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Acute Exposure to UV-B Sensitizes Cucumber, Tomato, and Arabidopsis Plants to Photooxidative Stress by Inhibiting Thermal Energy Dissipation and Antioxidant Defense

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Non-photochemical quenching/Reactive oxygen species/Ultraviolet-B/Xanthophyll cycle.

To characterize a change in NPQ upon exposure to ultraviolet-B (UV-B), the xanthophyll cycle-dependent and -independent NPQs were compared in *Cucumis sativus*, *Lycopersicum esculentum*, and *Arabidopsis thaliana* leaves. The xanthophyll cycle-dependent NPQ was dramatically but reversibly suppressed by UV-B radiation. This suppression was correlated more strongly with a marked decrease in photosynthetic electron transport rather than changes in xanthophyll cycle enzymes such as violaxanthin de-epoxidase and zeaxanthin epoxidase. Accordingly, the UV-B-induced suppression of NPQ cannot be attributed to changes in expressions of VDE and ZEP. However, suppression of the xanthophyll cycle-dependent NPQ could only account for the 77 K fluorescence emission spectra of thylakoid membranes and the increased level of $^1O_2$ production, but not for the decreased levels of $\bullet O_2^-$ production and H_2O_2 scavenging. These results suggest that a gradual reduction of H_2O_2 scavenging activity as well as a transient and reversible suppression of thermal energy dissipation may contribute differentially to increased photooxidative damages in cucumber, tomato, and Arabidopsis plants after acute exposure to UV-B radiation.

INTRODUCTION

Non-photochemical quenching (NPQ) of chlorophyll fluorescence indicates thermal dissipation of excitation energy absorbed for photosynthesis. NPQ protects PSII reaction centres against photoinhibitory damages by over excitation.[1)] NPQ is heterogeneous, consisting of a slowly reversible qI component and a rapidly reversible qE component.[2)] Generation of qE is associated with the enzymatic de-epoxidation of epoxy-xanthophyll violaxanthin to zeaxanthin via the xanthophyll cycle.[3)] Therefore, xanthophyll cycle pigments such as violaxanthin, antheraxanthin, and zeaxanthin, which are embedded in the light harvesting complexes (LHCs), have a critical role in NPQ. Their spatial distribution within PSII antenna complexes is important for the buildup and relaxation of NPQ. Recently, xanthophyll cycle pigments are known as allosteric effectors of NPQ.[4,5)]

Ultraviolet-B (UV-B) radiation (280–320 nm) is one of the most injurious environmental factors for photosynthetic organisms. The amount of UV-B radiation on the Earth has increased over the last few decades due to the destruction of the stratospheric ozone layer.[6)] Since UV-B radiation increases with solar radiation, change of thermal energy dissipation by UV-B might affect photodamage and photoprotection of photosystems. For instance, exposure to a high dose of UV-B radiation inhibited photosynthetic electron transport in higher plants, with PSII being the major site of UV-B damage.[7,8)] Moreover, UV-B treatment of *Pisum sativum* plants limited the amount of violaxanthin available for de-epoxidation by decreasing PSII activity rather than by reducing the pool size of xanthophyll cycle pigments.[9)] In contrast, growth under UV-B radiation increased tolerance to high light intensities and drought stress in pea and *Phaseolus vulgaris* (bean) and Arabidopsis plants.[10,11)] Therefore, it remains controversial whether the increased UV-B radiation associated with ozone depletion is a real threat to photosynthesis and plant productivity.[12,13)]

UV-B radiation also leads to production of reactive

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oxygen species (ROS) such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and superoxide anion ($\bullet O_2^-$) in the evolution of photosynthetic processes in plant cells, causing photooxidative stress.[14,15] Moreover, it has been suggested that UV-B-induced production of H_2O_2 was associated with the damage and degradation of PSII,[16] and thereby inhibition of electron transport chain due to degradation of D1 proteins in PSII might promote production of singlet oxygen from molecular oxygen by excited triplet chlorophylls.[17] However, there is still a lack of data about the photooxidative stress resistance of plants after UV-B exposure.

Therefore, considering the importance of NPQ and xanthophyll cycle pigments in protecting photosynthetic machineries from photooxidative stress, we attempted to characterize and compare changes in the xanthophyll cycle-dependent NPQ among cucumber, tomato, and Arabidopsis after UV-B exposure. Since cucumber and tomato are cultivated in the summer season with high solar radiation, they would depend on this defense mechanism more than Arabidopsis used as a model plant species. However, in the present study, it was revealed that reversible damages of photosystems and changes of photooxidative stress resistance after UV-B exposure are partly detached from different inhibition of NPQ among three plant species.

MATERIALS AND MTEHODS

Plant materials and treatment of UV-B or high light

Cucumber (*Cucumis sativus* L.), tomato (*Lycopersicum esculentum* Mill.) and wild-type and *npq1-2* mutant of *Arabidopsis thaliana* (ecotype Columbia) were cultivated in a plant growth chamber with a 16-h photoperiod and a day/night temperature regime of 22/18°C in a compound soil mixture (vermiculite:peat moss:perlite = 1:1:1). Lighting was adjusted to a photosynthetic photon flux density (PPFD) of 130 μmol m^{-2} s^{-1} supplied by six fluorescence lamps. The *npq1-2* seeds were obtained from the Arabidopsis Research Center (Ohio State University, Columbus, OH).

Cucumber and tomato plants were exposed to UV-B at 21 d after seeds were sown (DAS), while Arabidopsis plants at 29 DAS. UV-B radiation was irradiated to the plants at room temperature for 5 h with an intensity of 2.4 W m^{-2} from two UV-B lamps (XX-15B; Spectronics, Westbury, NY) under a PPFD of 40 μmol m^{-2} s^{-1} from two fluorescence lamps.

For high light treatment, all leaves were harvested from plants at 1 d after treatment with UV-B. The whole leaves or their leaf disks (5-mm diameter) were floated on distilled water, abaxial side down at 22°C for 5 h under a PPFD of 800 μmol m^{-2} s^{-1}, which was provided by six fluorescence and two tungsten lamps.

Chlorophyll fluorescence analysis

Chlorophyll (Chl) fluorescence was measured using a Chl fluorometer (IMAGING-PAM, Walz, Effeltrich, Germany) as reported previously.[18] Readings were taken after 5-mm diameter leaf disks were dark-adapted for 15 min at room temperature. Chl fluorescence and quenching parameters, e.g., Fv/Fm, ETR, qP and NPQ, were calculated as described by Moon *et al.*[19]

Pigment analysis

Separation of photosynthetic pigments was performed in a HPLC system (1200, Agilent Technologies, Santa Clara, CA) on a Spherisorb ODS-1 column (Alltech, Deerfield, IL), as previously described by Moon *et al.*[19] Concentrations of the pigments were estimated by using the conversion factors for the peak area to nanomoles, as determined by Gilmore and Yamamoto.[20] De-epoxidation state (DEPS) of the xanthophyll cycle pool was calculated as $(Ax \times 0.5 + Zx) \times 100 / (Vx + Ax + Zx)$, where Vx, Ax, or Zx is violaxanthin, antheraxanthin, or zeaxanthin, respectively.

Anthocyanin extraction and spectrophotometric quantification were performed as described by Thomas *et al.*[21] The amount of anthocyanin is presented as the values of A_{530}-$0.33A_{657}$ per gram fresh weight (FW).

Semi-quantitative RT-PCR analysis

Extraction and purification of total RNA from leaves and synthesis of cDNA were performed as previously described by Kim *et al.*[22] Sense/antisense primers for the subsequent PCR are listed in Table 1. Specific primers for VDE or ZEP of cucumber were designed from alignments of mRNA sequences available on the NCBI site. The PCR was carried out with 1 μl of a total 20 μl RT reaction mixture in a PCR system, Maxime™ PCR PreMix (iNtRON Biotechnology, Sungnam, Korea) as follows: denaturation at 94°C for 2 min, 40 cycles of 94°C (20 s) – 45°C (15 s) – 72°C (30 s) for cucumber or 30 cycles of 94°C (20 s) – 55°C (10 s) – 72°C (30 s) for tomato and *Arabidopsis*, and an extension at 72°C for 5 min. The resultant RT-PCR products were electrophoresed and analyzed on a 1.0% (w/v) agarose gel containing ethidium bromide (EtBr).

77 K fluorescence spectroscopy

Low-temperature (77 K) emission spectra were recorded from thylakoids using a fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan) and a custom-made apparatus. Thylakoids were isolated from freshly harvested leaves in a grinding buffer (pH 7.5) of 50 mM HEPES, 330 mM sorbitrol, 5 mM EDTA, 5 mM $MgCl_2$, 2 mM ascorbate and 0.1% (w/v) BSA, and subsequently in a resuspension buffer (pH 7.5) of 50 mM HEPES, 330 mM sorbitrol, 2.5 mM EDTA and 5 mM $MgCl_2$. All spectra were obtained after excitation at 440 nm and presented without correction for the spectral sensitivity of the photomultiplier.

EPR spectroscopy

Production of singlet state oxygen ($^1O_2$) in thylakoids

Table 1. Primer sequences used in semi-quantitative RT-PCR experiments. *LeVDE*, *LeZEP*, and *Le18srRNA* are gene names of VDE, ZEP, and 18sRNA in tomato and Arabidopsis (WT), while *CsVDE*, *CsZEP*, and *Cs18srRNA* are the corresponding names in cucumber. Specific primers for *CsVDE* and *CsZEP* were designed from alignments of mRNA sequences available on the NCBI site.

Gene name	Sense / antisense primers	Product size (bp)
LeVDE	5′ AGAGTGCAGGATAGAGCT TG 3′ / 5′ GATCAGGCACAGGAAAGTC 3′	223
LeZEP	5′ GGCTCTTCTGGTTTAGAAG C 3′ / 5′ CCAGGTACCATGTTCACTTC 3′	197
Le18srRNA	5′ GGAAACTTACCAGGTCCAG 3′ / 5′ GGCTAGTCCCTCTAAGAAGC 3′	196
CsVDE	5′ AAC CCTGCATGTGCAGCCAATGT 3′ / 5′ TACCAGTCATCTTGATAGTG 3′	610
CsZEP	5′ AAGTT TGATACGTTCACTCC 3′ / 5′ GAAGAAACAAAGTACTGTTT 3′	494
Cs18srRNA	5′ GTTGCTTTAAGGACTC CGCCA 3′ / 5′ AGGGGTACCTCCGCATAGCTAG 3′	200
AtVDE	5′GAGAGTGTTCTCCCTTCCTT 3′ / 5′ GAGTGGGCTTACTTCTCCTT3′	214
AtZEP	5′ GAGTACCACATCCAGGAAGA3′ / 5′ GTACGTTCAAGAGCATCGTC3′	186
At18srRNA	5′ GCCCAGAAGTCTTGTTCCAG3′ / 5′ CTTGGTGCAAGTGCTGTGAT3′	212

under high light was assayed by EPR spectroscopy with a specific chemical probe, 2,2,6,6-tetramethyl-4-piperidone (TEMP).[23] Thylakoids were isolated from pre-illuminated leaves at a PPFD of 500 μmol m^{-2} s^{-1} for 2 h as described by Färber and Jahns,[24] except for excluding ascorbate in the grinding buffer, and then they were placed under the same light for 1 h in the resuspension buffer supplemented with 50 mM TEMP and 100 μM methyl viologen. EPR spectra were measured within 1 h after treatment of high light with an ESR spectrometer (JES-FA200, JEOL Ltd., Tokyo, Japan) working at the X band. Spectra were recorded at room temperature with 9.4 GHz microwave frequency, 10 mW microwave power, 0.2 mT modulation amplitude, 100 kHz modulation frequency and 3.0×10 amplification. The amount of trapped $^1O_2$ was obtained as the area of EPR absorption spectra (double integral of measured spectra).

NBT staining

Production of superoxide ($\bullet O_2^-$) in leaves under photooxidative stress conditions was visualized by a dark blue insoluble formazan compound of nitroblue tetrazolium (NBT) as previously reported by Fryer *et al.*[25] All leaves harvested from plants at 1 d after treatment with UV-B, respectively, were excised into 1-cm disks. The leaf disks were infiltrated with 50 μM methyl viologen and 6 mM NBT at room temperature under vacuum and dim light for 2 h. To induce photooxidative stress, they were placed at 22°C for 3 h under a PPFD of 130 μmol m^{-2} s^{-1} supplied by six fluorescence lamps. Prior to imaging, pigments were removed from the leaf disks by boiling in lactic acid-glycerol-ethanol (1:1:4 by vol.) for 5 min. All images were presented without correction for the background.

H_2O_2 scavenging assay

One gram of leaf material was collected and immediately frozen in liquid nitrogen. Leaves were crudely extracted with 0.1 M potassium phosphate buffer (pH 7.0). The leaf extract was centrifuged to remove cell debris for 5 min at 300 g and 4°C. The supernatant was mixed with 20 mM H_2O_2 (1:1 by vol.) and incubated at 25°C for 10 min. After 5-min centrifugation at 15,000 g and 4°C, the supernatant was further incubated at 25°C with 50 mM 3,3'-diaminobenzidine (DAB) for 30 min to quantify the remaining H_2O_2. Finally, polymerization products of DAB were estimated by obtaining the absorbance at 485 nm. H_2O_2 scavenging efficiency was expressed as percentages of $(\Delta A_{485}(\text{Blank})-A_{485}(\text{Sample}))/\Delta A_{485}(\text{Blank})$.

RESULTS

Xanthophyll cycle-dependent NPQ is inhibited by UV-B radiation

The kinetics of NPQ was analyzed in leaves detached from cucumber, tomato, and Arabidopsis plants after treatment with UV-B radiation. NPQ occurred more slowly in plants subjected to UV-B than in the control, and the maximum amplitude of NPQ was 45 to 66% that of the control (Fig. 1A–C). The relaxation of NPQ was also affected differentially in the three plant species by UV-B. The inhibition of NPQ buildup by UV-B was fully relieved in the three plant species within four days (Fig. 1D). However, the recovery of NPQ buildup in cucumber was not initiated within two days. This trend was similarly observed in the maximal photochemical efficiency of photosystem II (PSII) as shown in Fv/Fm (Fig. 1E).

To test whether xanthophyll cycle-dependent or xanthophyll cycle-independent NPQ was affected by treatment with UV-B radiation, the NPQ kinetics was further analyzed after infiltration of leaves with a violaxanthin de-epoxidase (VDE) inhibitor, dithiothreitol (DTT). The NPQ buildup was substantially suppressed in the presence of DTT, indicating inhibition of xanthophyll cycle-dependent NPQ (Fig. 2). Pigment analysis of the DTT-infiltrated leaves revealed no detectable increase in zeaxanthin content during the NPQ buildup phase upon illumination (data not shown). The DTT-

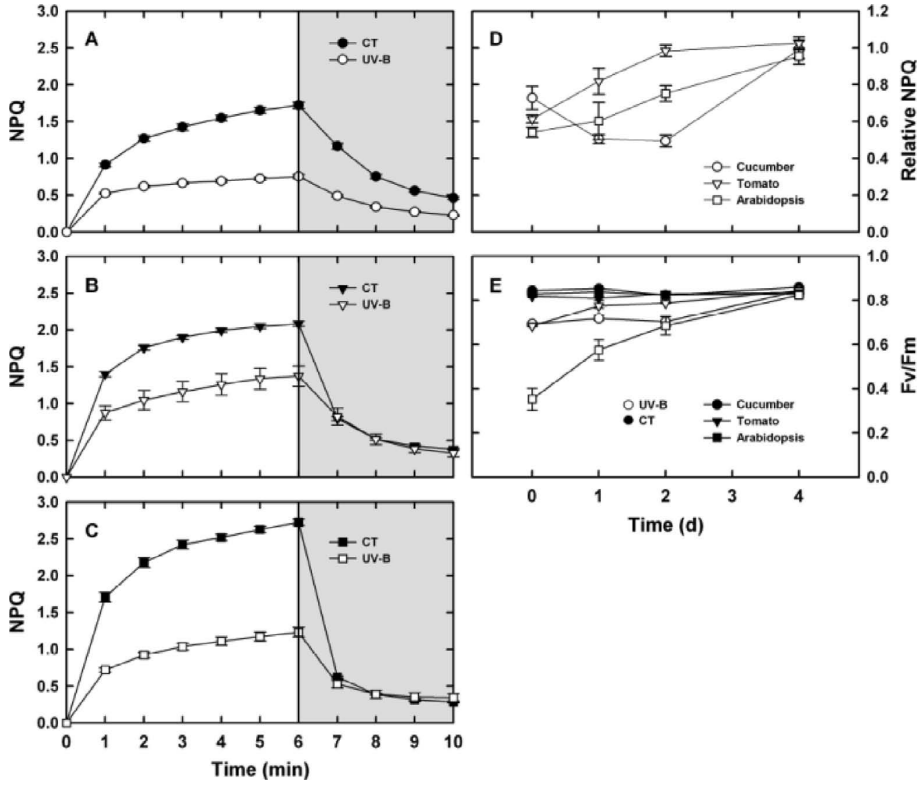

Fig. 1. NPQ kinetics and Fv/Fm of cucumber, tomato, and Arabidopsis (WT) leaves after treatment with UV-B. After dark-adaptation for 15 min, leaf disks (5-mm diameter) were exposed to continuous actinic illumination of 1,210 μmol quanta m^{-2} s^{-1} for 6 min and then to darkness for 4 min. Saturation pulses applied during the analysis were equivalent to 2,400 μmol quanta m^{-2} s^{-1}. **A–C**, Buildup and release of NPQ; **D**, Maximum amplitude of NPQ after a 6-min actinic illumination; and **E**, Maximal photochemical efficiency (Fv/Fm). **A**, Cucumber; **B**, Tomato; and **C**, Arabidopsis (WT). In **D**, relative NPQ values represent ratios of the NPQ values of the UV-B-treated samples to the NPQ values of the respective controls. Data are means ± SE of 15 measurements from three different experiments. CT, control; and WT, wild type.

inhibitable NPQ or the xanthophyll cycle-dependent NPQ in the three plant species was the main portion of the NPQ affected by UV-B (Fig. 2). However, a considerable portion of the xanthophyll cycle-independent NPQ was also suppressed in tomato and Arabidopsis.

Ratio of violaxanthin to total chlorophyll and anthocyanin content are increased by UV-B radiation

Xanthophyll cycle-dependent NPQ is generated by conversion of violaxanthin into zeaxanthin by VDE. Based on the above data, it could be predicted that the amount of violaxanthin available for de-epoxidation would be affected by UV-B. Although it has been suggested that the pool size of violaxanthin available for de-epoxidation is not directly affected by UV-B radiation,[9] the ratio of violaxanthin to total chlorophyll at two days after treatment with UV-B radiation was 120–158% that of the control in the three plant species (Table 2). This change was due to an increase of violaxanthin content and/or a decrease of total chlorophyll content. Similarly, the production of anthocyanins, which is stimulated by UV-B radiation and contributes to effective photoprotection of photosystems,[26] increased to 150–420% that of the control in the three plant species. The increase of anthocyanin content was the most remarkable in the UV-B-treated Arabidopsis leaves. In contrast, the ratio of another xanthophyll, lutein, increased only in the UV-B-treated cucumber leaves.

Expressions of xanthophyll cycle enzymes are altered against UV-B-induced suppression of NPQ

To determine whether the suppression of the NPQ buildup in the UV-B-treated leaves was associated with the xanthophylls cycle enzymes such as violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZEP), transcriptional levels of VDE and ZEP were analyzed in the control and UV-B-treated leaves of cucumber, tomato and Arabidopsis (WT). VDE and ZEP expressions were differentially affected in the three plant species after treatment with UV-B (Fig. 3). Especially, the expression of ZEP was oppositely affected in cucumber and tomato leaves by

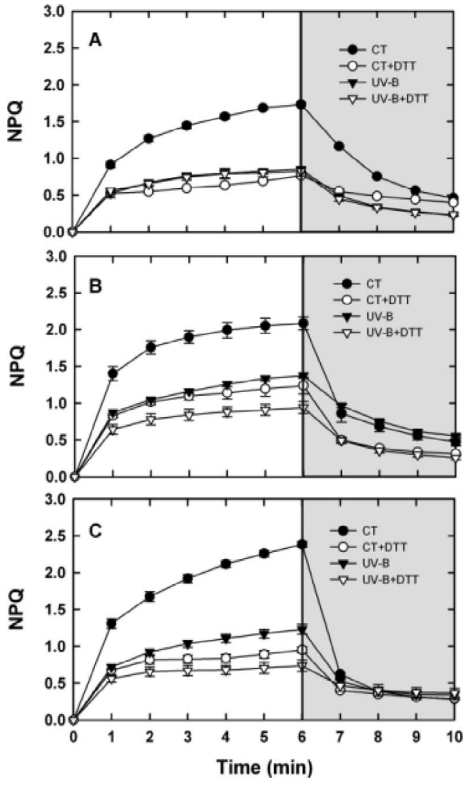

Fig. 2. NPQ kinetics of UV-B-treated leaves in the presence of DTT. Leaf disks (7.5-mm diameter) were incubated with 1 mM dithiothreitol (DTT), an inhibitor of violaxanthin de-epoxidase (VDE), under the growth light for 2 h and then in the dark for 2 h. Analysis of NPQ was performed as described in Fig. 1. **A**, Cucumber; **B**, Tomato; and **C**, Arabidopsis (WT). Data are means ± SE of 10 measurements from two different experiments. CT, control.

UV-B radiation. However, the expression ratio of VDE to ZEP, which can be associated with the xanthophyll cycle-dependent NPQ, seemed to increase commonly in the UV-B-treated leaves of cucumber, tomato and Arabidopsis (WT) within four days. This result may indicate more enzyme activities for de-epoxidation of violaxanthin to zeaxanthin in the UV-B-treated leaves. Therefore, the UV-B-induced suppression of NPQ cannot be attributed to changes in expressions of VDE and ZEP.

UV-B radiation has a major inhibitory effect on photosynthetic activity

Chlorophyll (Chl) fluorescence quenching analysis was performed in leaves detached from cucumber, tomato, and Arabidopsis plants after treatment with UV-B. As well as the NPQ buildup observed with high actinic illumination, the maximum amplitude of NPQ recorded under relatively low actinic illumination of the growth light intensity was reversibly reduced by UV-B radiation (Figs. 1D and 4Aa). In parallel, photochemical quenching of PSII (qP) and photosynthetic electron transport (ETR) were sharply decreased by UV-B and fully relieved within four days (Figs. 4Ab and c). However, the recovery of NPQ and qP was the most slow in cucumber, while the decrease of ETR was the most remarkable in Arabidopsis.

Changes in the ratio of 680/700-nm fluorescence emission for isolated chloroplasts at low temperature (77 K) are thought to reveal the presence of structural changes in the pigment-protein complexes of photosystems, which are associated with qE.[27] Therefore, photosystem damage after treatment with UV-B was identified in cucumber, tomato,

Table 2. Composition of xanthophyll pigments and anthocyanins in cucumber, tomato, and Arabidopsis leaves after treatment with UV-B radiation. Vx, Ax + Zx, Lut, and Ant represent violaxanthin, antheraxanthin + zeaxanthin, lutein, and anthocyanin, respectively. Values for Vx, Ax + Zx, and Lut, were calculated as a ratio of each pigment to total chlorophyll. Those for Ant represent A_{530}-0.33A_{657} as described in Material and Methods. Total chlorophyll (Chl *a* + *b*) contents were expressed as a concentration per leaf area (nmoles/cm^2). C, U, and Day imply control, UV-B, and time after treatment with UV-B, respectively. Data are means ± SE of one or three different experiments for anthocyanins or xanthophyll pigments, respectively.

Plant	Day	Vx		Ax + Zx		Lut		Chl *a* + *b*		Ant	
		C	U	C	U	C	U	C	U	C	U
Cucumber	0	11.1 ± 0.3	11.6 ± 0.2	3.3 ± 0.2	2.7 ± 0.0	49.9 ± 0.9	51.6 ± 0.1	26.4 ± 0.8	23.3 ± 0.5	0.03 ± 0.0	0.04 ± 0.0
	1	11.1 ± 0.4	10.7 ± 0.1	3.4 ± 0.0	3.6 ± 0.0	49.2 ± 0.1	54.4 ± 0.3	29.4 ± 0.0	24.9 ± 0.4	0.03 ± 0.0	0.08 ± 0.0
	2	10.1 ± 0.1	12.8 ± 0.1	4.0 ± 0.2	5.3 ± 0.0	50.0 ± 0.3	54.4 ± 0.1	29.5 ± 0.9	26.1 ± 0.1	0.03 ± 0.0	0.07 ± 0.0
	4	10.9 ± 0.2	13.9 ± 0.1	4.6 ± 0.0	5.5 ± 0.0	50.2 ± 0.4	57.4 ± 0.1	25.6 ± 0.1	26.5 ± 0.1	0.03 ± 0.0	0.07 ± 0.0
Tomato	0	10.2 ± 0.9	11.9 ± 0.0	1.0 ± 0.8	0.3 ± 0.0	48.3 ± 0.6	48.4 ± 0.2	18.8 ± 0.0	13.7 ± 0.0	0.21 ± 0.0	0.28 ± 0.0
	1	11.0 ± 0.9	14.9 ± 0.0	1.1 ± 0.7	0.4 ± 0.0	48.9 ± 0.9	49.6± 0.0	17.2 ± 0.0	11.7 ± 0.1	0.20 ± 0.0	0.30 ± 0.0
	2	10.6 ± 0.5	15.4 ± 0.1	1.2 ± 0.8	0.3 ± 0.0	48.1 ± 0.6	50.2 ± 0.1	16.4 ± 0.2	13.6 ± 0.1	0.20 ± 0.0	0.30 ± 0.0
	4	11.0 ± 0.5	15.0 ± 0.2	1.0 ± 1.2	0.3 ± 0.0	48.9 ± 0.4	50.6 ± 0.3	16.6 ± 0.0	14.9 ± 0.1	0.20 ± 0.0	0.28 ± 0.0
Arabidopsis	0	11.4 ± 0.6	10.7 ± 0.1	1.0 ± 0.1	0.5 ± 0.0	50.3 ± 0.2	48.9 ± 0.7	9.5 ± 0.5	9.7 ± 0.2	0.05 ± 0.0	0.07 ± 0.0
	1	11.1 ± 0.1	11.2 ± 0.1	0.8 ± 0.0	0.5 ± 0.0	49.8 ± 0.7	49.8 ± 0.1	10.9 ± 0.4	11.2 ± 0.1	0.05 ± 0.0	0.08 ± 0.0
	2	10.9 ± 0.4	11.4 ± 0.2	0.8 ± 0.0	0.5 ± 0.0	49.2 ± 0.6	48.9 ± 0.0	11.1 ± 0.2	11.0 ± 0.3	0.05 ± 0.0	0.21 ± 0.0
	4	10.7 ± 0.6	11.8 ± 0.1	0.8 ± 0.0	0.5 ± 0.0	49.8 ± 0.0	49.0 ± 0.9	10.9 ± 0.1	10.8 ± 0.2	0.05 ± 0.0	0.25 ± 0.0

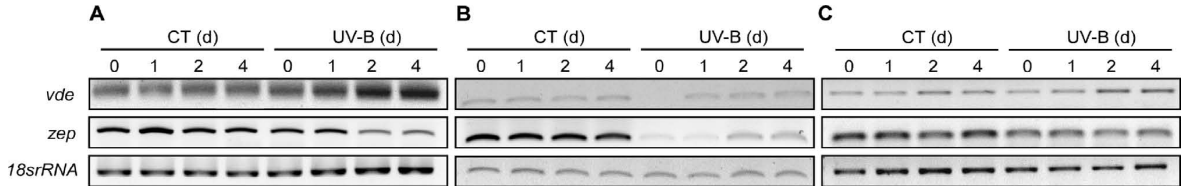

Fig. 3. Semi-quantitative RT-PCR analysis of the expression of VDE and ZEP in leaves of cucumber, tomato and Arabidopsis (WT) plants after treatment with UV-B. **A**, Cucumber; **B**, Tomato; and **C**, Arabidopsis (WT). *18srRNA* was used as an endogenous control gene. All expression patterns were confirmed by three different experiments.

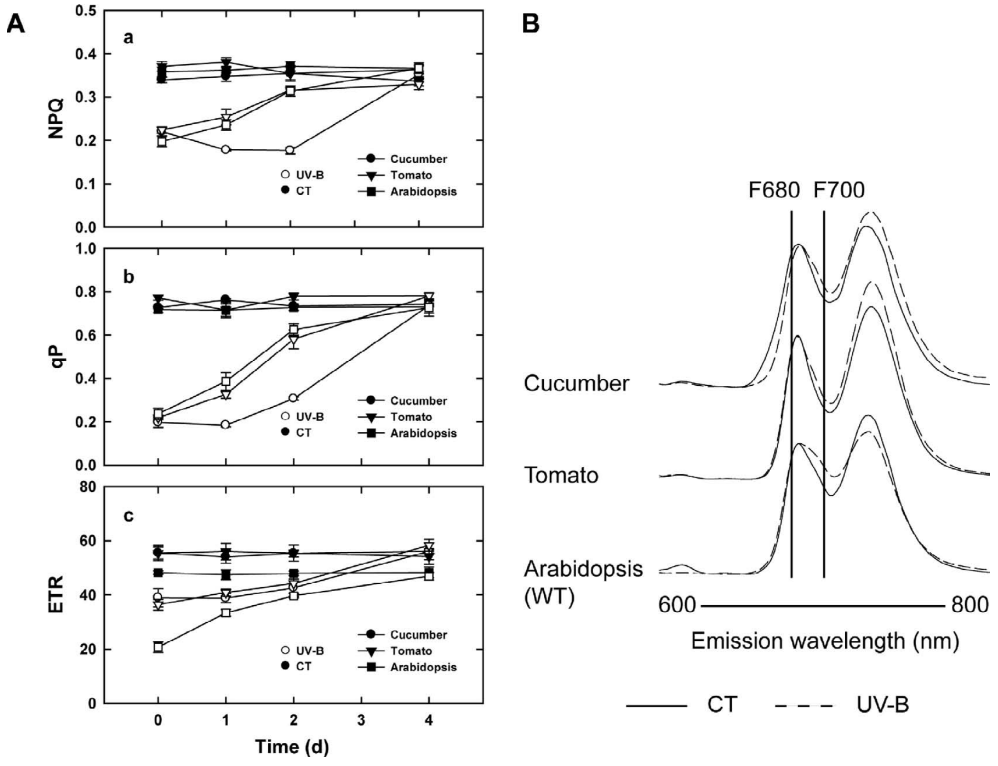

Fig. 4. Changes in Chl quenching parameters and low-temperature (77 K) fluorescence emission spectra of UV-B-treated leaves. In **A,** Chl quenching analysis was performed on 15-min-dark-adapted leaf disks (5-mm diameter); actinic illumination was 135 µmol quanta m^{-2} s^{-1}, which is almost equivalent to the photosynthetic photon flux density (PPFD) of the growth light. Saturation pulses applied during the analysis were equivalent to 2,400 µmol quanta m^{-2} s^{-1}. Data are means ± SE of six measurements. In **B,** 77 K fluorescence analysis was carried out with thylakoids as described in Materials and Methods. Spectra were obtained as a relative fluorescence at the emission wavelengths of 600 nm to 800 nm and normalized to their 685-nm emissions. Data are means ± SE of three measurements. CT, control.

and Arabidopsis (WT) leaves by emission fluorescence spectra of thylakoid membranes at 77 K. The ratio of 680/700-nm fluorescence emission at 77 K was noticeably less in the UV-B-treated leaves than in non-treated leaves, indicating preferential damage in PSII (Fig. 4B).

Photosystem damages under high light conditions are increased in the UV-treated leaves

Suppression of the NPQ buildup due to depletion of VDE increases photosystem damages in Arabidopsis under high light conditions.[3)] In the UV-B-treated leaves of cucumber and Arabidopsis (wild type and VDE-deficient *npq1-2* mutant), the decrease in Fv/Fm under high light conditions (800 µmol m^{-2} s^{-1}) was significantly enhanced (Figs. 5Aa and c). As expected, this trend was more pronounced in the *npq1-2* than in the wild type. In contrast, the high light-induced decrease of Fv/Fm was not significantly different between the control and UV-B-treated leaves of tomato. Therefore, the less but significant decrease of NPQ in the UV-B-treated tomato leaves seemed to be somewhat tolerated

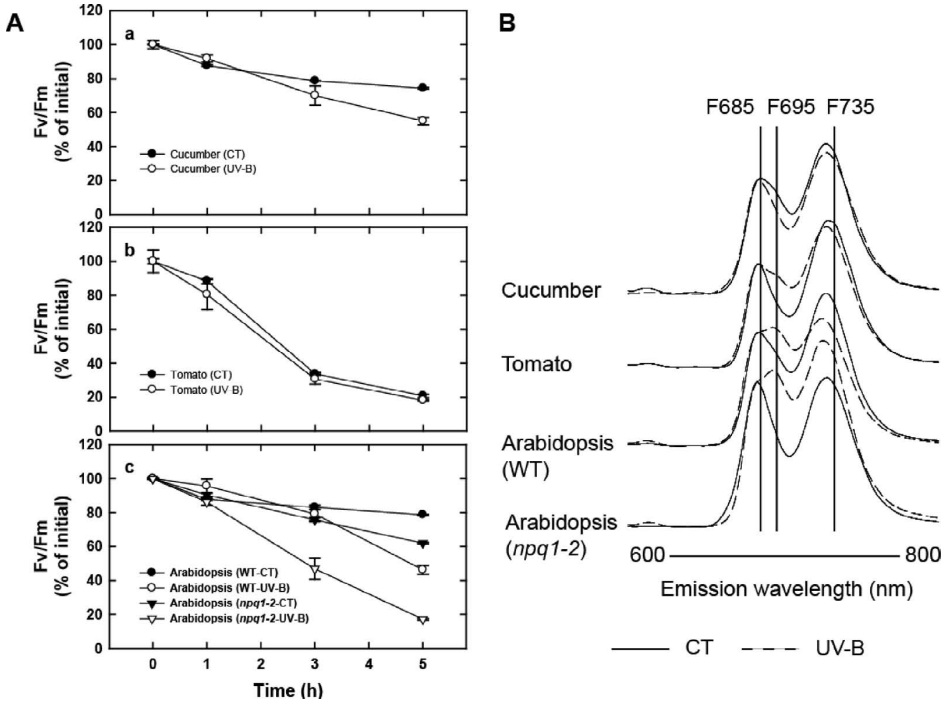

Fig. 5. Changes in Fv/Fm and 77 K fluorescence emission spectra of UV-B-treated leaves under high light conditions. Leaves were floated on distilled water, abaxial side down, at 22°C for 5 h under a PPFD of 800 μmol m^{-2} s^{-1}. In **A**, All values were obtained after 15 min of dark adaptation and are expressed as percentages of the initial values. Data are means ± SE of six measurements. In **B**, 77 K fluorescence emission spectra were generated from thylakoids in the same way as described in Fig. 4. Data are means ± SE of three measurements. CT, control.

under high light illumination (Figs. 1D and 5Ab).

To compare high-light-induced structural changes of photosystems between the control and UV-B-treated leaves, the 77 K emission fluorescence spectra were obtained from thylakoid membranes of the control and UV-B-treated leaves exposed to high light illumination. Emissions near 685 and 695 nm or near 735 nm, which are attributed to PSII (CP43 and CP47) or PSI, respectively,[28–30] were evaluated. The emission spectra were dramatically altered in the UV-B-treated leaves of tomato and Arabidopsis, indicating that this treatment increased photodamage of photosystems under high light conditions (Fig. 5B). This result was more pronounced in the *npq1-2* than in the wild type. In contrast, the emission spectra did not indicate more high light-induced photosystem damages in the UV-B-treated leaves of cucumber than in the control.

Production and scavenging of reactive oxygen species are altered in the UV-B-treated leaves under high light or oxidative stress conditions

Singlet oxygen ($^1O_2$) is the main reactive oxygen species (ROS) to damage photosystems under high light conditions.[31,32] When the xanthophyll cycle and/or the photosynthetic electron transport are impaired, the production of $^1O_2$ is expected to increase. Therefore, the level of $^1O_2$ produced by thylakoid membranes under high light conditions was determined in the control and UV-B-treated leaves using electron paramagnetic resonance (EPR) spectroscopy. As expected, thylakoid membranes from the UV-B-treated leaves of cucumber, tomato and Arabidopsis (WT) showed significantly higher EPR signals than did those from the control (Fig. 6). However, the signal was much less in the UV-B-treated leaves of VDE-deficient *npq1-2* mutant compared to the control, suggesting that the suppression of xanthophyll-dependent NPQ rather than the decrease of photosynthetic electron transport would increase the production of $^1O_2$ in the UV-B-treated leaves of cucumber, tomato, and Arabidopsis plants under high light illumination. This hypothesis was further evidenced by a close correlation between the differential suppression of NPQ and the production level of $^1O_2$ in the three plant species (Figs. 1 and 6).

Methyl viologen-induced photooxidative stress is mediated via the production of another ROS, $\bullet O_2^-$, at PSI sites.[25] As expected from the noticeable decrease in ETR after treatment with UV-B, the production of $\bullet O_2^-$ by methyl viologen was substantially decreased in the UV-B-treated leaves of tomato and Arabidopsis (WT and *npq1-2* mutant) (Fig. 7B–D). However, this production was reversely increased in the UV-B-treated leaves of cucumber, probably due to an

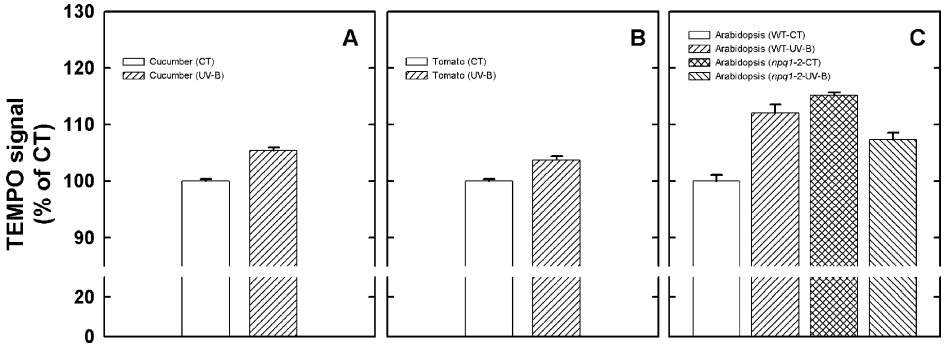

Fig. 6. EPR analysis of 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TEMPO) radicals to evaluate the production of $^1O_2$ by thylakoids under high light conditions. $^1O_2$ converts 2,2,6,6-tetramethyl-4-piperidone (TEMP) to 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TEMPO) radical, which is detectable by EPR spectroscopy. TEMPO signals are presented as percentages of the respective controls in cucumber, tomato, and Arabidopsis (WT). However, in Arabidopsis (*npq1-2*), the signals represent percentages of the WT control, and not of the *npq1-2* control. This presentation allows us to compare the production of $^1O_2$ by WT and *npq1-2* leaves under high light conditions. Experimental procedures are described in Materials and Methods. Data are means ± SE of six measurements from two different experiments. CT, control.

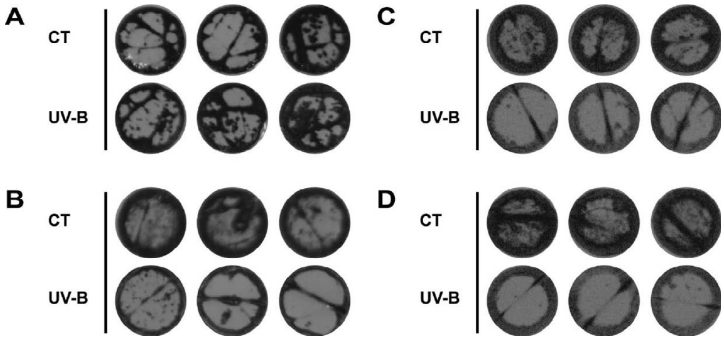

Fig. 7. Analysis of $\bullet O_2^-$ production by photooxidative stress in leaf disks infiltrated with nitroblue tetrazolium (NBT). Leaf disks (1-cm diameter) were infiltrated with 50 μM methyl viologen and 6 mM nitroblue tetrazolium (NBT) as described in Materials and Methods. They were exposed to a PPFD of 130 μmol m^{-2} s^{-1} for 3 h to induce photooxidative stress. Images were obtained after the leaf disks were boiled in lactic acid-glycerol-ethanol to remove pigments. **A**, Cucumber; **B**, Tomato; **C**, Arabidopsis (WT); and **D**, Arabidopsis (*npq1-2*). CT, control.

inhibition of superoxide dismutase (SOD) activity for scavenging $\bullet O_2^-$ (Fig. 7A). This result may imply that the UV-B-induced suppression of NPQ wouldn't directly and exclusively increase methyl viologen-induced photooxidative stress in the UV-B-treated leaves.

Besides $\bullet O_2^-$, H_2O_2, is a key contributor to induce oxidative damages in plants. The most toxic ROS, •OH, is produced from $\bullet O_2^-$ and H_2O_2 via the Fenton reaction.[15,33] Therefore, the whole H_2O_2 scavenging activity including both enzymatic and non-enzymatic components was compared between the control and UV-B-treated leaf extracts as described in Fig. 8. The H_2O_2 scavenging efficiency gradually decreased in cucumber, tomato, and Arabidopsis within four days after treatment with UV-B. Considering the almost full recovery of thermal energy dissipation and photosynthetic activity, this decrease in the H_2O_2 scavenging activity can be correlated with the increased photosystem damages in the UV-B-treated leaves under high light conditions.

DISCUSSION

UV-B radiation inhibits thermal dissipation of excess excitation energy mainly via a xanthophyll cycle-dependent manner

Thermal dissipation of excess excitation energy absorbed is necessarily required for protection of photosynthetic machineries. This process is called NPQ and consists of xanthophyll cycle-dependent and -independent components. In the present study, the NPQ buildup was found to be differentially suppressed in cucumber, tomato, and Arabidopsis plants after treatment with UV-B, and this suppression to be dependent mainly on the inhibition of violax-

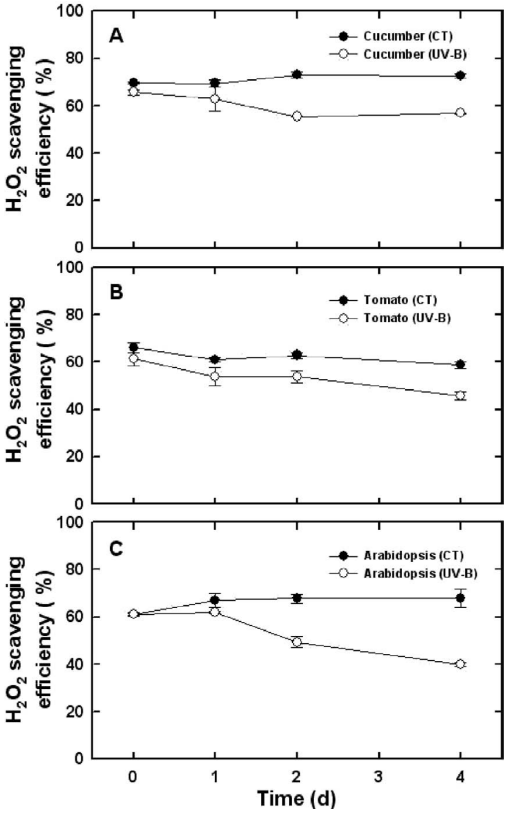

Fig. 8. Analysis of H_2O_2 scavenging efficiency using 3,3'-diaminobenzidine (DAB). Leaf extracts were prepared from the control and UV-B-treated leaves as described in Materials and Methods. They were mixed with 20 mM H_2O_2 (1:1 by vol.) and incubated at 25°C for 10 min. After 5-min centrifugation at 15,000 g and 4°C, the supernatant was further incubated at 25°C with 50 mM 3,3'-diaminobenzidine (DAB) for 30 min to quantify the remaining H_2O_2. Finally, polymerization products of DAB were estimated by obtaining the absorbance at 485 nm. H_2O_2 scavenging efficiency was expressed as percentages of $(\Delta A_{485}(\text{Blank})\text{-}A_{485}(\text{Sample}))/\Delta A_{485}$ (Blank). Data are means ± SE of three measurements. CT, control.

anthin de-epoxidation (Figs. 1 and 2; data not shown). In contrast, the xanthophyll cycle-independent NPQ, which is responsible for a large DTT-insensitive NPQ in higher plants,[34] was not a common component of the NPQ buildup affected by UV-B (Fig. 2). Unlike in tomato and Arabidopsis, this DTT-insensitive NPQ buildup was relatively constant in cucumber. These results suggest that UV-B suppress the NPQ buildup by preferentially inhibiting the xanthophyll cycle-dependent NPQ.

UV-B radiation increases the relative pool size of violaxanthin to total chlorophyll and the expression ratio of VDE to ZEP for de-epoxidation despite suppression of xanthophyll cycle-dependent NPQ via inhibition of photosynthetic activity

The inhibition of violaxanthin de-epoxidation by UV-B radiation has been reported in isolated chloroplasts and intact leaves of pea plants.[9] Although the pool size of violaxanthin available for de-epoxidation was not directly affected by UV-B radiation, a decrease in violaxanthin availability mediated by a decrease in PSII activity or by general membrane damage was suggested to inhibit VDE activity. This hypothesis has been partly supported by numerous reports of exposure to UV-B radiation causing damage to photosynthetic processes in plants.[35,36] Here, we demonstrated that the NPQ levels suppressed by UV-B radiation were almost fully restored within four days, showing correlative increases in the Fv/Fm, qP, and ETR (Figs. 1E and 4Aa–c). This result strongly supports that the sub-optimal pH for VDE activity due to a decrease in the ETR,[7,9,37] can be the main reason for the suppression of NPQ in plants exposed to UV-B radiation.

In contrast, the main components for violaxanthin de-epoxidation, e.g., violaxanthin and VDE, showed a constant trend for four days after treatment with UV-B. The relative pool size of violaxanthin to total chlorophyll and the expression ratio of VDE to ZEP for de-epoxidation were steadily increased in the three plant species and kept significantly higher than those of the control (Table 2 and Fig. 3). Taken together with the UV-B-induced suppression of xanthophyll cycle-dependent NPQ, these data support that syntheses of xanthophyll cycle pigments and enzymes could be controlled via a redox system depending on photosynthetic electron transport. An increase in the relative pool size of violaxanthin to total chlorophyll as well as in the content of anthocyanin is suggested to be an alternative defense mechanism against over-excitation and damage of photosytems due to inhibition of photosynthetic activity after treatment with UV-B. This suggestion may also hold for the increased relative pool size of lutein in cucumber after treatment with UV-B (Table 2). Recently, it was known that an increased level of lutein is substituted for zeaxanthin in qE, the major component of NPQ, in the *Arabidopsis thaliana suppressor of zeaxanthin-less1* (*szl1*) *npq1* double mutant, which has a partially restored qE but lacks zeaxanthin.[38] However, the VDE protein and activity levels during developmental maturation in tobacco leaves were not consistent with the level of VDE transcript.[39] Also, the steady-state transcript and protein levels of VDE and ZEP in Arabidopsis did not contribute to the short-term induction of the xanthophyll cycle in high light conditions.[40] Therefore, it seems that the transcript or protein level of VDE cannot represent VDE activity *in vivo*. In the present study, the differential suppression of the xanthophyll cycle-dependent NPQ in the UV-B-treated leaves of cucumber, tomato, and Arabidopsis should be alternatively attributed to distinct photosystem damages (Figs. 4 and 5).

UV-B-induced suppression of NPQ is partly responsible for increased photosystem damages of UV-B-treated leaves under high light or oxidative stress conditions

Arabidopsis plants grown in the presence of UV-B radiation are more able to down-regulate the photochemical efficiency of PSII under saturating light conditions.[11] However, the induction of photooxidative stress in the control and UV-B-treated leaves indicated that the suppression of NPQ by the inhibition of photosynthetic activity would cause a further decrease in Fv/Fm in the treated leaves under high light conditions, discriminating among the three plant species (Figs. 1, 2, and 5A). Additionally, the ratios of 685/695-nm and 685/735-nm emission, which are associated with PSII and PSI, respectively,[28–30] demonstrated the consistent high light-induced damage of photosystems in the UV-B-treated leaves, which was more pronounced than that in the control (Fig. 5B). Taken together with the increased levels of $^1O_2$ production in the VDE-deficient *npq1-2* and UV-B-treated leaves under high light conditions (Fig. 6), $^1O_2$ may be one of the main ROS to induce these photosystem damages as previously reported.[41] Especially, the increased $^1O_2$ production in the UV-B-treated leaves of three plant species can be attributed to the suppression of xanthophyll-dependent NPQ rather than the decrease of ETR.

In contrast, this suppression of NPQ is unlikely to be mainly responsible for the known photosystem damages of UV-B radiation under other oxidative stress conditions such as drought condition.[42,43] This hypothesis is based on the fact that methyl-viologen-induced photooxidative stress produced less $\bullet O_2^-$ in the UV-B-treated leaves of tomato and Arabidopsis plants than in the control, probably due to the decrease of ETR, and that H_2O_2 scavenging efficiency decreased gradually and substantially in cucumber, tomato, and Arabidopsis after treatment with UV-B (Figs. 7 and 8). Therefore, as the suppression of NPQ shows under high light conditions, a decrease in the whole H_2O_2 scavenging activity including both enzymatic and non-enzymatic components may involve in increased damages of UV-treated leaves under other photooxidative stress conditions.

In relation to the observed differences among the three plant species investigated, UV-B-inhibitable NPQ was more xanthophyll cycle-dependent in cucumber than in tomato and Arabidopsis, showing a distinct delay in recovery with the photochemical efficiency of PSII (Figs. 1, 2, and 3). The xanthophyll cycle-independent proportion of the UV-B-inhibitable NPQ was the highest in tomato, which revealed the highest level of anthocyanins before and after treatment with UV-B (Fig. 2 and Table 2). In Arabidopsis, the UV-B-inhibitable NPQ seemed to be much more important in protection from singlet oxygen-mediated photosystem damages under high light conditions than in cucumber and tomato (Figs. 5 and 6). Nevertheless, sensitization of the UV-B-treated plants to photooxidative stress should be partly attributed to changes in the antioxidant defense system including both enzymatic and non-enzymatic components (Table 2, Figs. 7 and 8).

In conclusion, UV-B treatment of cucumber, tomato, and Arabidopsis plants induces a substantial suppression of the xanthophyll cycle-dependent and/or -independent NPQ in leaves. The former suppression can be correlated with a marked decrease in photosynthetic electron transport more than xanthophyll cycle enzymes. Furthermore, our findings indicate that a reduction of antioxidant defense systems such as H_2O_2 scavenging activity as well as a transient and reversible suppression of thermal energy dissipation may contribute to distinctly increased photooxidative damages of leaves after acute exposure to UV-B radiation, discriminating among cucumber, tomato, and Arabidopsis.

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