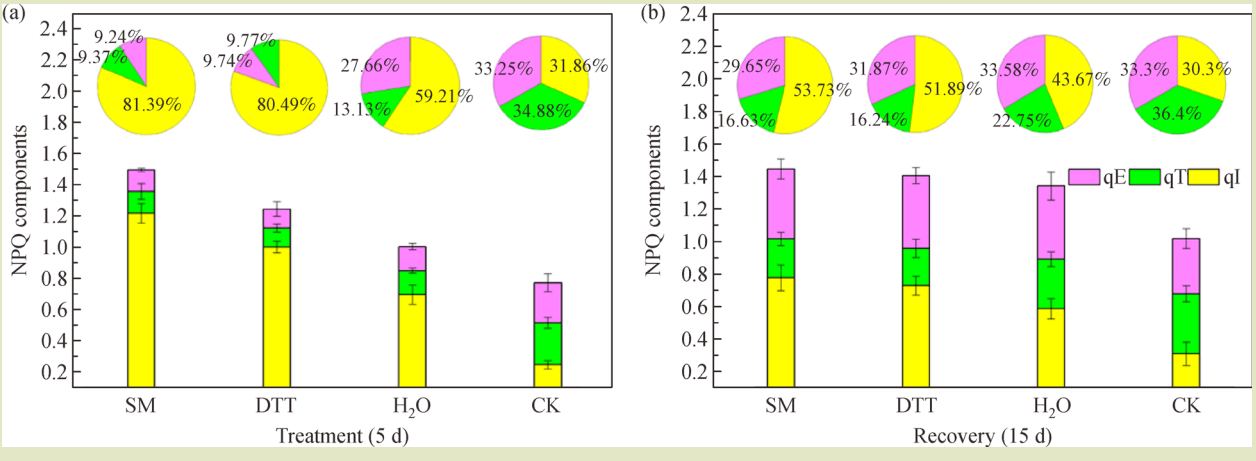

Fig. 4 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on transition state of tomato leaves under sub-high temperature and high light stress. CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. Data are mean values of five replicates with standard errors shown as vertical bars.

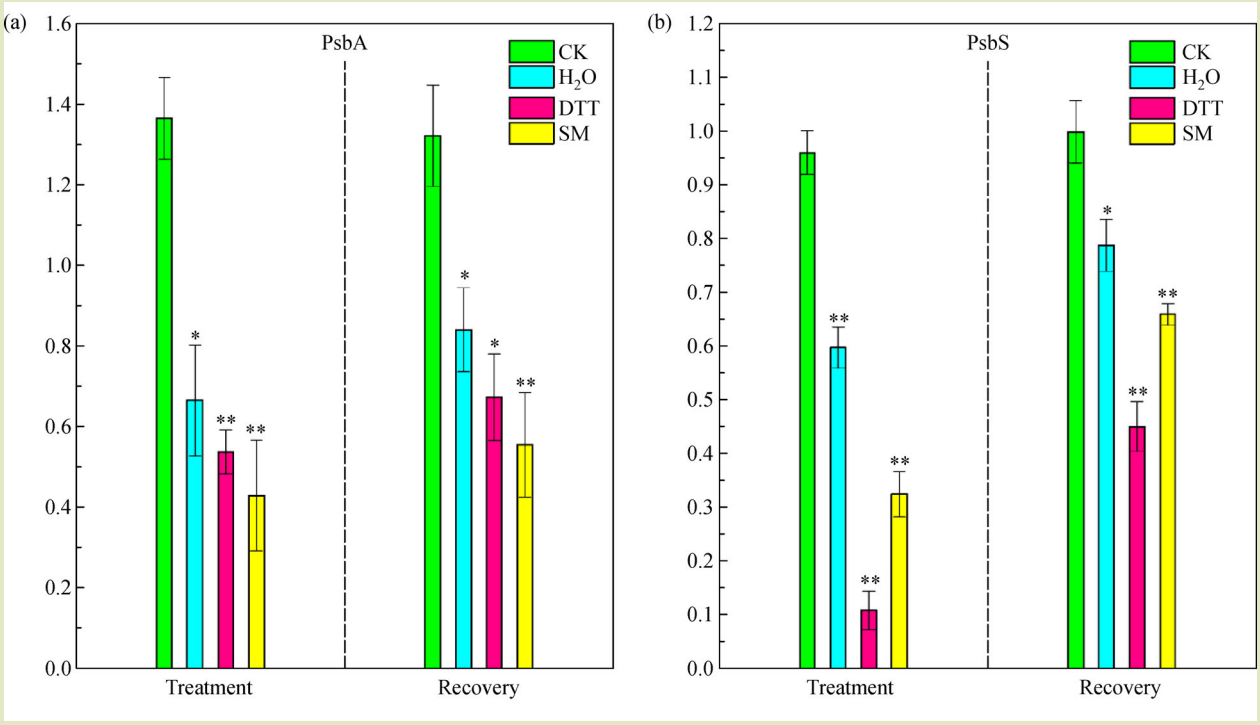

Fig. 5 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on PS II reaction center protein related gene expressions in tomato leaves under sub-high temperature and high light. CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. The left side of the dotted line shows different treatments and the other side shows the treatments of recovery. Data are mean values of five replicates with standard errors shown as vertical bars. *, significant difference ($P \leqslant 0.05$); and **, highly significant diffe-rence ($P \leqslant 0.01$).

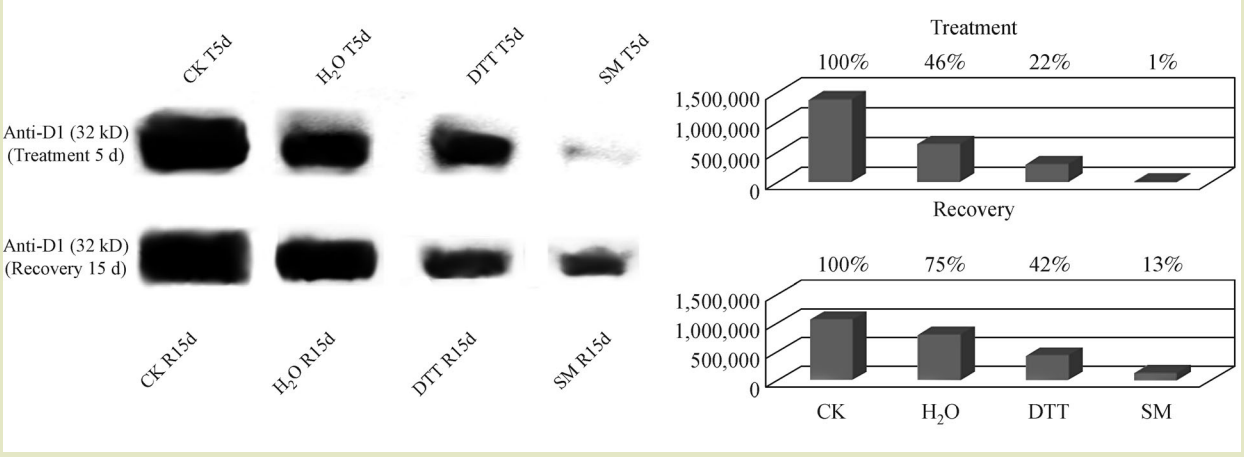

Fig. 6 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on the amount of D1 protein in thylakoids in tomato leaves under sub-high temperature and high light stress. Amounts of D1 were determined by immune-blotting with specific D1-N-terminal antibodies (32 kD). Relative densitometric values of D1 are presented on the right. CK T5d and CK R15d samples represent 100%. CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH.

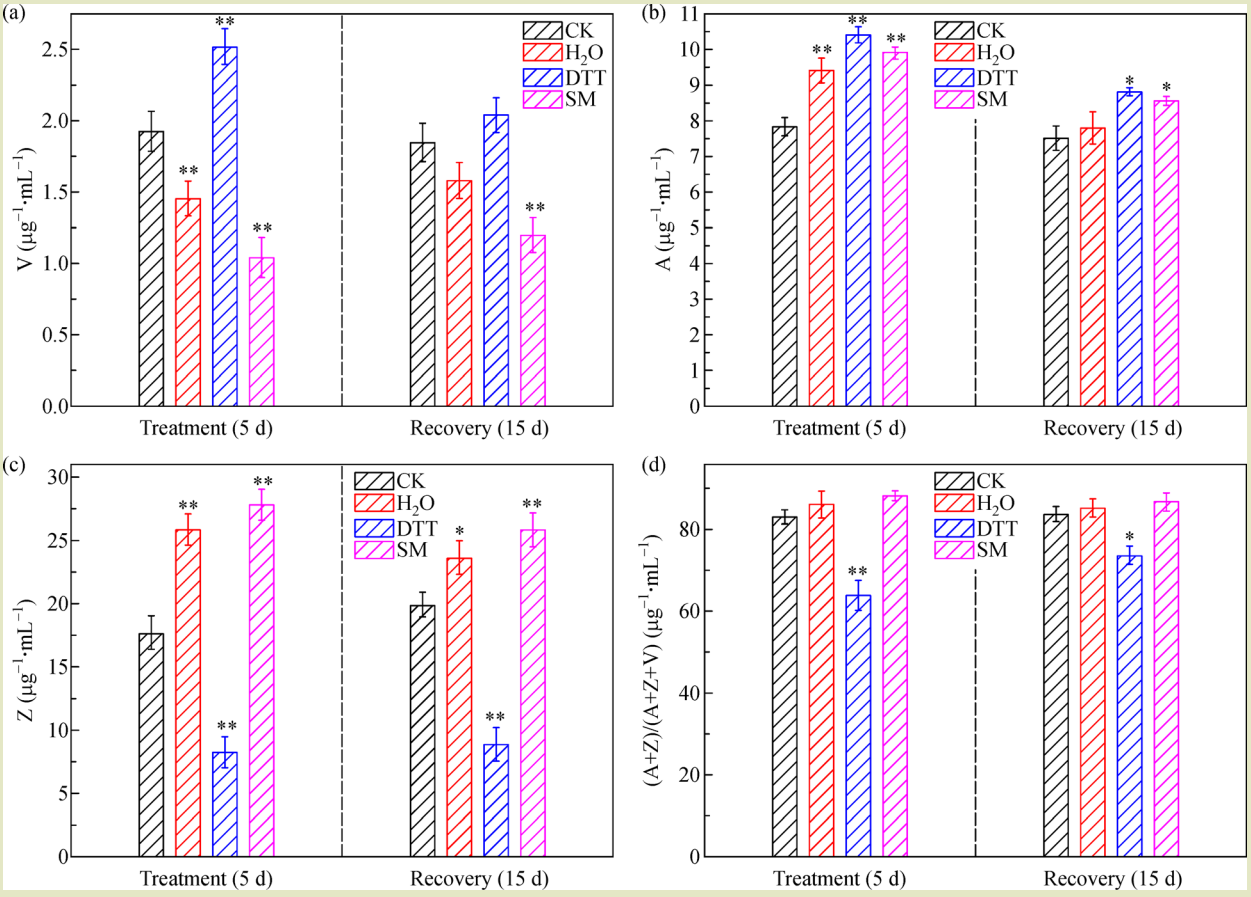

Fig. 7 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on xanthophyll cycle in tomato leaves under sub-high temperature and high light stress. V, Violaxanthin; A, Antheraxanthin; Z, Zeaxanthin; CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. The left side of the dotted line shows different treatments and the other side shows the treatments of recovery. Data are mean values of five replicates with standard errors shown as vertical bars. *, significant difference ($P \leqslant 0.05$); and **, highly sig-nificant difference ($P \leqslant 0.01$).

leaves with DAB and NBT staining were in agreement with fluorescence labeling observations for detecting guard cell changes with cell-permeative probes DCFH-DA and DHE. A marked increase in the fluorescence intensity of H_2O_2 and O_2^- production in SM- and DTT-stressed chloroplasts of stomatal cells was observed compared to the control after 5 d (Fig. 8(a–d)). Likewise, the H_2O_2 contents of H_2O-treated plants under HH were significantly higher than the control, and the accumulation of H_2O_2 was exacerbated by the application of SM and DTT to HH-treated plants (Fig. 8(e)).

There are several ROS-scavenging enzymes in plants, including SOD, POD, CAT and APX. DTT and SM treatment significantly inhibited the activities of AOC, SOD, CAT and POD (Fig. 9). At the same time, the gene expressions of *APX* and *$_{(Cu/Zn)}$SOD* were strongly correlated with their enzymatic activities (Fig. 10). Both SM- and DTT-treated plants had H_2O_2 and MDA levels that did not return to those seen in the control even after a 10-d recovery period (Fig. 11 and Fig. 8(e)). Additionally, *$_{(Cu/Zn)}$SOD* and *APX* expression levels of SM-treated plants recovered to only 45% and 73%, respectively. However, the gene expression levels of DTT-treated plants recovered up to 64% and 81%, respectively (Fig. 10).

4 DISCUSSION

4.1 Effects of D1 turnover and xanthophyll cycle on the photoprotection of photosystem under HH

Leaf photosynthesis is substantially affected, often lethally, by high temperatures and high light stress, as plants are not capable

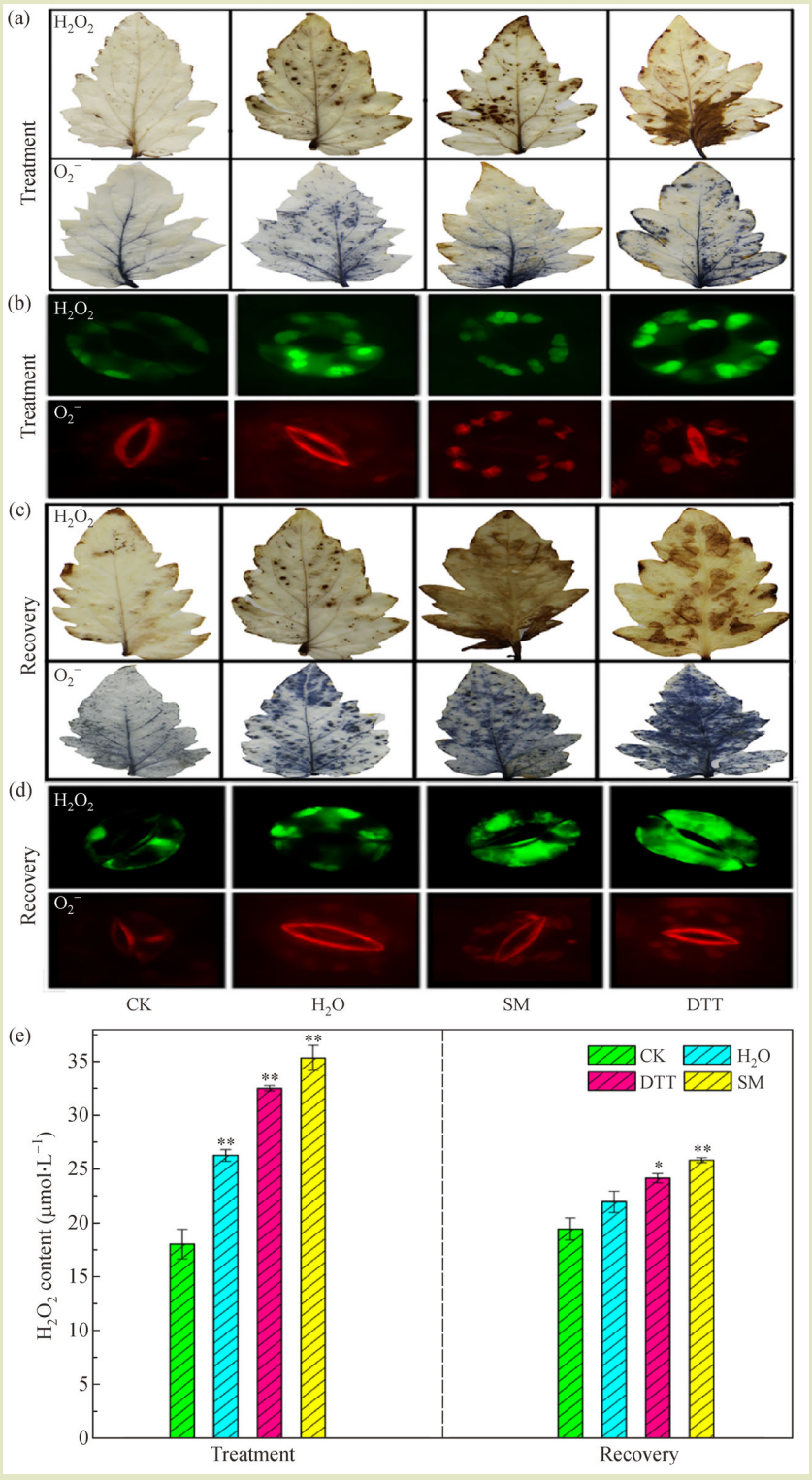

Fig. 8 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on DAB-stained H_2O_2 and NBT-stained O_2^- levels of tomato leaves; DCFH-DA-stained H_2O_2 and DHE-stained O_2^- levels in guard cells and free radical species (mainly H_2O_2, E) in tomato leaves under sub-high temperature and high light stress. CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. The left side of the dotted line shows different treatments and the other side shows the treatments of recovery. Data are mean values of five replicates with standard errors shown as vertical bars. *, significant difference ($P \leqslant 0.05$); and **, highly significant difference ($P \leqslant 0.01$).

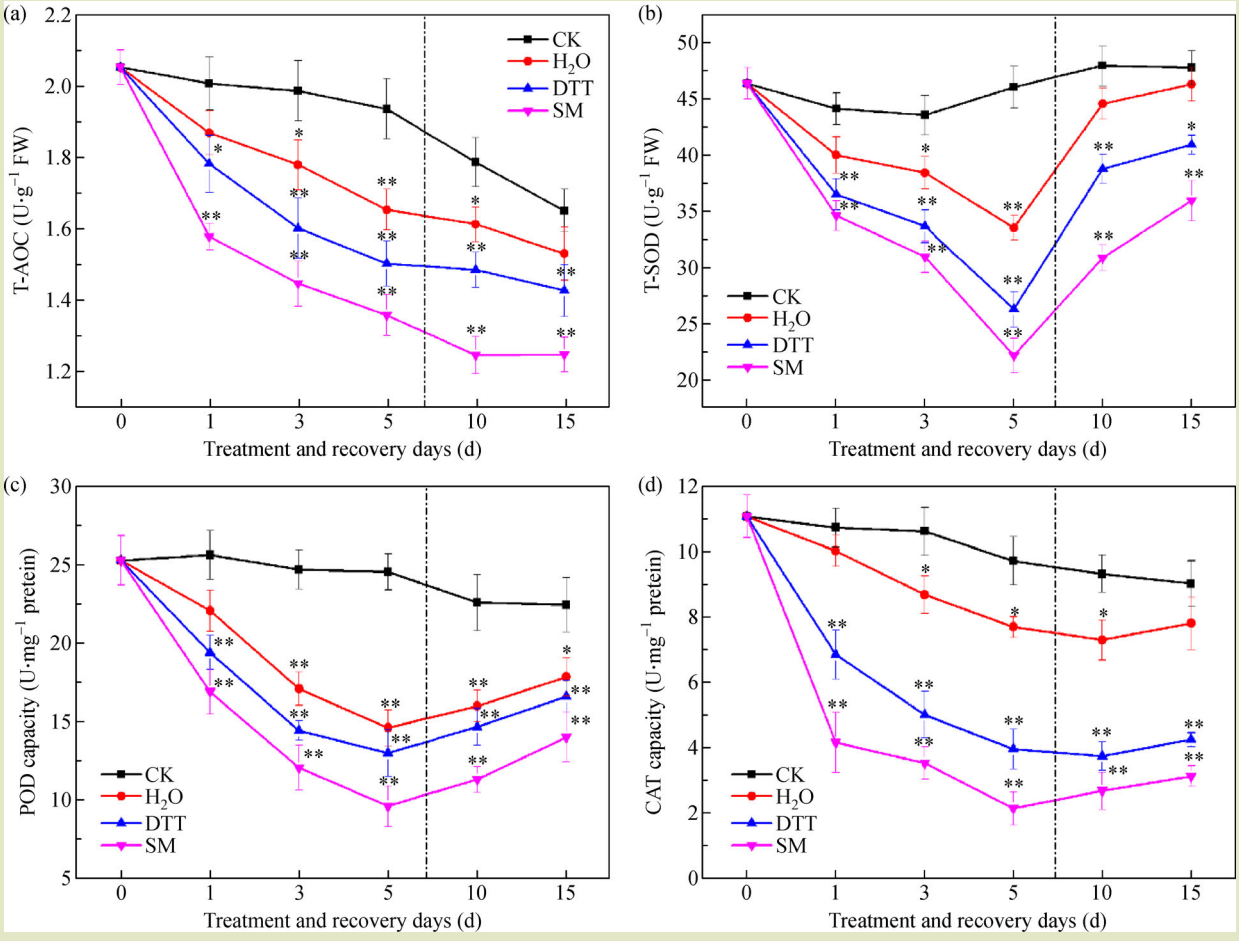

Fig. 9 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on the activities of antioxidant enzymes (AOC, SOD, POD and CAT) in tomato leaves under sub-high temperature and high light stress. CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. The left side of the dotted line shows different treatments and the other side shows the treatments of recovery. Data are mean values of five replicates with standard errors shown as vertical bars. *, significant difference ($P \leqslant 0.05$); and **, highly significant diff-erence ($P \leqslant 0.01$).

of moving to more favorable environments[26]. Environmental stress enhances the extent of photoinhibition, a process that is determined by the balance between the rate of photodamage to PSII and the rate of its repair[27]. D1 protein turnover and XC are essential for PSII self-protection. Here, we used the specific inhibitors SM and DTT to suppress D1 protein synthesis and turnover and XC, respectively. Our results reveal that F_v/F_m was significantly decreased by SM and DTT under HH (Fig. 2), indicating that the photosynthetic efficiency was reduced. Similarly, the F_v/F_o was also significantly suppressed by SM and DTT, indicating that the potential activity of PSII was inhibited. Reduced carbon fixation ability (Fig. 1) and blocking of linear electron transportation (Fig. 2) led to excess excitation of PSII reaction centers under HH conditions. qP and qN showed significant decreases 5 d after SM and DTT treatment, indicating a blockage of PSII reaction center opening and an inhibition of photosynthetic activities as well as XC alone not being adequate to cope with HH (Table 3). D1 protein western blot analysis also shows that the content of D1 protein decreased significantly 5 d after SM and DTT treatment (Fig. 6). These results are consistent with *PsbA* gene expression profiles (Fig. 5), further confirmed photodamage of tomato cells. The photodamage of tomato seedlings implies that photosynthesis was hindered by SM and DTT application and extended inhibition of D1 protein turnover and XC eventually led to cell death. Additionally, SM treatment causes more severe photoinhibition than DTT treatment, indicating that D1 protein turnover contributes to photoprotection under HH more than XC.

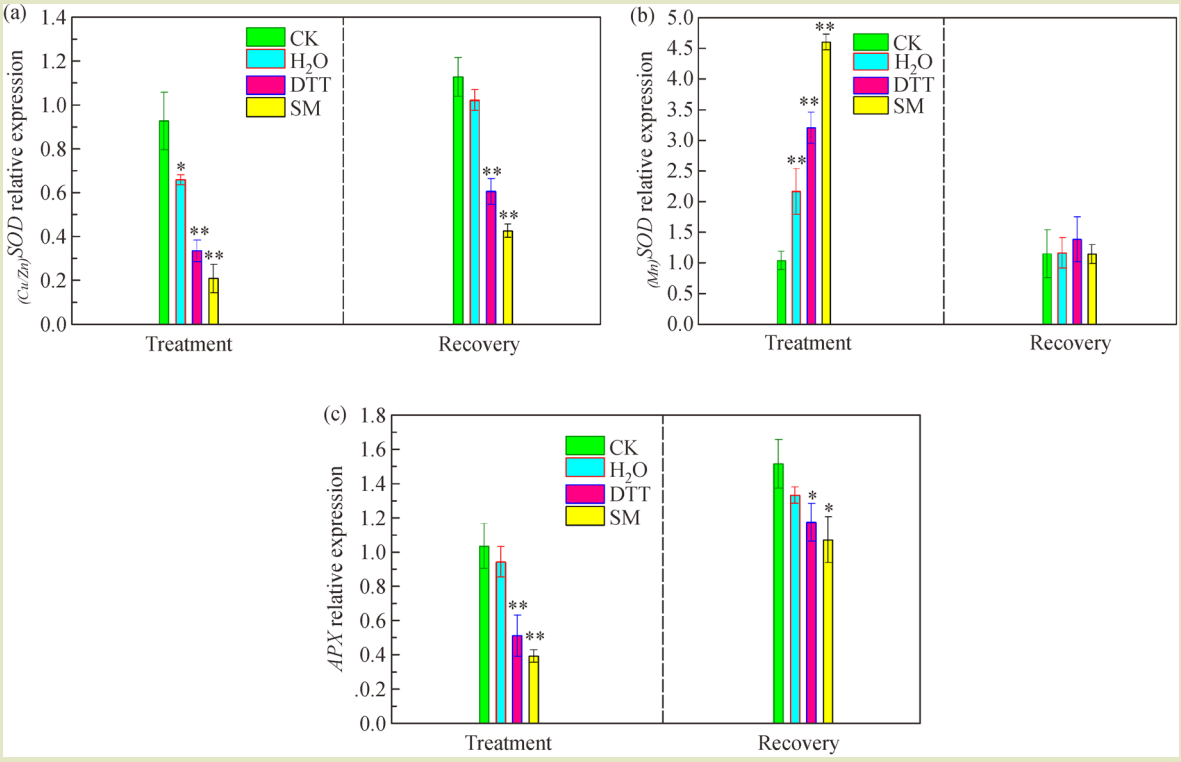

Fig. 10 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on genes expression of antioxidant enzymes in tomato leaves under sub-high temperature and high light stress. CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. The left side of the dotted line shows different treatments and the other side shows the treatments of recovery. Data are mean values of five replicates with standard errors shown as vertical bars. *, significant difference ($P \leqslant 0.05$); and **, highly significant difference ($P \leqslant 0.01$).

Photosynthetic pigments are the central components of photosynthesis and are important in light-harvesting and photosynthetic reactions. When tomato plants were sprayed with SM and DTT, apparent declines in Chl a and Chl b contents were detected. Chl a/Chl b ratio also changed significantly (Fig. 3), indicating a destruction in the pigment structures. F_o is related to the concentration of Chl a. Therefore, the significant inhibition of PSII activities under HH were caused by an imbalance of light absorption and conversion which produced excess light energy and inhibited the activity of PSII. In addition, the high Chl a/Chl b ratio in SM- and DTT-treated plants explains the greater susceptibility to injury of light-harvesting complexes by oxidative stress than of photosynthetic antenna complexes[28]. The influence of DTT and SM on photosynthesis also included inhibition of stomatal opening (Table 2). This indicates that D1 protein turnover and XC significantly affected the photosynthetic characteristics of tomato leaves under HH through maintaining the morphology of guard cells and the opening of stomata.

A reversible NPQ and a functional XC are essential to dissipate excess energy. In plants, NPQ activity is modulated by zeaxanthin, and constitutive zeaxanthin accumulation, as in the *npq2* mutant, makes the onset of qE faster, implying that zeaxanthin is required for the full activation of qE[29]. The NPQ of SM-treated plants was significantly higher than that of control plants (Fig. 4). This is different from previously reported results[30] which show similar levels of anther anxanthin and zeaxanthin (Fig. 7). This is an indication that XC was not inhibited by SM in plant cells. However, when there was no functional XC in DTT-treated plants, the NPQ level was lower than in SM-treated plants (Fig. 4). Also, the formation of singlet oxygen was lower than in SM-treated plants (Fig. 8). These results demonstrate that a relatively low level of NPQ without XC combined with other photoprotective mechanisms such as ROS scavenging and excited states of triplet chlorophyll quenching, were adequate to protect against photoinhibition in DTT-treated plants. qE development is associated with the accumulation of XC carotenoid zeaxanthin and PsbS protein[31].

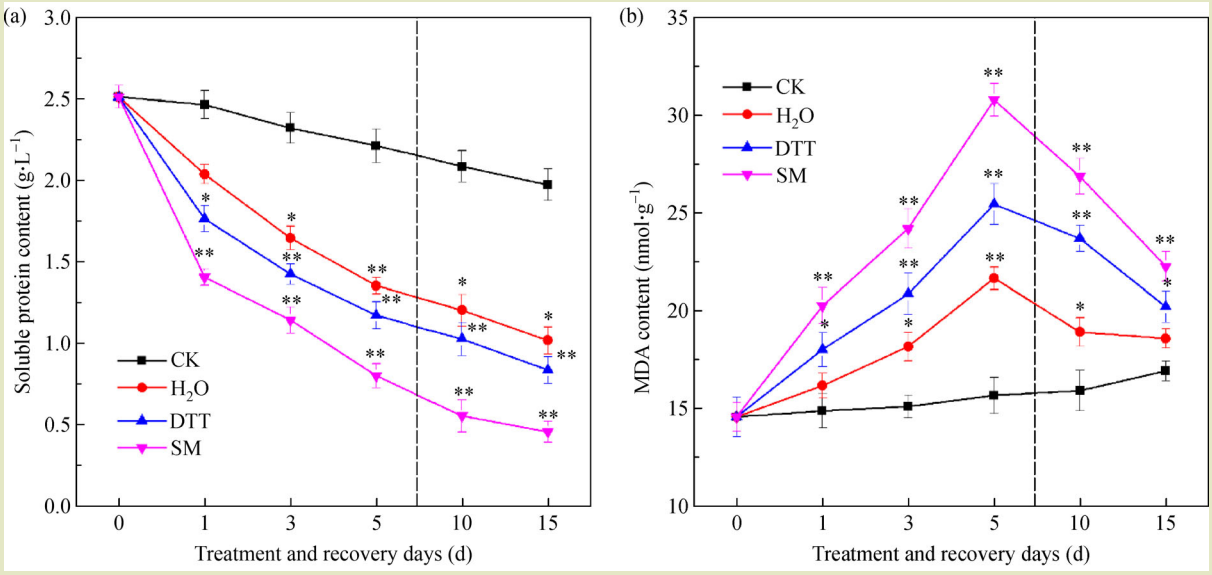

Fig. 11 Effects of streptomycin sulfate (SM) and dithiothreitol (DTT) treatment and recovery on (a) soluble protein content and (b) lipid peroxidation (expressed as MDA content) in tomato leaves under sub-high temperature and high light stress. CK, control plants grown at optimum temperature; H_2O, plants treated with H_2O (distilled water) and grown at HH; DTT, plants treated with DTT (10 mmol·L^{-1}) and grown at HH; SM, plants treated with SM (3 mmol·L^{-1}) and grown at HH. The left side of the dotted line shows different treatments and the other side shows the treatments of recovery. Data are means of five replicate values with standard errors shown as vertical bars. *, significant difference ($P \leqslant 0.05$); and **, highly significant difference ($P \leqslant 0.01$).

Here, once D1 turnover was inhibited, the decrease in qE was mainly caused by the suppression of *PsbS* expression, not zeaxanthin. However, when XC was severely impaired, the decrease in qE capacity was due to the inhibition of the synthesis of zeaxanthin and the photoprotection protein PsbS. However, DTT inhibited qE levels by decreasing the expression of the *PsbS* gene and accumulating zeaxanthin (Fig. 4 and Fig. 5). Since PsbS protein behaves in a dose-dependent manner in leaves, increasing PsbS should result in a higher qE[12]. We speculate that the SM treatment intensified the sensitivity of tomato leaves to HH following the DTT treatment. When inhibitors and adverse environments were removed, the NPQ components and XC pigments recovered to some extent (Fig. 4(b) and Fig. 7), but D1 protein remained depleted (Fig. 5 and Fig. 6). Recovery of the PSII reaction center is a complicated process that is affected by several factors such as protein assembly, proton gradient throughout the thylakoid membrane and electron transporter redox state. Further investigation is required to better understand the interactions among these mechanisms.

4.2 Effects of D1 turnover and xanthophyll cycle on reactive oxygen species under HH

ROS are common byproducts of aerobic metabolism and are generated predominantly within the mitochondria and chloroplasts of photosynthetic plants. Nevertheless, the accumulation of excessive levels of ROS under environmental stresses can cause metabolic imbalance and oxidative damage[28]. A clear boost of ROS was similarly detected in our study under HH (Fig. 8), which agrees with previous studies[22]. Additionally, application of SM and DTT was found to significantly increase ROS levels, suggesting that the tomato seedlings have suffered more severe oxidative stress. The DCFH-DA-derived H_2O_2 signals were observed primarily in the chloroplasts of guard cells in SM- and DTT-treated plants (Fig. 8), and this further supports the notion that the chloroplasts are a major ROS source in plant cells. The production of ROS in chloroplasts depends on the transfer efficiency of electron flow in the PSI and PSII reaction centers and their metabolic oxidative nature[32].

Soluble protein is an important osmotic regulator, and its increase can promote water retention as well as membrane stability[33]. MDA is a final metabolic product of membrane lipid peroxidation and is an indicator of membrane damage[34]. Excess ROS causes peroxidation of lipids and pigments on the cell membrane, resulting in increased cell membrane permeability and destruction of its functions[34]. As shown in Fig. 11, we found that there was a marked decrease in soluble protein contents and a significant increase in MDA levels simultaneously under HH 5 d after SM and DTT treatment. The increase in

MDA content indicates that the antioxidant system of tomato cells cannot remove excess ROS and protect plant leaves from oxidative damage (Fig. 11). Similar observations have been reported[28]. These results indicate that the reduction of D1 turnover and XC resulted in ROS bursts which severely injured the membrane system and weakened the resistance capacity of the tomato plants.

Higher plants remove excess ROS via antioxidant systems and increased production of ROS is typically accompanied by increased activities of AOC, SOD, POD and CAT under high temperatures or high light stress[28]. Here, exogenous spraying of SM and DTT further exacerbated the accumulation of ROS while reducing the antioxidant enzyme activity (Fig. 9), resulting in weakened effective protection of photosynthetic organs, and eventually led to photobleaching and damage to photosynthetic organs and loss of photosynthetic function under HH[35]. Additionally, SM and DTT also downregulated the transcriptional expression of several key photoprotective genes. Zelko et al.[36] suggested that light stress hindered the expression of $_{(Cu/Zn)}SOD$ and $_{(Mn)}SOD$. Here, the transcription of $_{(Cu/Zn)}SOD$ and *APX* was downregulated by SM and DTT (Fig. 10). These results imply that SM- and DTT-treated plants lack the ability to enhance the activities of antioxidant enzymes under HH, leading to significant impairment of photosynthesis. While decreased SOD activity supports this hypothesis, the decrease in CAT activity was caused by decreased hydrogen peroxide production by the SOD disproportionation reaction *in vivo*[37]. In summary, the absence of an increase in antioxidant enzyme activity in SM- and DTT-treated tomato plants under HH suggests that hindering D1 protein turnover or XC can adversely affect the ability of plants to respond to stress conditions.

5 CONCLUSIONS

Here, SM and DTT were used to assess the effects of D1 protein turnover and XC on PSII function in tomato leaves under HH. We found that D1 protein turnover had a more important role in photoprotection than XC. Under HH stress, D1 protein turnover and XC avoided photooxidative damage of the photosystem through regulating stomatal movement, maintaining photosynthetic pigment contents and inducing changes in NPQ core components. In addition, SM and DTT induced a substantial increase in ROS and thylakoid membrane lipid peroxidation in tomato leaves and disrupted the ROS scavenging system (Fig. 12).

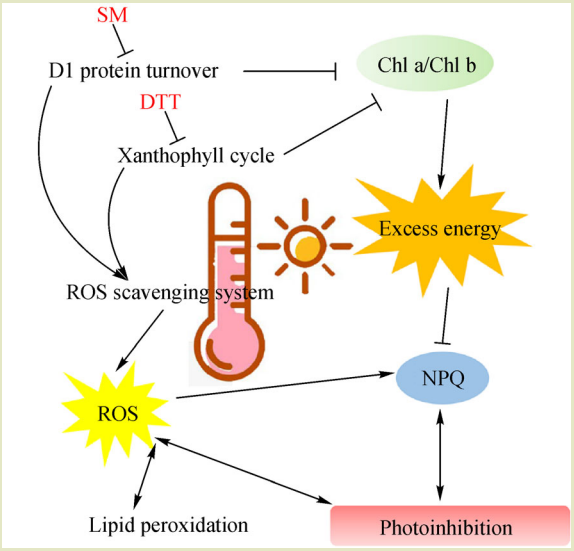

Fig. 12 Schematic representation of the effects of D1 protein turnover and xanthophyll cycle (XC) on PSII function in tomato leaves under sub-high temperature and high light (HH) stress. D1 turnover plays a more important role than XC in photoprotection under HH in tomato. Under HH stress, D1 protein turnover and XC avoided the accumulation of excess energy and photooxidative damage of the photosystem through maintenance of photosynthetic pigment contents and inducing changes in NPQ core components. In addition, D1 protein turnover and XC also avoided accumulation of ROS and membrane lipid peroxidation by maintaining the activity of the ROS scavenging system. Chl a/Chl b, chlorophyll a/ chlorophyll b; ROS, reactive oxygen species; NPQ, non-photochemical fluorescence quenching.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31772356) and the China Agriculture Research System (CARS-25).

Compliance with ethics guidelines

Tao Lu, Jiazhi Lu, Mingfang Qi, Zhouping Sun, Yufeng Liu, and Tianlai Li declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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