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Decrease of Photochemical Efficiency Induced by Methyl Viologen in Rice (*Oryza sativa* L.) Leaves is Partly due to the Down-Regulation of PSII

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In the rice leaves treated with methyl viologen (MV), the photochemical efficiency of PSII (or F_v/F_m) was significantly decreased, and significant portion of the photoinactivation process was reversible during the dark-recovery. The dark-reactivation process was relatively slow, reaching its plateau after 2-2.5 h of dark incubation. The damaged portion of functional PSII was 13%, based on the value of $1/F_0$-$1/F_m$ after this dark-recovery period. The reversible photoinactivation process of PSII function in the MV-treated leaves consisted of a xanthophyll cycle-dependent development of NPQ and a xanthophyll cycle-independent process. The latter process was reversible in the presence of nigericin. As well as the increase in the values of Chl fluorescence parameters, the epoxidation process during the dark-recovery after the MV-induced photooxidation was very slow. These results suggest that the photooxidative effect of MV is partly protected by the down-regulation of PSII before inducing physical damages in core proteins of PSII.

key words: methyl viologen, chlorophyll fluorescence, down-regulation of PSII, reversible photoinactivation, dark-recovery, photosynthesis

INTRODUCTION

Methyl viologen (MV), a potent herbicide, has been long used as a model chemical to study photooxidative stress [1,2]. According to Fujii *et al.* [3], MV produces superoxide ($\cdot O_2^-$) at photosystem I (PSI) sites through photoreduction of dioxygen using electrons from the iron-sulfur clusters Fe-S_A/Fe-S_B of PSI, causing depletion of NADPH and the inhibition of CO_2 fixation [4,5]. Reactive oxygen species (ROS) cascade, which is responsible for photooxidative stress, starts with dismutation of $\cdot O_2^-$ into H_2O_2 by superoxide dismutase. When catalytic free metals like Fe(II) or Cu(I) exist near to the production site, the most toxic form of ROS, hydroxyl radical ($\cdot$OH), will be unavoidably produced from both ROS. These ROS affect the entire photosynthetic machinery, causing the membrane leakage by the lipid peroxidation and/or inducing the disassembly of photosystems by the degradation of chlorophylls and proteins. Because of this action mechanism of MV, the photooxidative effect of MV has been thought to be mainly irreversible damages in photosystems through the ROS-mediated reactions.

Therefore, little attention has been given to the reversible mechanism after MV-induced photooxidative stress except our recent report by Kim *et al.* [6]. However, the results in this study suggest that reversible mechanisms are also partly involved in the photoinactivation of PSII by MV, in addition to the well-known direct damage to core proteins of PSII.

In general, reversible photoinactivation or down-regulation of PSII has been related with reversible phosphorylation of major light harvesting complex II (LHCII) [7] or a minor inner antenna chlorophyll-protein complex (CP29) [8,9], xanthophyll cycle dependent non-photochemical quenching (NPQ) [10,11,12,13], a reversible conformational change of D1 proteins [14] and the energy-dependent NPQ [15]. MV will provide favorable conditions for the xanthophyll cycle de-epoxidation by enhancing the photosynthetic electron transport rate (ETR) and accelerating the trans-thylakoid proton gradient [16,17,18]. Reversible recovery of PSII from photoinhibiton has been reported in various experimental conditions and materials [12,10,19,20,21]. Generally, the recovery mechanisms have been associated with a direct reactivation of PSII possibly through a reversible conformational change of D1 proteins [14] or related to the relaxation of energy-dependent NPQ [15]. There are also numerous reports that in leaves exposed to excess light, the development of NPQ is correlated with the accumulation of de-epoxidized xanthophylls [22,23]. Moreover, in the recovery from severe photoinhibition, a close relationship between epoxidation of zeaxanthin and PSII recovery was reported in pea [12] and in shade leaves of *Scheffera arboricola* [13].

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MATERIALS AND METHODS

Plant Material

Rice (*Oryza sativa* L. cv. Dongjin-byeo) plants were grown in a growth chamber with a photosynthetic photon flux density (PPFD) at pot level of 100 μmol photons m^{-2} s^{-1} supplied by metal halide lamps. The growth chamber was maintained at 28/

23°C (day/night) with a 14 h photoperiod. For all experiments, rice leaf segments in length of 3 cm were excised from fully expanded leaves of 4-6-week-old seedlings.

Chemical treatment and dark-recovery

Rice leaf segments were floated on distilled water or a chemical solution with abaxial side down under a PPFD of 50 μmol $m^{-2} s^{-1}$ at 28°C for 1 h and then in darkness for 1 h (preincubation). The latter dark-preincubation was applied to eliminate the zeaxanthin produced during the former light-preincubation. After 2 h pre-incubation, the leaf segments were further exposed to 150 μmol light $m^{-2} s^{-1}$ for 3 h at 28°C to induce photooxidative stress (photooxidative treatment). Finally, after 3 h photooxidative treatment, the leaf segments were allowed to recover in darkness at room temperature for 2 h (dark-recovery), except for 3 h in the recovery kinetics experiment. Chemical solutions used are: 10 μM MV, 20 μM nigericin (Nig), and their mixture (MV/Nig).

Measurement of chlorophyll (Chl) fluorescence

The photooxidative effect of MV to PSII was monitored by measuring the ratio of variable fluorescence (F_v) to maximum yield of fluorescence (F_m) with a portable fluorometer (Plant Efficiency Analyzer, Hansatech Instrument, Norfolk, UK) after dark adaptation for 10 min at room temperature. The variable fluorescence was obtained by subtraction of the initial Chl fluorescence (F_0) from the maximum yield of fluorescence. To use $1/F_0 - 1/F_m$ as a measure of functional PSII units, all fluorescence yields (F_0 and F_m) were normalized to the mean F_0 value of controls according to Kim *et al.* [24]. The physical meaning of this value is true only when the two parameters were measured after a full-dark-recovery period.

Pigment Analysis

Photosynthetic pigments were analyzed according to Gilmore and Yamamoto [25]. Five leaf segments were frozen in liquid nitrogen and ground with a mortar and pestle in ice-cold 100% acetone. The pigment extracts were filtered through a 0.2 μm syringe filter. Pigment separation was performed in a HPLC system (HP 1100 series, Hewlett Packard, Waldbronn, Germany) on a Spherisorb ODS-1 column (Alltech, USA) as described by Gilmore and Yamamoto [25]. Concentrations of the pigments were estimated by using the conversion factors for peak area to nanomoles as determined for this solvent mixture by Gilmore and Yamamoto [25]. The de-epoxidation index (D_i) was expressed as a percent value of $A \times 0.5+Z$ of V+A+Z, where A is antheraxanthin, Z is zeaxanthin, and V is violaxanthin.

RESULTS

Decrease of photochemical efficiency of PSII by MV

The photooxidative damage in PSII was monitored by the decrease of photochemical efficiency or F_v/F_m, which is frequently used to imply PSII activity. The treatment of MV at 2.5 μM, a relatively low concentration, was enough to induce a significant decrease of the photochemical efficiency, showing the saturated effect of MV (Figure 1). This decrease may be closely related to the irreversible inactivation of PSII or the loss of D1 protein. However, when the loss of D1 proteins was measured, this effect of MV was not saturated at 2.5 μM but largely dependent on the concentration of MV used [6]. This result implies that the decrease of F_v/F_m caused by MV can be partly attributed to a different mechanism from the loss of D1 proteins.

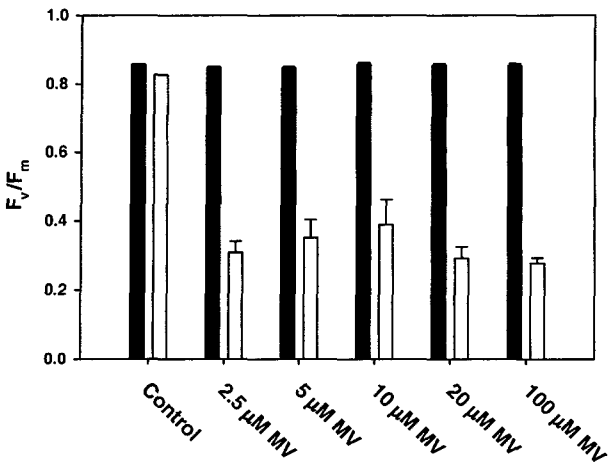

Figure 1. Decrease of photochemical efficiency of PSII by MV. Leaf segments were floated on distilled water or MV solutions with different concentrations (2.5, 5, 10, 20 and 100 μM) during the preincubation and photooxidative treatment as described in Materials and Methods. Black and white bars imply F_v/F_m values after the preincubation and the photooxidative treatment, respectively. The F_v/F_m values were measured after 10 min dark-adaptation at room temperature. Error bars indicate SE (n = 3).

Changes in Chl fluorescence parameters after the dark-recovery

In Figure 2, the reduced F_v/F_m by photooxidative treatment could be significantly recovered after 2 h dark-recovery, confirming that a considerable proportion of the photo-inactivation of PSII caused by MV is reversible. But a significant proportion still remains not recovered after the dark-recovery, implying the irreversible damage to D1 proteins.

As shown in Figure 2A and B, the maximum yield of fluorescence (F_m) contributed to the recovery of F_v/F_m more than did the initial Chl fluorescence yield (F_0). A Chl fluorescence parameter, $1/F_0-1/F_m$, also increased during the dark-recovery (Figure 2D). This parameter can be used as a measure of functional PSII units when the down-regulated PSII is fully reactivated in darkness [24].

Time course of Chl fluorescence parameters during the dark-recovery

The kinetics of Chl fluorescence parameters was investigated

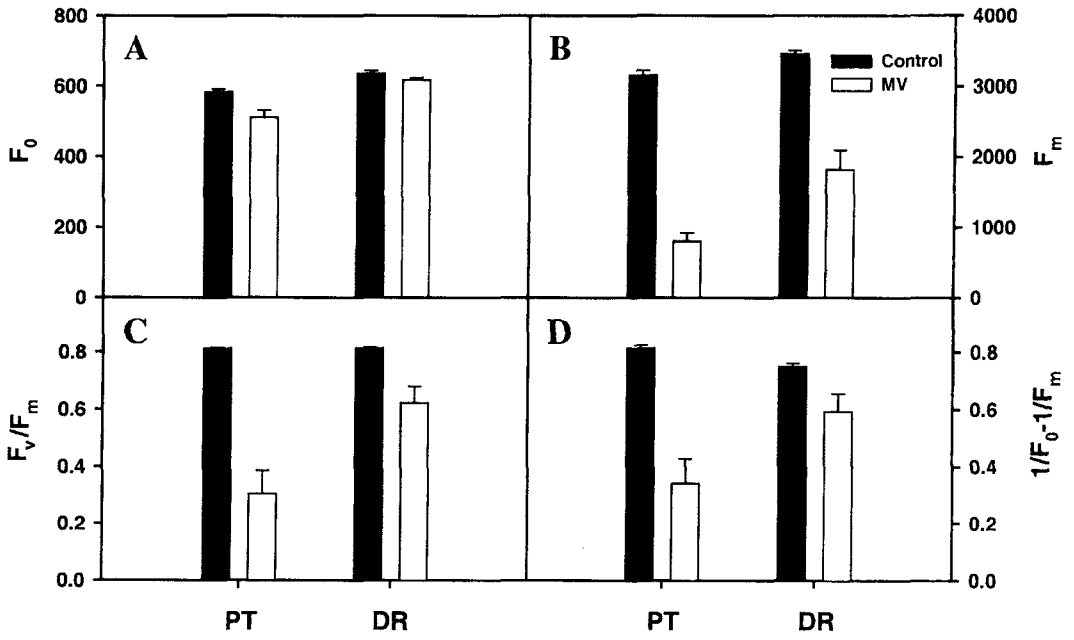

Figure 2. Changes in Chl fluorescence parameters (F_0, F_m, F_v/F_m and $1/F_0-1/F_m$) after the dark-recovery. Leaf segments were floated on distilled water or a 10 μM MV solution during the preincubation, photooxidative treatment and dark-recovery as described in Materials and Methods. A, F_0; B, F_m; C, F_v/F_m; D, $1/F_0-1/F_m$. PT, photooxidative treatment; DR, dark-recovery. The initial Chl fluorescence (F_0) and the maximum yield of fluorescence (F_m) were measured after dark-adaptation for 10 min at room temperature. Error bars indicate SE (n=5).

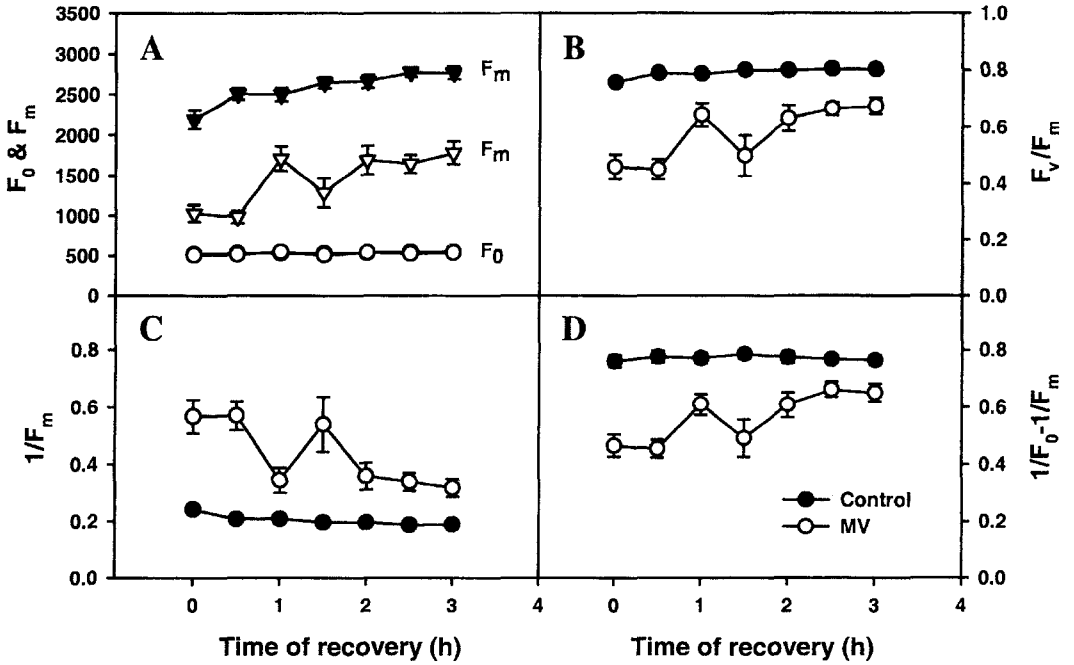

Figure 3. Time course of the changes in the values of Chl fluorescence parameters (F_0, F_m, F_v/F_m, $1/F_m$ and $1/F_0-1/F_m$) during the dark-recovery. The experimental conditions (preincubation, photooxidative treatment and dark-recovery) are the same as in Figure 2. However, the time for the dark-recovery was extended to 3 h. A, F_0 and F_m; B, F_v/F_m; C, $1/F_m$; D, $1/F_0-1/F_m$. The F_0 and F_m values were measured after dark adaptation for 10 min at room temperature. Error bars indicate SE (n=10-12 from two different experiments).

during the recovery in darkness for 3 h. The F_0 did not significantly change, but a gradual increase of F_m was observed in both control and MV-treated leaves (Figure 3A). However, the increase of F_m was much more pronounced in the MV-treated leaves, and therefore the increase of F_v/F_m during the dark-recovery depended mainly on the kinetics of F_m. This increase

of F_m reflects the relaxation of NPQ, as shown in the changes of an alternative parameter, $1/F_m$ (Figure 3C), that is known as an actual measure of NPQ [26,27,28,29]. In Figure 3, the recovery kinetics reached a plateau after 2-2.5 h. Supposed that after this time, the down-regulated PSII was fully reactivated, the parameter, $1/F_0 - 1/F_m$, can be used to imply the functional PSII content. Therefore, it is thought that the damaging effect of MV in PSII function was only as low as 13% (Figure 3D).

Change in the de-epoxidation index (D_i) by MV

One of the mechanisms for the PSII down-regulation is related to the accumulation of zeaxanthin, which can dissipate the excess light under photoinhibitory conditions as a fluorescence quencher. In Figure 4, the D_i increased up to 52% in the MV-treated leaves after the photooxidative treatment, implying a significant accumulation of zeaxanthin, but it decreased only slightly to 42% after the dark-recovery. The accumulation of zeaxanthin can be explained by the rapid and sustained development of proton gradient across thylakoid membranes during the photooxidative treatment as discussed by Kim *et al.* [6]. Our preliminary results show that the increase of the D_i could be reached to a plateau within 30 min (data not shown). Compared with an about 30% decrease of the D_i during the dark-recovery after light-chilling by Xu *et al.* [30], there was only 10% decrease for 2 h dark-recovery in MV treated leaves (Figure 4).

Effect of nigericin (Nig) on the increase of F_v/F_m during the dark-recovery

As we have mentioned, the reversible decrease of F_v/F_m is probably due to the down-regulation mechanisms closely related with the development of inter-thylakoid proton gradient. However, when the conversion of violaxanthin (V) into the de-epoxidized forms, antheraxanthin (A) and zeaxanthin (Z),

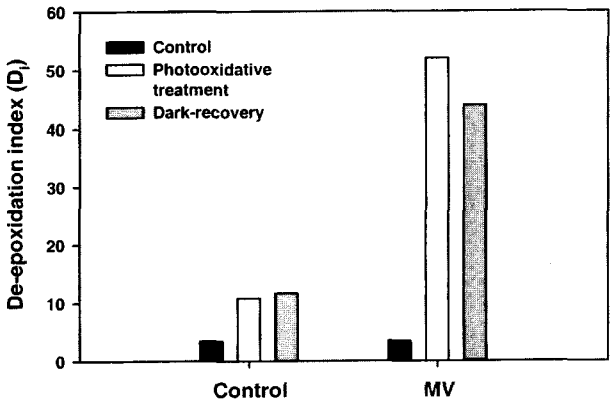

Figure 4. Changes in the de-epoxidation index (D_i) after the photooxidative treatment and after the subsequent dark-recovery. The experimental conditions (preincubation, photooxidative treatment and dark-recovery) are the same as in Figure 2. The D_i was expressed as a percent value of A× 0.5+Z of V+A+Z, where A is antheraxanthin, Z is zeaxanthin, and V is violaxanthin.

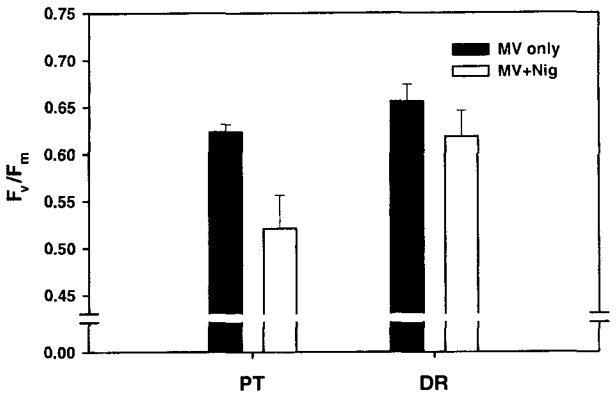

Figure 5. Effect of nigericin (Nig) on the increase of F_v/F_m during the dark-recovery after MV-induced photooxidation. The experimental conditions (preincubation, photooxidative treatment and dark-recovery) are the same as in Figure 2. PT, photooxidative treatment; DR, dark-recovery. Error bars indicate SE (n=3).

was blocked by the treatment of Nig, the photochemical efficiency was more depressed during the photooxidative treatment, and the decreased photochemical efficiency could be recovered significantly, but not to the level in the control leaves treated with MV only (Figure 5). During the dark-recovery, the increase of F_v/F_m in the MV treated leaves could probably be attributed to the xanthophyll cycle-dependent PSII down-regulation. In the MV/Nig-treated leaves, the recoverable portion of F_v/F_m by dark-recovery could probably be attributed to the xanthophyll cycle-independent PSII down-regulation. This xanthophyll cycle-independent process is supposed to occur in the presence of Nig (Figure 5). The rest unrecoverable portion is probably due to irreversible damage in PSII reaction centers.

DISCUSSION

From the increase in Chl fluorescence parameters showing the reactivation of PSII during the dark-recovery, we noticed the existence of reversible mechanisms in the photoinactivation of PSII by MV, in addition to the well-known direct damage in core proteins of PSII.

This result is also supported by our recent report [6], showing that the decrease of F_v/F_m by MV was not consistent with the degree of the loss of D1 proteins, a core protein of PSII. In this experiment, the treatment of 10 μM MV in rice leaves for 3 h caused only 13% loss of the functional PSII content. However, there were few reports about the recovery from the MV-induced photoinactivation of PSII.

The increase of F_v/F_m observed during the dark-incubation in the MV-treated leaves could be associated with a direct reactivation of PSII possibly through a reversible conformational change of D1 proteins [14], the relaxation of energy-dependent fluorescence quenching [15], or the relaxation of other NPQs

developed during the photooxidation process, while the unrecovered portion of F_v/F_m is thought to be dependent on the D1 metabolism [19,31]. The involvement of the latter possibility is skeptic, because the *de novo* synthesis of D1 protein requires the presence of light.

The reversible part of MV-induced photoinactivation is probably due to the down-regulation mechanisms closely related with the development of inter-thylakoid proton gradient. In the presence of MV, the increased efficiency of electron capture will enhance the photosynthetic electron transport rate (ETR) and accelerate the acidification of the thylakoid lumen, providing favorable conditions for xanthophyll cycle de-epoxidation [16,17,18] as proved in Figure 4.

The recovery process was relatively slow in the MV-treated leaves, and the full recovery process took about 2-2.5 h of dark incubation (Figure 3) as compared with 30 min for the recovery from light-chilling in cucumber leaves [24]. The recovery kinetics was also observed in the recovery from low temperature photoinhibition [10,12,19,20,21]. Although an initial major recovery process may be finished within this short period of time, the complete dark-recovery process took often more than several hours. However, leaf senescence also takes place during the dark-recovery, and therefore the duration of the dark-recovery process should be less than a day. In this study, the value of $1/F_0$-$1/F_m$ measured with the dark-recovery more than 2 h can only be used to imply the functional PSII content. Interestingly, the relaxation of de-epoxidation state in the MV-treated leaves during the dark-recovery was also very slow (Figure 4).

Several candidates for the slow relaxation of the de-epoxidation state in the MV-treated leaves during the dark-recovery could be found from the literatures explaining slow epoxidation during the dark-recovery after light-chilling. In the recovery from low temperature photoinhibition, the inhibition of epoxidation could be attributable to a sustained trans-thylakoid proton gradient [32], or to the limitation of co-substrates, NADPH and FAD, for epoxidase [33]. However, the change in the de-epoxidation index was relatively small, compared with the changes in Chl fluorescence parameters shown in Figure 3. Recently, we have shown that the reactivation of PSII could occur even after blocking the epoxidation of zeaxanthin during the dark-recovery after light-chilling [30]. Therefore, we suggested non-direct causal relationship between the epoxidation of zeaxanthin and the reactivation of PSII during the dark-recovery after light-chilling, and the same would be true for the recovery process after the MV-induced photooxidation.

After the dark-recovery, the recoverable portion of PSII photoinactivation in the MV/Nig-treated leaves was attributed to the xanthophyll cycle-independent PSII down-regulation (Figure 5). As a candidate for this mechanism, many reports have supported that reversible phosphorylation of LHCII, CP29, or D1 protein could be implicated in down-regulation of PSII [7,8,9,14]. However, the xanthophyll cycle-independent PSII down-regulation process seemed to be Nig-independent.

In conclusion, we suggest that 1) the photooxidative effect of MV is partly protected by the down-regulation of PSII before the damage in PSII core proteins occurs, 2) the reversible photoinactivation process of PSII function in the MV-treated leaves involves the xanthophyll cycle-dependent development of NPQ and a xanthophyll cycle-independent process, possibly phosphorylation of thylakoid phosphoproteins, 3) the recovery process was relatively slow, reaching its plateau after 2-2.5 h of dark-recovery period. These results suggest that the photooxidative effect of MV is partly protected by the down-regulation of PSII before inducing physical damages in core proteins of PSII. The reason for this slow recovery remains for further investigation.

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ABBREVIATIONS

Chl, Chlorophyll; PSII, photosystem II; F_m, maximal fluorescence; F_0, minimal or initial fluorescence; F_v, variable fluorescence; MV, methyl viologen