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Inactivation of Photosystem I in Cucumber Leaves Exposed to Paraquat-Induced Oxidative Stress

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Cucumber leaves subjected to light chilling stress exhibit a preferential inactivation of photosystem (PS) I relative to PSII, resulting in the photoinhibition of photosynthesis. In light-chilled cucumber leaves, Cu/Zn-Superoxide dismutase (SOD) is regarded as a primary target of the light chilling stress and its inactivation is closely related to the increased production of reactive oxygen species. In the present study, we further explored that inactivation of PSI in cucumber leaves is not a light chilling specific, but general to various oxidative stresses. Oxidative stress in cucumber leaves was induced by treatment of methylviologen (MV), a producer of reactive oxygen species in chloroplasts. MV treatment decreased the maximal photosynthetic O_2 evolution, resulting in the photoinhibition of photosynthesis. The photoinhibition of photosynthesis was attributable to the decline in PSI functionality determined in vivo by monitoring absorption changes around 820 nm. In addition, MV treatment inactivated both antioxidant enzymes Cu/Zn-superoxide dismutase and ascorbate peroxidase known sensitive to reactive oxygen species. From these results, we suggest that chloroplast antioxidant enzymes are the primary targets of photooxidative stress, followed by subsequent inactivation of PSI.

Key words: Methylviologen, photosystem I, photoinhibition, Cu/Zn-superoxide dismutase, Ascorbate peroxidase, cucumber

INTRODUCTION

Low temperature is a major factor limiting the productivity and geographical distribution of many species. Chilling (0 – 12ºC) is common during the growing season in temperate regions and can substantially decrease plant productivity. Many temperate-climate crops such as tomato, rice, cucumber, and mango come from a tropical and subtropical evolutionary background [1].

In chilling-sensitive plants as in chilling-resistant plants, the combination of low temperature with high light enhances the induction of photodamage [2], resulting from the inhibition of photosynthetic capacity, repair mechanisms, xanthophyll-cycle-related energy dissipation, or antioxidant enzyme activity [3]. While PSII is a photoinhibitory site for chilling resistant plants [4,5], PSI in chilling sensitive plants, at least in cucumber leaves, is regarded as the main site for photoinhibition at chilling temperatures [4,6]. However, the evidence that PSI activity declines to a greater extent than PSII is not necessarily sufficient to identify PSI as a primary target of chilling [7]. Indeed, the antioxidant system downstream of photosynthetic electron transport is the primary target to light chilling stress. Further, the photoinhibitory mechanism of PSI is likely to be related to the reduction state of the photosynthetic electron transport chain, suggesting photoinactivation of PSI is more or less similar to that of PSII [7].

Chloroplast is the major source of reactive oxygen species (ROS) in leaf cells. The rate of O_2^- production is determined by the rate of light absorption into, and the consumption of electrochemical energy. Under the conditions where various abiotic stresses such as drought, low temperature, air pollutants, or chemicals hinder the consumption of reducing power, the fraction of electron flux to oxygen is increased. When these unfavorable conditions are combined with high light, the production of superoxide and then H_2O_2 catalyzed by superoxide dismutase (SOD) is enhanced. These ROS are highly reactive and toxic, and hence quite capable of damaging various cellular components, especially photosynthetic components and various antioxidant enzymes. Chloroplasts are equipped with multiple defense systems for detoxifying such oxidative species [8]. ROS are scavenged (1) chemically by antioxidant molecules such as ascorbate, glutathione, tocopherols, carotenoids, polyphenols and flavonoids which chemically scavenge free radicals and reactive oxygen species, and (2) enzymatically by SOD and ascorbate peroxidase (APX) which scavenge O_2^- and H_2O_2, respectively.

Recently, we showed in cucumber leaves subjected to light chilling stress that the increase in leaf H_2O_2 contents is directly proportional to the reduction state of PSII, and might play a role in the inactivation of Cu/Zn-SOD [7]. Accordingly, it was

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suggested that light chilling-induced oxidative stress would lead to photoinhibition of PSI via dysfunction of antioxidant enzymes [7]. In the present study, we addressed further this view that both PSI and antioxidant enzymes of cucumber leaves are vulnerable to another form of oxidative stress induced by methylviologen (MV). In chloroplasts, MV catalyzes the photoreduction of O_2 at PSI and hence accelerates the production of O_2^- and H_2O_2 [9]. Here, we report that MV inactivates Cu/Zn-SOD and APX activities. As a result of inactivation of these antioxidant enzymes, PSI activity is degenerated and leads to the photoinhibition of photosynthesis.

MATERIALS AND METHODS

Plant Material

Cucumber (*Cucumis sativus* L. cv Eunhwa) was grown from seed in a soil used in horticulture that contains various inorganic and organic compounds for plant growth (Sangto, Bunong Co, Korea) and watered every second day. Cucumber plants were grown in growth chambers (16 h light/28°C; 8 h dark/25°C) under fluorescent light (FL40D, WooRi Co, Korea). Light intensity at the upper surface of leaves was 100 mmol photons m^{-2} s^{-1}. To ensure uniformity of leaves, the primary leaf pairs, representing the youngest fully expanded pair of leaves of 20 – 22 days old plants, were collected in the morning at about one to two hours into the photoperiod.

Paraquat treatments

Leaf disks (each 0.79 cm^2) were floated on 10 μM methyl viogoen under the low light illumination (100 μmol photons m^{-2} s^{-1}) at 25°C. The treatment light was provided by a halogen lamp (MH250WB, Mia Co. Korea) and the heat was eliminated by a 3 cm-layer of water between the light source and samples.

Determination of chlorophyll fluorescence parameters

The maximum efficiency of PSII was estimated from the chlorophyll fluorescence ratio, Fv/Fm, at room temperature. After light-chilling treatment at 100 μmol photons m^{-2} s^{-1} at various low temperatures, leaf discs were dark-treated for 30 min in leaf clips of a Hansatech Plant Efficiency Analyzer and excitation light was then given at 80% of maximum (red light, peak at 650 nm, 2800 μmol photons m^{-2} s^{-1}) for 2 s by using a Hansatech Plant Efficiency Analyser. Analysis of fluorescence quenching parameters during illumination (qP and NPQ) was conducted with a Xe-PAM fluorometer (Heinz Walz, Effeltrich, Germany). Excitation pressure on PSII was calculated as (1 – qP), where qP is the coefficient of photochemical quenching as defined [10]. The non-photochemical quenching parameter (NPQ) was calculated as (Fm/Fm' – 1), where Fm and Fm' are maximum fluorescence yields after dark incubation for 30 min and during illumination at 100 μmol photons m^{-2} s^{-1}, respectively. Electron flux through PSII was estimated as (1 – F/Fm') x 0.5 x irradiance x leaf absorptance, where F is the steady state fluorescence, 0.5 is a factor assuming that PSII receives half of the absorbed photons and leaf absorptance was taken as 0.9.

Determination of photosynthetic O_2 evolution

Light-response curves of photosynthetic O_2 evolution during illumination were determined with a leaf-disc O_2 electrode (Oxygraph system, Hansatech) in air with 5% CO_2 at 25°C. Various irradiances were provided using neutral density filters and the temperature was kept constant at 25°C. The chlorophyll in leaf discs was determined from aqueous buffered 80% acetone extracts (25 mM Hepes, pH 7.5), as in [11].

P700 measurements

The redox state of P700 leaf disk was determined *in vivo* using a PAM-101 modulated fluorometer (Heinz Walz GmbH, Effeltrich, Germany) equipped with ED P700DW emitter-detector and PAM Data Acquisition System (PDA-100). Far red light (FR; λ_{max} = 735 nm, 10 W m^{-2}, Schott filter RG 715) was provided by a 102-FR light source. At approximately 10 minutes following the cessation of a light-chilling treatment, the content of photooxidizable P700 was evaluated as the absorbance change around 820 nm (reference wavelength 860 nm) in a leaf-disc electrode (LD2, Hansatech) at the growth temperature 25°C. The signals were recorded using a PDA-100. Data analysis was performed using a Microcal™ Origin™ Version 6.0 software package (Microcal Software Inc., Northampton, MA, USA).

Isozymes of superoxide dismutase and ascorbate peroxidase

For SOD enzyme extraction, 20 leaf discs (each 0.79 cm^2) were frozen in liquid nitrogen and ground to power in pre-cooled mortars. The powder was homogenized in 0.5 m*l* of extraction buffer containing 50 mM potassium phosphate buffer (pH 7.8), 10 mg ml^{-1} ascorbate, 5 μ*1* ml^{-1} 2-mercaptoethanol, and 1 mM phenylmethylsulfonylfluoride (PMSF). The resulting homogenate was transferred to pre-cooled Eppendorf tubes and centrifuged at 12,000 g at 4°C for 5 min. SOD isozymes were separated by non-denaturating PAGE. Enzyme crude extracts (100 μg per well) were loaded on 12% gels and run at 4°C. SOD isozymes on the gels were detected by the nitroblue tetrazolium (NBT) reduction by superoxide radicals generated photochemically as described [12]. After electrophoresis, the gels were covered with a solution containing 0.25 mg ml^{-1} NBT and 0.1 mg ml^{-1} riboflavin, and then exposed to light. The two types of SOD, Mn-SOD and Cu/Zn-SOD were identified using inhibitors. Mn-SOD was diagnosed by its insensitivity to 5 mM H_2O_2 and 1 mM KCN, while Cu/Zn-SOD was sensitive to 1 mM KCN [13]. Activity staining patterns of the enzyme isozymes were analyzed using a GELDOC2000 Biorad densitometer and a computer-aided image analysis system. The isozyme activity as a percentage relative to the control was quantified by recording the transmittance of the gels.

Zymograms of ascorbate peroxidase (APX) activity were measured mainly based on the method [14]. Crude extracts obtained for measuring SOD isozymes was used and loaded at 150 μg protein per well on 12% gels. After separating proteins,

APX isozymes were detected by equilibrating the gels in a solution composed of 50 mM Na-phosphate buffer (pH 7.0), 4 mM ascorbate and 2 mM H_2O_2 for 20 min. After washing with 50 mM sodium phosphate buffer (pH 7.0), gels were submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8) with 28 mM TEMED and 1.25 mM NBT for 10 min at room temperature.

RESULTS

Electron flux through PSII, excitation pressure on PSII, NPQ and photosynthesis to MV-induced stress

Photosynthetic performance of cucumber leaves treated with 10 μM MV under the low light illumination (100 μmol $m^{-2}s^{-1}$) was investigated. Following MV treatments for 2 hrs, cucumber leaves were dark adapted for 30 min in the leaf disk chamber and then subjected to 5 min of illumination (100 μmol m^{-2} s^{-1}) in air. The fate of absorbed light in MV-treated cucumber leaves was estimated by analyzing chlorophyll fluorescence quenching under steady-state photosynthesis. Chlorophyll fluorescence parameters F, Fm', and Fm were obtained and then used for the calculation of noncyclic electron transport flux through PSII, excitation pressure, nonphotochemical quenching and the photosynthetic O_2 evolution rate (Table 1). Compared to control leaves, MV-fed cucumber leaves showed significant decreases in the PSII quantum yield, electron flux at PSII, and hence maximal photosynthetic O_2 evolution. However, the amount of the reduced primary electron acceptor of PSII, Q_A and the nonradiative dissipation of excitation energy as heat were slightly affected by MV-treatment.

PSII and PSI activities to MV-induced stress

In order to see this MV-induced photoinhibition of photosynthesis in cucumber is closely related to photoinhibition of PSI rather than PSII as in light chilling stress [7], we determined the quantum yield of PSII and photooxidizable P-700 in vivo (Fig. 1A and 1B). The maximal quantum yield of PSII in vivo was estimated as a fluorescence parameter, Fv/Fm. As shown in Fig. 1A, photoinhibition of PSII in cucumber leaves hardly occurred under MV-stressed conditions. Instead, the relative amount of $P700^+$ determined in vivo by monitoring the leaf absorptance change around 820 nm upon far-red excitation ($\Delta A_{820}/A_{820}$) was decreased greatly compared to PSII (Fig. 1B). When cucumber leaves were, for instance, illuminated by low light at 100 μmol $m^{-2}s^{-1}$ for 2 hrs at 25ºC, the maximum quantum yield of PSII decreased by less than 4.5%, but that of PSI decreased by 33.8%.

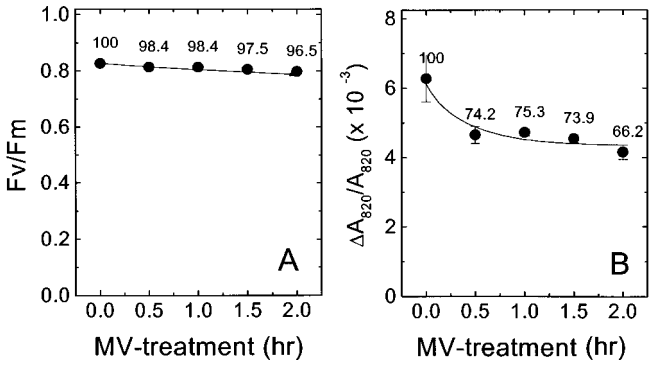

Figure 1. A, Maximal quantum yield of PSII (Fv/Fm) and B, the extent of $P700^+$ ($\Delta A_{820}/A_{820}$) as a function of MV treatment (hr) at an irradiance of 100 μmol m^{-2} s^{-1} in cucumber leaves. Mean values (± SE) for 5 – 7 leaf disks are shown.

SOD and APX activities to MV-induced stress

Active oxygen species generated in chloroplasts are effectively scavenged by scavenger enzymes Cu/Zn-SOD and APX, which are prone to paraquat-induced oxidative stress in wheat [15] and spinach [16]. In order to see any changes in Cu/Zn-SOD and APX under MV-induced stress, gel assay systems were employed using crude enzymes isolated from leaves exposed to various stress conditions as mentioned above. As shown in Fig. 2A, changes in SOD activities from cucumber leaves were observed in native PAGE and subsequent activity staining. Band I was identified as Mn-SOD by its insensitive to both inhibitors, KCN and H_2O_2. Bands 2, and 3 were inhibited by KCN and H_2O_2, and hence identified as Cu/Zn-SOD. Fe-SOD, an abundant isoform in tobacco [17], was not expressed in detectable amounts in cucumber leaves [7]. In contrast with the lack of any effect of light chilling on the activity of mitochondrial Mn-SOD, MV-feeding apparently decreased chloroplastic Cu/Zn-SOD activity.

Ascorbate peroxidase (APX) is an H_2O_2-scavenging peroxidase that uses ascorbate as an electron donor and is inactivated by a suicide mechanism by free radicals generated by H_2O_2 and by itself at nanomolar levels when the ascorbate concentration is low [18]. Ascorbate peroxidase was also significantly affected by MV treatment (Fig. 2B) though light-chilling stress hardly induced any significant changes in activity [7].

Table 1. MV-induced changes in the actual quantum yield of PSII (1 - F/Fm'), electron flux at PSII [(1 – F/Fm')× 100× 0.5× 0.9], PSII excitation pressure (1 – qP), nonphotochemical quenching (NPQ), and maximal photosynthetic O_2 evolution (Pmax, μmol O_2 $m^{-2}s^{-1}$) of cucumber leaves. Leaf disks were floated on DW (CO) or 10 μM methylviologen (MV) for 2 hrs at an illumination of 100 μmol m^{-2} s^{-1} at 25ºC. Mean values (± SE) for 7 – 9 leaf disks are shown.

Parameter	1 – F/Fm'	Electron flux at PSII	1 – qP	NPQ	Pmax
CO	0.63 ± 0.03	45.63 ± 1.26	0.21 ± 0.04	0.51 ± 0.09	14.92 ± 0.62
MV	0.49 ± 0.03	22.19 ± 1.40	0.29 ± 0.04	0.35 ± 0.04	10.75 ± 0.38

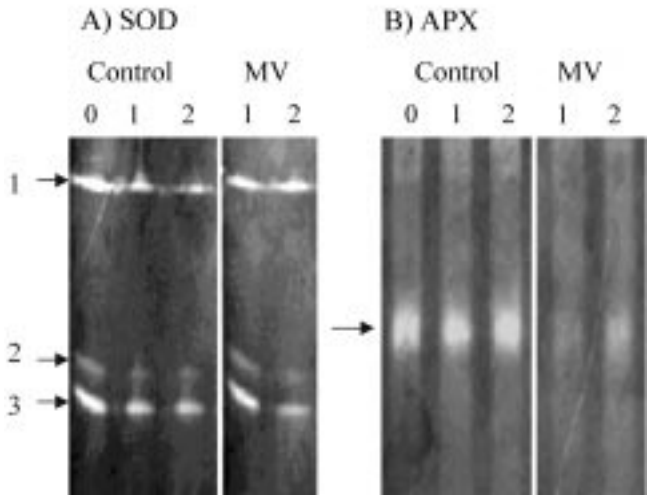

Figure 2. Zymograms for superoxide dismutases (A) and ascorbate peroxidase (B) from the cucumber leaves exposed MV for 2 hrs at an irradiance of 100 μmol m^{-2} s^{-1}. In A, Band 1 was identified as Mn-SOD, and Bands 2 and 3 as Cu/Zn-SODs. Each number in the panel represents period (hr) for MV treatment.

DISCUSSION

Photoinhibition of photosynthesis in MV-treated cucumber leaves with respect to the photosynthetic O_2 evolution (Table 1) is largely accompanied by inactivation of PSI (Fig. 1B) not of PSII (Fig. 1A). The inhibition of PSI activity appears due to the inactivation of PSI protective mechanism, chloroplast-scavenging enzymes. The most probable candidate for oxidative stress-sensitive step is thylakoid-bound APX and SOD since they scavenge superoxide and hydrogen peroxide, and are located near the PSI reaction center [8]. As expected, Cu/Zn-SOD and APX activities were severely inhibited by MV-treatment.

Given that Cu/Zn-SOD and APX could be vulnerable targets of MV-stress, their inactivation could initiate events that lead to dysfunction in PSI. For example, as the dismutation of superoxide radicals slowed down, the concentration of O_2^- would increase, favoring the reaction

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + HO^{\cdot}$$

The hydroxyl radicals, being much more reactive than O_2^- and H_2O_2 , might then cause widespread damage to PS I [6,8].

This finding is contrast to the previous result for light chilling stress that inactivates Cu/Zn-SOD activities without significant effect on APX [7]. We do not know the reason for these differences between the two findings at present, but it is likely that the ratio of NADPH/$NADP^+$ would explain this discrepancy. Though both paraquat treatment and light-chilling stress induce the production of highly ROS, there is some difference in terms of generating reducing power, NADPH, which is required for the regeneration of reduced ascorbic acid in addition to its involvement in the CO_2 fixation pathway. That is, NADPH production in paraquat-fed conditions hardly occurs as electrons from PSI mainly flow to methyl viologen rather than $NADP^+$, but in light-chilling stress leaves, the NADPH level remains higher as the Calvin cycle is not properly operating. It is well known that the sensitivity of APX to H_2O_2 highly depends on ascorbic acid [16]. Therefore, the lowered ascorbic acid content, due to inhibited regeneration of NADPH in paraquat-induced oxidative stress would lead to the preferentially inactivation of APX as reported.

In conclusion, we showed that Cu/Zn-SOD and APX are primarily inactivated in cucumber leaves upon expose to MV-induced photooxidative stress. With the loss of ability to scavenge O_2^- and H_2O_2 by SOD and APX, respectively, photoprotection of PSI by those enzymes would be dysfunctional, resulting in the preferential inactivation of PSI relative to PSII.

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