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Chloroplastic ascorbate peroxidase is the primary target of methylviologen-induced photooxidative stress in spinach leaves: its relevance to monodehydroascorbate radical detected with in vivo ESR

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Abstract

Methylviologen (MV) induces oxidative damages in leaves. In order to understand its mechanism we studied initial biochemical events under light in MV-fed spinach leaves. When isolated chloroplasts were illuminated in the presence of MV, both stromal and thylakoid-bound ascorbate peroxidases (APX) were inactivated rapidly at the same rates, and their inactivation was retarded by ascorbate (AsA) at higher concentrations. Since MV accelerates the photoproduction of O_2^- in Photosystem (PS) I and simultaneously inhibits the photoreduction of monodehydroascorbate (MDA) to AsA, the inactivation of APX was attributed to the loss of AsA and accumulation of H_2O_2 in the stroma. Following APX, superoxide dismutase and $NADP^+$-glyceraldehyde 3-phosphate dehydrogenase, both of which are vulnerable to H_2O_2, were inactivated by MV plus light. Dehydroascorbate reductase, monodehydroascorbate reductase, PS II, PS I and ferredoxin-$NADP^+$ reductase were far less sensitive to the treatment. In the treated leaves, cytosolic APX and guaiacol-specific peroxidase were also inactivated, but slower than chloroplastic APXs were. Catalase was not inactivated. Thus the MV-induced photooxidative damages of leaves are initiated with the inactivation of chloroplastic APXs and develop via the inactivation of other H_2O_2-sensitive targets. The decay half-life of the MDA signal after a short illumination in the leaves, as determined by in vivo electron spin resonance spectrometry (ESR), was prolonged when the H_2O_2-scavenging capacity of the leaf cells was abolished by the inactivation of chloroplastic and cytosolic APXs. The measurement of MDA in leaves by ESR, therefore, allows to estimate in vivo cellular capacity to scavenge the photoproduced H_2O_2.

Keywords: Ascorbate peroxidase; Chloroplast; Electron spin resonance; Monodehydroascorbate radical; Methylviologen; Oxidative stress

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; cAPX, cytosolic APX; Chl, chlorophyll; DHA, dehydroascorbate; DMBQ, 2,6-dimethylbenzoquinone; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; ESR, electron spin resonance spectrometry; FBPase, fructose-1,6-bisphosphatase; Fd, ferredoxin; FNR, Fd-$NADP^+$ reductase; GAPDH, $NADP^+$-glyceraldehyde phosphate dehydrogenase; GuPX, guaiacol peroxidase; MDA, monodehydroascorbate; MV, methylviologen; PS, Photosystem; SOD, superoxide dismutase; sAPX, stromal APX; tAPX, thylakoid-bound APX

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1. Introduction

Photosynthetic electron transport is inevitably accompanied by the reduction of dioxygen to superoxide radical (O_2^-) on the reducing side of Photosystem (PS) I. The electron flux to dioxygen through this pathway constitutes 10% of the total electron flux through the thylakoidal electron transport, even when the supply of $NADP^+$ saturates [1]. Production rate of O_2^- in chloroplasts is determined by the balance between the influx of light energy into, and the consumption of electrochemical energy (as NADPH and ATP) in chloroplasts. Under extreme environmental conditions, such as temperature stress, drought and pollutants, the energy consumption for CO_2-assimilation is suppressed because the Calvin cycle enzymes are inhibited (e.g., at low temperatures) and the CO_2 supply decreases (e.g., by stomata closure due to drought). Especially when these unfavorable conditions are combined with high light, the resulting 'light-excess' status in chloroplasts would lead to an enhanced production of O_2^-, and subsequently H_2O_2 and O_2, catalyzed with superoxide dismutase (SOD). If not properly scavenged, these reactive oxygen species inactivate metalloproteins such as aconitase, CuZnSOD, and ascorbate peroxidase (APX). H_2O_2 and O_2^- also produce highly reactive hydroxyl radicals ($HO^\bullet$) via the Haber-Weiss reaction, which is catalyzed by transition metal ions. Facilitated production of $HO^\bullet$ under severe environmental stresses is detrimental to cells because $HO^\bullet$ indiscriminately decomposes proteins, lipids, DNA and polysaccharides, and also initiates radical chain reactions.

Plant cells have multiple defense systems against such photooxidative stress [1–3]. Antioxidants such as ascorbate (AsA), glutathione, tocopherols, carotenoids, polyphenols and flavonoids chemically scavenge radicals and reactive oxygen species. O_2^- is specifically scavenged by SOD, and H_2O_2 by APX and catalase. In the chloroplast, which is the major source of reactive oxygen species in leaf cells, the water–water cycle efficiently scavenges the photoproduced O_2^- and H_2O_2 and regenerates AsA from its oxidized forms [4]. The rates of the SOD-catalyzed disproportionation of O_2^-, the APX-catalyzed reduction of H_2O_2 to H_2O and the reduction of MDA to AsA in the water-water cycle are 1000-fold or higher than the photoproduction rate of O_2^-, because of the high turnover rates of the participating enzymes and of their microcompartmentation in PS I complex [4,5]. The scavenging of H_2O_2 via APX produces monodehydroascorbate (MDA) radical and stimulates electron transport because MDA is an excellent electron acceptor for PS I [6]. Increased electron flux relaxes the overreduced state of the intersystem electron carriers and facilitates the downregulation of PS II [7] by building up ΔpH across the thylakoid membrane. As a result, PS II acceptors are more oxidized and the possible generation of $^1O_2$ due to the charge-recombination in the PS II reaction center is suppressed.

Methylviologen (MV) catalyzes the photoreduction of O_2 at PS I, and thereby accelerates the production of O_2^- and H_2O_2. MV has been often used as an inducer of photooxidative stress [8–10], which is similar to environmental stress-induced ones [11]. Since MV induces the photoaccumulation of H_2O_2 in chloroplasts [12], H_2O_2-sensitive enzymes such as fructose 1,6-bisphosphatase (FBPase), $NADP^+$-glyceraldehyde phosphate dehydrogenase (GAPDH) and ribulose-5-phosphate kinase, APX and SOD are inactivated [13,14]. In addition, MDA reductase and dehydroascorbate (DHA) reductase are also inactivated in MV-stressed leaves [10]. Release of ferredoxin-$NADP^+$ reductase (FNR) from thylakoid membranes by MV is also reported [9]. Thus, MV extensively inactivates various enzymes, but the very initial biochemical process of the damage development has not been identified.

Here we report that the chloroplastic APXs are the primary targets of MV. As a result of APX inactivation, the H_2O_2-scavenging capacity of chloroplasts was abolished, leading to oxidative damage of cells. We also describe an intimate correlation between the loss of cellular H_2O_2-scavenging capacity and the decay half-life of the photoproduced MDA signal in leaves, as determined by in vivo ESR. Part of this work has been presented in the XIth International Congress on Photosynthesis in a preliminary form [15].

2. Materials and methods

2.1. Preparation of chloroplasts and thylakoids

Intact chloroplasts were prepared at 4°C from spin-

ach leaves from a local market and were purified by Percoll (Amersham–Pharmacia Biotech Japan, Tokyo) density gradient centrifugation [16]. The chloroplasts were suspended in 1 mM AsA, 0.3 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 0.5 mM diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA), 0.5 mM sodium pyrophosphate and 50 mM Hepes–KOH, pH 7.6 (chloroplast medium). Thylakoid membranes were prepared at 4°C as follows: spinach leaves were homogenized in 0.4 M sucrose, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM AsA, and 50 mM Hepes–KOH, pH 7.8 (thylakoid medium), filtered through a layer of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at $6000 \times g$ for 5 min. The resulting pellet was suspended in the thylakoid medium. Chlorophyll (Chl) concentration was determined according to Arnon [17].

2.2. Stress treatment of leaves

Spinach leaves, removed of midribs and veins thicker than 1.5 mm, and also of 1 cm region from leaf edge, were cut into pieces of 7.5 mm square with a razor blade and floated, with adaxal side up, on either distilled water or 0.5 mM MV in petri dishes. In order to minimize leaf-dependent variation of the stress response, leaf disks from one leaf were distributed to all the petri dishes for treatments, so that the population in each petri dish represented the leaf population tested. Leaf pieces floated on these media were incubated at 25°C in darkness for 12 h, and then illuminated with white fluorescent lamps (100 μmol $m^{-2}\,s^{-1}$) at 25°C. After illumination leaf pieces in one dish were immediately frozen in liquid nitrogen and was used for APX isozyme assay. Leaf pieces in another dish was dark-adapted for 5 min, Chl fluorescence determined, then frozen and used for catalase and guaiacol peroxidase (GuPX) assays.

2.3. Preparation of leaf extract

All procedures were done at 4°C. Frozen leaf pieces were homogenized in 20% (w/v) sorbitol, 50 mM potassium phosphate (pH 7.0), 1 mM AsA and 0.5 mM DTPA, filtered through a layer of Miracloth and then centrifuged at $12\,000 \times g$ for 5 min. The pellet was washed with and suspended in the homogenizing medium and used for the assay of thylakoid-bound APX. The supernatant was passed through a Sephadex G-25 (Amersham-Pharmacia) column (0.9 cm in diameter × 2.8 cm), which had been equilibrated with 50 mM potassium phosphate (pH 7.0), 1 mM AsA and 20% (w/v) sorbitol, and then was used for assays of APX isozymes. Leaf extract for assays of catalase and GuPX was prepared separately by the same procedure, but AsA and sorbitol were omitted. Protein was determined by the Bradford method [18], with bovine serum albumin as the standard. AsA in protein samples was depleted by adding a small amount of AsA oxidase before protein determination.

2.4. Separate assay of APX isoforms

Activities of stromal and cytosolic APXs in the leaf extract were separately determined as previously reported [19], utilizing the different sensitivities of these isoforms to a low-AsA condition established with AsA oxidase. APX was assayed based on the rate of AsA oxidation at 290 nm (absorption coefficient 2.8 $mM^{-1}\,cm^{-1}$) upon addition of 0.1 mM H_2O_2 to the reaction mixture containing 0.5 mM AsA, 50 mM MES–NaOH (pH 6.5), and enzyme.

2.5. Assays of catalase, DHA reductase, GAPDH, GuPX, MDA reductase, SOD and electron transport

Catalase was assayed polarographically with a Clark-type oxygen electrode (Hansatech, King's Lynn, UK). Reaction mixture contained 50 mM potassium phosphate (pH 7.0) and 1 mM H_2O_2. Reaction was started by adding leaf extract and the initial rate (5–30 s) of oxygen evolution was determined. DHA reductase was determined based on the rate of AsA production from DHA at 265 nm (absorption coefficient 14 $mM^{-1}\,cm^{-1}$) [20]. GAPDH was determined by following the oxidation of NADPH at 340 nm by 1,3-bisphosphoglycerate that was produced from 3-phosphoglycerate with 3-phosphoglycerate kinase [21]. GuPX was determined with pyrogallol as the electron donor [19]. MDA reductase was determined based on the rate of NADH oxidation by MDA, generated by ascorbate oxidase [22]. SOD was assayed based on the inhibition of the O_2^--dependent reduction of ferricytochrome *c* with the xanthine–

xanthine oxidase system [23]. The PS II activity, H_2O to 2,6-dimethylbenzoquinone (DMBQ), in the presence of 0.5 μM nigericin, was determined by the O_2 evolution [24]. The PS I activity was determined by the O_2 consumption in a reaction mixture containing thylakoids (10 μg Chl ml^{-1}), 50 mM Hepes–KOH (pH 7.4), 0.1 mM MV, 50 μM 2,6-dichlorophenolindophenol, 0.5 mM AsA, 1 mM NaN_3 and 10 μM 3-(3,4-dichlorophenyl)-1,1′-dimethylurea. Electron transport activity of the whole chain (H_2O to $NADP^+$) was determined spectrophotometrically [25] in a reaction mixture containing 5.4 μg Chl ml^{-1} of chloroplasts, 50 mM Hepes–KOH (pH 7.4), 10 μM ferredoxin (Fd), 0.5 mM $NADP^+$, and 0.5 μM nigericin. Red actinic light above 640 nm was used.

2.6. Chlorophyll fluorescence measurement

The chlorophyll fluorescence parameter F_v/F_m of leaf pieces, which represents the PS II activity [26], was determined with a pulse-amplitude modulation Chl fluorometer (MINI-PAM, Walz, Effeltrich, Germany), using a plastic fiberoptics (2 mm in diameter). Leaf pieces were dark-adapted at least for 5 min prior to the measurement.

2.7. Assay of MDA by ESR

A 5 mm × 3 cm section cut from a spinach leaf was infiltrated with 0.1 mM MV under vacuum and incubated in darkness at room temperature for 3 h, when the photogeneration of the MDA signal became maximal. The MV-fed leaf segment was pasted with silicone grease on a quartz tissue cell of ESR (JEOL, Tokyo, Japan) and was illuminated with a tungsten lamp at 1000 μmol photons $m^{-2}\,s^{-1}$ for the indicated periods (MV-phototreatment). After an incubation in darkness for 5 min, the treated leaf segment was illuminated in the cavity of an X-band ESR spectrometer (JES-RE2X, JEOL) for 10 s at 440 μmol photons $m^{-2}\,s^{-1}$ and the MDA signal was monitored. ESR conditions were: microwave power, 3 mW; microwave frequency, 9.40 GHz; modulation amplitude, 0.032 mT; and sweep rate, 2.5 mT min^{-1}. The MDA signal was observed in MV-treated leaf under illumination, overlapping a photoinduced broad signal as previously described [27]. Kinetic data of the MDA radical in leaves were recorded with a computer-aided A/D converter for subtracting the kinetic trace of the overlapping signal from that of the lower field peak of the MDA signal as described later.

2.8. Sources of chemicals and enzymes

Ascorbate oxidase was obtained from Toyobo Co. (Tokyo, Japan). Catalase, nigericin and 3-phosphoglycerate kinase were from Sigma Japan (Tokyo). Fd was prepared from spinach leaves [28]. Xanthine oxidase from cow milk was a product of Boehringer Mannheim (Tokyo).

3. Results and discussion

3.1. Inactivation of APX by MV due to the inhibition of the AsA-photoregeneration in chloroplasts

The photoreduction of MV at PS I accelerates O_2^- production and simultaneously inhibits the photoreduction of Fd. Since the reduction of the oxidized forms of AsA, both MDA and dehydroascorbate (DHA), totally depends on the reduced form of Fd [6], MV inhibits the photoregeneration of AsA in chloroplasts. Upon illumination, the concentration of AsA in chloroplasts in MV would rapidly drop due to the accelerated AsA consumption via the APX-catalyzed H_2O_2 reduction and the inhibited AsA regeneration. The chloroplastic isoforms of APX, stromal and thylakoid-bound APXs (sAPX and tAPX, respectively) are rapidly inactivated below 20 μM AsA [29,30]. This is because the Compound I of APX, unless it is reduced by AsA to Compound II, is oxidatively decomposed by nanomolar levels of H_2O_2, which is generated by the autooxidation of trace amounts of AsA [31]. These chloroplastic APXs are stabilized only when the AsA concentration is kept over 20 μM. Therefore, it is expected that sAPX and tAPX are inactivated when chloroplasts are illuminated in MV, which inhibits the regeneration of AsA from MDA.

When intact chloroplasts were illuminated in 0.1 mM MV, APX activity was lost rapidly, as expected (Fig. 1). In the absence of MV, APX was not inactivated by a 10-min illumination if the AsA concen-

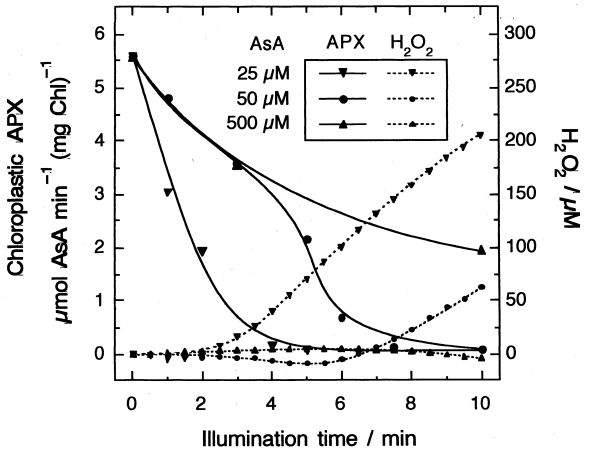

Fig. 1. Effects of AsA concentration on the MV-dependent inactivation of APX and production of H_2O_2 in intact chloroplasts. Intact chloroplasts (50 μg Chl ml^{-1}) were suspended in the chloroplast medium containing 0.1 mM MV and AsA at the indicated concentrations, and illuminated with a white tungsten lamp at 1000 μmol m^{-2} s^{-1} at 25°C. At the indicated points of time the reaction mixture was centrifuged within 15 s. The pellet was suspended in 50 mM MES–NaOH (pH 6.5), 0.5 mM AsA and 0.5 mM DTPA (APX medium) and the chloroplastic APX was assayed. H_2O_2 production was determined independently from the difference of O_2 consumption, using the O_2 electrode, in the presence and absence of saturating amount of catalase, multiplied by 2. Solid lines, APX activity; broken lines, H_2O_2 production.

tration was 50 μM or higher (data not shown). The MV-dependent inactivation of APX was delayed by higher concentrations of AsA, indicating that the inactivation was caused by the lowered AsA concentration [31]. On the other hand, H_2O_2 started to accumulate in the MV-fed chloroplasts after a lag period, which was elongated by the increasing concentration of AsA added to the medium. These observations indicate that H_2O_2 photogenerated via the MV-mediated reaction was reduced to water, catalyzed by APX, as far as AsA was available in chloroplasts, and that when AsA was decreased to lower than 20 μM, APX was inactivated and H_2O_2 could not be reduced to water by the APX reaction. Thus, the H_2O_2-scavenging capacity of chloroplasts depends on both the AsA concentration and the APX activity.

It should be noted that APX was inactivated even when there was no apparent accumulation of H_2O_2. In 500 μM AsA, H_2O_2 did not accumulate in a 10-min illumination, but 40% of APX activity was lost. This suggests that the local concentration of AsA in chloroplasts was lowered to below 20 μM at a site where APX was microcompartmented, that is, in the PS I complex [3], because the regeneration of AsA was inhibited. In the case where AsA was added to 500 μM, concentration gradient between the bulk medium and the microcompartmented site of APX could be more than 480 μM. In other words, AsA did not uniformly distributed within the MV-fed chloroplasts during illumination, when AsA is rapidly consumed for the APX-catalyzed reduction of H_2O_2. Probably the supply of AsA from the cytosol via the facilitated diffusion across the chloroplast envelope [32–34] could not compensate the rapid consumption of AsA. The photoreduction systems of MDA and DHA in chloroplasts are thus essential for maintaining the chloroplastic APX activity.

3.2. *APX is the primary target among the H_2O_2-sensitive enzymes in chloroplasts*

We compared the sensitivity of the potential targets to the MV-induced photooxidative stress with that of APX, using intact chloroplasts (Fig. 2). Both sAPX and tAPX were inactivated simultaneously with a half-life of 20 s (Fig. 2A), similar to the situation where H_2O_2 was added exogenously to chloroplasts in darkness [30]. Thus, both APXs experience similar microenvironments when H_2O_2 is either photoproduced endogenously on the surface of thylakoids or added exogenously. GAPDH was also inactivated by MV-phototreatment, but much slower than APX; 50% inactivation was 10 min. Miyagawa et al. [14] has also observed that in MV-stressed tobacco leaves the chloroplast APXs were inactivated before the thiol-enzymes GAPDH, FBPase and ribulose-5-phosphate kinase were. DHA reductase and MDA reductase (Fig. 2B) were less sensitive; they were unaffected by a 10-min illumination. SOD was inactivated by MV-phototreatment with a similar sensitivity to that of GAPDH (Fig. 2B). The major SOD isozyme in spinach chloroplasts is the stromal CuZnSOD [35], which is sensitive to H_2O_2. The inactivation observed here therefore can be ascribed to H_2O_2, which accumulated up to 200 μM in 10-min illumination (Fig. 1). All these enzymes were stable when either MV or light was omitted (data not shown).

Thylakoid electron transport system is also a po-

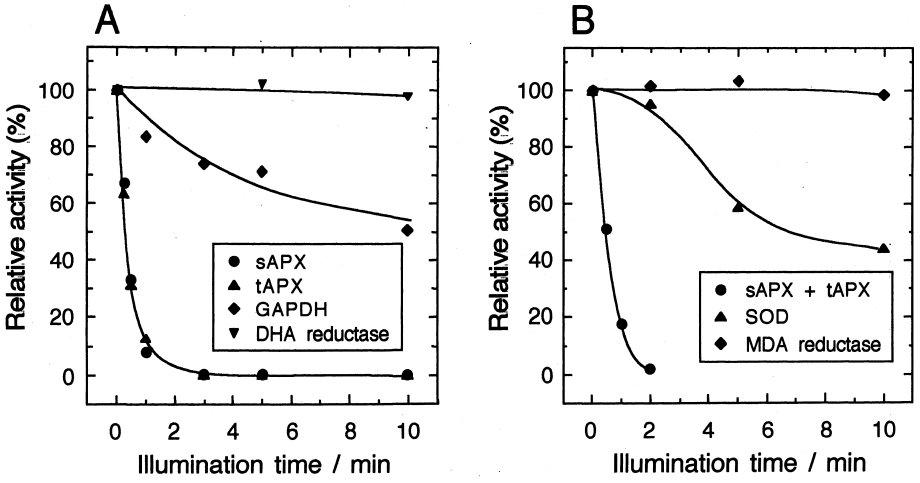

Fig. 2. Activities of APX isoforms, DHA reductase and GAPDH (A) and SOD and MDA reductase (B) in chloroplasts during illumination in MV. Intact chloroplasts were suspended at 50 μg Chl ml^{-1} in chloroplast medium containing 0.1 mM MV and 25 μM AsA. The reaction mixture was then illuminated for the indicated period and centrifuged within 15 s. The pellet was suspended in the APX medium as in Fig. 1. Activities of GAPDH, DHA reductase, MDA reductase and SOD were determined as described in Section 2. AsA in the samples for SOD assay was removed by adding a small amount of AsA oxidase. For separation of sAPX and tAPX, the illuminated chloroplasts were suspended in the APX medium and centrifuged again, and the supernatant (stroma fraction) and the pellet (thylakoids) were separated. Thylakoids were re-suspended in the APX medium. In the absence of MV, APX activity was unaffected by illumination for 10 min. In A, activities for 100% were 5.6 and 10.1 μmol AsA oxidized min^{-1} (mg Chl)$^{-1}$ for sAPX and tAPX, respectively, 49 μmol NADH min^{-1} (mg Chl)$^{-1}$ for GAPDH and 0.70 μmol AsA oxidized min^{-1} (mg Chl)$^{-1}$ for DHA reductase. In B, activities for 100% were 8.2 μmol AsA oxidized min^{-1} (mg Chl)$^{-1}$ for APX, 412 units mg $protein^{-1}$ for SOD, and 0.35 μmol NADH min^{-1} (mg Chl)$^{-1}$ for MDA reductase. Enzyme activities were averages of two runs.

tential target of the MV-induced photooxidative stress. In MV-treated leaves, FNR is released from thylakoid membranes [9] and the PS II activity, as determined by F_v/F_m is inhibited [14,36]. Activities of PS II (H_2O to DMBQ), PS I (AsA to MV) and the whole chain (H_2O to $NADP^+$) in the thylakoids were all unaffected up to 10 min illumination in 0.1 mM MV, while chloroplast APX was completely inactivated in 5 min (Fig. 3). FNR would not be released from the thylakoids since the whole chain electron transport activity remained intact. Thus, thylakoid electron transport activities were much less sensitive to MV-phototreatment than was chloroplastic APX.

The present results clearly demonstrate that sAPX and tAPX are the primary targets of the MV-induced photooxidative stress in chloroplasts. Inactivation of other H_2O_2-sensitive proteins were most likely the secondary effects of the APX inactivation.

3.3. *Chloroplastic APX is the primary target also in leaves*

Leaf cells contain cytosolic APX (cAPX) and peroxisomal APX in addition to chloroplast APXs. These APXs are also possible targets of MV-induced photooxidative stress. cAPX is inactivated in low AsA concentrations, though it is more tolerant than sAPX is [19]. GuPX can scavenge H_2O_2 when phenolics are the electron donors. Although GuPX has a relatively low specificity for AsA, AsA can donate electrons to the GuPX reaction indirectly via the reduction of the phenoxyl radicals [37]. GuPX is inactivated by high concentrations of H_2O_2 when the concentrations of electron donors are low [38]. Catalase is localized mainly in peroxisomes and responsible for the scavenging of H_2O_2 produced via photorespiration. Catalase is inactivated by SO_2 [39] or sulfite [40]. Since SO_2 induces the photoproduction of H_2O_2 and $HO^\bullet$ via radical chain reactions [41], similar targets and damaging mechanisms for SO_2- and MV-induced photooxidative stresses could be expected.

When MV-fed leaf pieces were illuminated, sAPX and tAPX were simultaneously inactivated (Fig. 4), like the observation for chloroplasts in Fig. 3. cAPX was also inactivated but at a slower rate than the

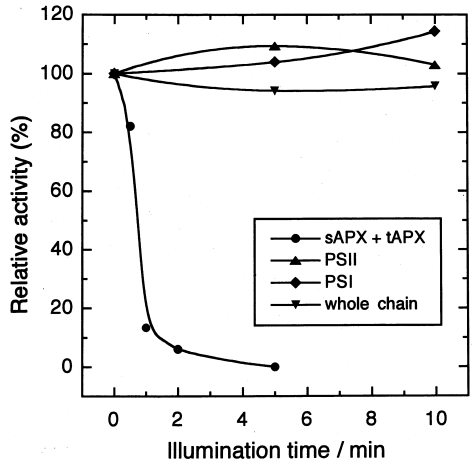

Fig. 3. Activities of APX, PS II, PS I, and the whole-chain electron transport in illuminated chloroplasts. Intact chloroplasts were suspended in the chloroplast medium containing 25 μM AsA and 0.1 mM MV, as in Fig. 2. The reaction mixture was illuminated, centrifuged and the pellet suspended in the APX medium for APX assay as in Fig. 1. Thylakoids were pelleted by centrifugation and suspended in the thylakoid medium. Electron transport activities of PS II (H_2O to DMBQ, triangle), PS I (AsA to MV, diamond) and whole-chain (H_2O to $NADP^+$, reverse triangle) were determined as in Section 2. Control rates were 11.2 μmol AsA oxidized min^{-1} (mg Chl)$^{-1}$ for APX, 307 μmol O_2 h^{-1} (mg Chl)$^{-1}$ for PS II, 1018 μmol O_2 h^{-1} (mg Chl)$^{-1}$ for PS I and 188 μmol NADPH h^{-1} (mg Chl)$^{-1}$ for whole chain. Activities were averages of two runs.

chloroplastic APXs. At a slower rate, GuPX was also inactivated. At 5-min illumination, when the chloroplastic APXs were inactivated by more than 80%, the loss of cAPX activity was about 50% and that of GuPX 30%. In the control leaf pieces without MV, all these enzymes were not inactivated by the illumination up to 20 min (data not shown). Catalase was not inactivated by illumination within 20 min both in the presence and absence of MV. Damage to PS II, as judged by F_v/F_m, was much smaller than those to APXs and GuPX in MV-fed leaves. Thus, the chloroplastic APXs are the earliest targets in the MV-stressed leaves as well as in intact chloroplasts.

As demonstrated above, the MV-induced damages of leaf cells develops sequentially, starting with the loss of chloroplastic APXs. The possible scenario of the MV-induced cellular damages is as follows: MV accelerates the photoproduction of O_2^- at PS I, and simultaneously inhibits the regeneration of AsA from MDA. Chloroplastic APXs are rapidly inactivated when AsA concentration is decreased to less than 20 μM. As the H_2O_2-scavenging capacity in chloroplasts is abolished, the photoproduced H_2O_2 accumulates in chloroplasts and diffuses from chloroplasts to other compartments. Among potential targets of H_2O_2 in chloroplasts, the thiol-enzyme GAPDH and CuZnSOD are relatively sensitive, while catalase, DHA reductase, MDA reductase, FNR and thylakoid components of the electron transport chain are rather tolerant to the stress. Increased concentrations of H_2O_2 in cytosol inactivates cAPX and GuPX, further decreasing the cellular capacity to scavenge H_2O_2. Inactivated APX and CuZnSOD will release Fe and Cu ions. Iron–sulfur proteins such as aconitase, Fd and PsaB protein in the PS I reaction center would be also oxidized by O_2^- and H_2O_2 to release Fe ions. These metal ions serve as potent catalysts of the Haber–Weiss reaction, producing $HO^\bullet$ from H_2O_2. The reducing equivalents in cells will be exhausted via the radical chain reactions caused by $HO^\bullet$, leading to extensive oxidative damages of cellular components.

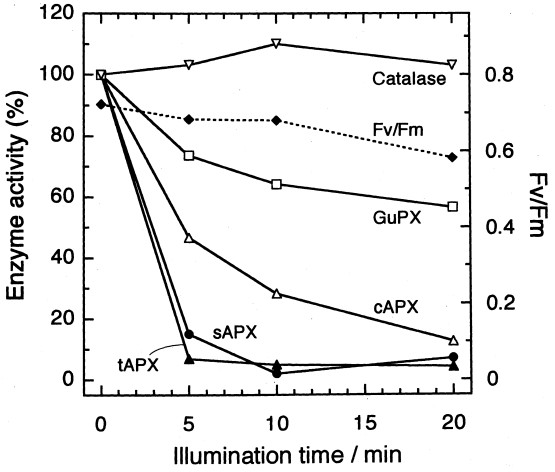

Fig. 4. Changes due to illumination in F_v/F_m and the activities of sAPX, tAPX, cAPX, GuPX and catalase in MV-fed leaf pieces. Leaf pieces preincubated in 0.5 mM MV was illuminated for various periods and frozen in liquid nitrogen. Extracts and thylakoids were prepared as in Section 2. Activities for 100% were 6.7 μmol AsA min^{-1} (mg Chl)$^{-1}$ for tAPX, 1.0 and 0.61 μmol AsA min^{-1} (mg protein)$^{-1}$ for sAPX and cAPX, respectively, 0.25 μmol min^{-1} (mg protein)$^{-1}$ for GuPX and 7.0 μmol min^{-1} (mg protein)$^{-1}$ O_2 for catalase. AsA-oxidizing activity due to GuPX, determined after the inactivation of APX with *p*-chloromercuribenzoic acid [19], was negligible. Enzyme activities were averages of three runs.

3.4. ESR signal of MDA in leaves is a probe of H_2O_2-scavenging capacity of chloroplasts

Various environmental stresses induce photogeneration of the MDA radical in leaves, as detected by in vivo ESR [27,40,42–44]. The ESR signals observed in MV-fed leaves are shown in Fig. 5, left panel. The MDA radical, appearing at 337.05 mT as a doublet spaced by 0.14 mT, was observed only in a MV-fed leaf under illumination (trace D), but not in darkness or in the absence of MV. This is inferred as a result of the total inhibition of the photoreduction of MDA due to the exclusive electron partitioning from PS I to O_2 through MV. Very little MDA signal in the absence of MV suggests that the MDA radical, which is generated by the APX reaction, is immediately reduced to AsA either directly by the reduced Fd [6] or by NAD(P)H with MDA reductase [45]. Thus, the MDA signal intensity is enhanced by either an increase in the production or a decrease in the scavenging of the MDA radical, or both.

When the MV-fed leaves are illuminated for several minutes to hours, the photoinduced level of the MDA signal is lowered [42,44]. Since the chloroplast APXs are primarily inactivated by MV-light treatment as presented above, we expected a correlation between the MDA signal and the chloroplast APX activities in leaves. A close correlation between the MDA signal intensity and the APX activity has been demonstrated in an in vitro experiment with thylakoids [46]. We here analyze the changes in the MDA signal in the MV-fed leaves in detail.

In order to follow the actual changes of the MDA radical, we corrected for the photoinduced, broad background ESR signal (Fig. 5, left panel B) [27]. Time courses of ESR signal at the lower field peak of MDA radical and the trough of the broad background signal (*a* and *b*, respectively, in trace D in the left panel of Fig. 5) are shown in A′ and B′ in the right panel, respectively. The correction for the broad background signal was necessary for actual MDA radical because the MDA signal and the background signal exhibited distinct kinetics. The time course C′ represents the corrected trace, and it

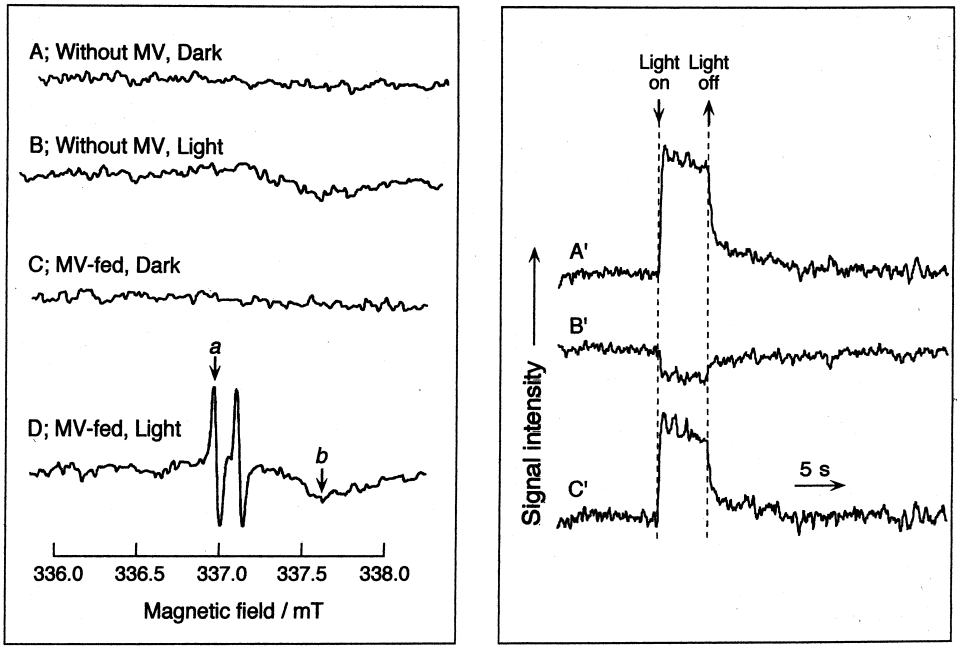

Fig. 5. ESR traces of MDA in MV-treated leaves. Left panel: Spectra for leaf pieces from spinach leaves without MV (A,B) and the leaves fed with 1 mM MV in a dark place overnight (C,D). While scanning at 2 mT min^{-1} from low to high field (left to right), actinic light at 200 μmol $m^{-2}\,s^{-1}$ was illuminated (B,D). Right panel: Time courses of the ESR signals at the magnetic fields *a* and *b* in the left panel D (A′ and B′, respectively). Trace C′ shows the actual MDA signal after the correction for the broad photoinduced signal.

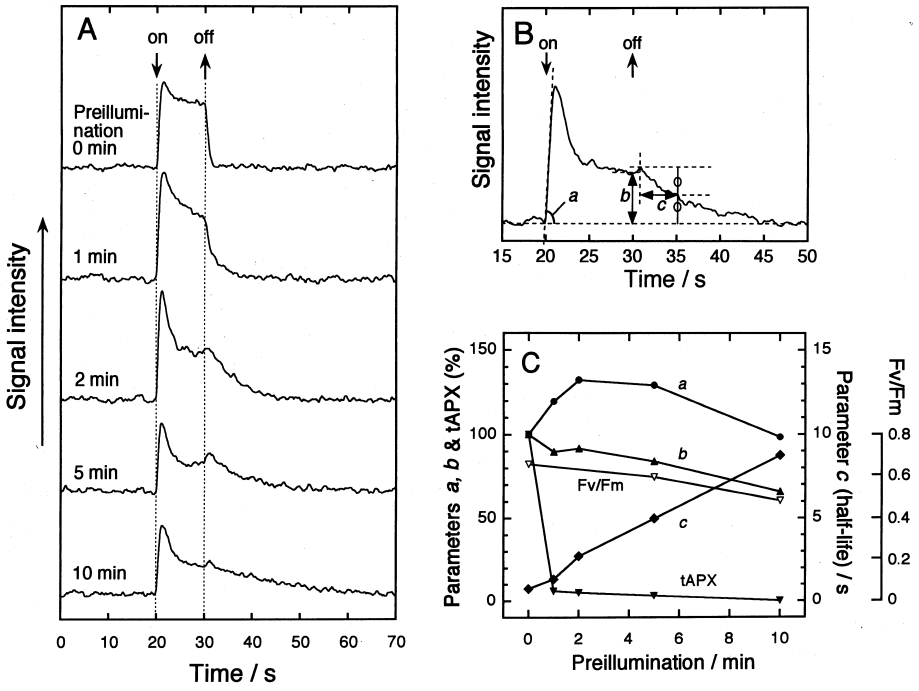

Fig. 6. Effects of preillumination on the light–dark kinetics of MDA signal in MV-fed leaves. (A) A leaf segment fed with 100 μM MV was preilluminated for the indicated periods with white light at 440 μmol $m^{-2} s^{-1}$ through a heat-absorbing water filter. The kinetics of the production and decay of MDA signal were monitored 5 min after preillumination in darkness and 10 s actinic light (440 μmol $m^{-2} s^{-1}$), turned on and off as indicated and corrected as in Fig. 5. (B) Extraction of the kinetic parameters to represent the MDA signal rise and decay; the initial slope upon actinic illumination (*a*), signal intensity at 10-s illumination (*b*) and half-life of the signal after turning off of actinic light (*c*). (C) Changes in the MDA signal kinetic parameters (*a*, *b*, *c*), tAPX activity and F_v/F_m in MV-fed leaf pieces. tAPX activity for 100% was 5.5 μmol AsA min^{-1} (mg Chl)$^{-1}$. Data are averages of four runs.

showed a rapid change, e.g., in seconds, of the MDA radical in leaf cells by turning on and off of actinic light. The profile of the corrected MDA radical change is characterized as follows: only little or no MDA was detectable in darkness, but upon illumination the MDA signal increased rapidly. Afterwards the signal intensity went down to a semi-steady-state level during illumination. Upon the cessation of actinic light the signal intensity was decreased to the initial dark level within 2 s. This complex profile of the MDA signal would represent changes in the balance between the production and scavenging of MDA.

Preillumination of the MV-fed leaves altered the production and scavenging kinetics of MDA on several aspects, i.e., (i) the rise steepness of the signal upon actinic light, (ii) the semi-steady-state level, and (iii) the decay rate after turning off of the actinic light (Fig. 6A). We extracted the kinetic parameters corresponding to the above three features (parameters *a*, *b* and *c*, respectively; Fig. 6B) and plotted against the preillumination time (Fig. 6C). Under the present conditions, tAPX was inactivated by 95% in 1 min. This represented the loss of chloroplast APXs, since tAPX and sAPX are simultaneously inactivated by the MV-phototreatment (Figs. 2 and 4). The F_v/F_m value was lowered by 10% in 10 min. Thus, this stress treatment was comparable with that in Fig. 4, and hence we can expect that cAPX also was largely inactivated in 10 min. Considering such biochemical changes together with the known reactions for the production and scavenging of MDA, we interpret the changes in the above kinetic parameters of in vivo MDA signal as below.

Parameters *a* and *b*: An in vitro ESR analysis with thylakoids in the absence of MV revealed that the photoproduced MDA is mostly ascribable to the APX reaction [46]. In the MV-fed leaves the chloroplast APX activity was completely lost in 2 min (Fig. 6C), but the light-induced MDA signal was observed

even after 10-min preillumination (Fig. 6A). Thus, the photoproduced MDA is not solely accounted for by the APX reaction. We tentatively infer that this APX-independent MDA signal could be ascribed to the non-enzymic oxidation of AsA by O_2^- (ca. 10^5 $M^{-1}\,s^{-1}$ at pH 7.0 [47,48]). The direct reaction between O_2^- and AsA in illuminated chloroplasts is largely suppressed by the millimolar level of CuZn-SOD microcompartmented in the O_2^--producing site, i.e., PS I [49]. When MV is added, however, the photoproduction rate of O_2^- is enhanced by 10–20-fold (data not shown), and at a constant concentration of SOD the steady-state concentration of O_2^- would be increased proportionally to the O_2^- production rate [49]. The oxidation of AsA by O_2^- is thus expected to be enhanced, and because of the inhibited photoreduction of MDA, such an increase in the MDA production could be detected by ESR. Another possible source of APX-independent MDA production is the oxidation of AsA by H_2O_2, if a catalyst such as transition metal ions are available. The light-induced MDA signal in MV-fed leaves is therefore largely dependent on the availability of AsA in chloroplasts.

During the preillumination AsA in chloroplasts is rapidly consumed. However, parameter *a*, the initial slope of the MDA production, was relatively insensitive to the preillumination. This was probably because the AsA in chloroplasts, which had been consumed by the preillumination, was supplemented by the cytosolic AsA pool during the 5-min dark incubation prior to the ESR measurement.

The supplemented AsA in chloroplasts is rapidly consumed upon the actinic illumination by the enzymic and non-enzymic oxidation by H_2O_2 and O_2^-, leading to a decrease in the MDA production rate. This situation is reflected in the MDA profile as a 'drop' of the signal intensity after the initial peak (Fig. 6A). Because AsA is supplied only from outside of chloroplasts, the semi steady-state level (parameter *b*) reflects the equilibrium between the intrachloroplastic oxidation rate of AsA and the supply rate of AsA into the chloroplasts. The semi-steady-state level of the MDA signal decreased linearly on the increasing preillumination time, with a similar extent to the F_v/F_m value (Fig. 6C). The decreased MDA level could be recovered to some extent by a longer incubation in darkness [42], probably due to the supply of AsA to chloroplasts during the dark incubation. However, if the stress is given extensively, the AsA pool outside of chloroplasts will be exhausted and the MDA signal level is irreversibly lowered [44]. Thus, the parameter *b* appears to represent cellular damages at rather later stages of the stress, i.e., loss of reducing equivalents.

Parameter *c*: The decay of the MDA signal in the dark after 10-s illumination was elongated by the preillumination, appearing as the increase in parameter *c*. This was caused by either an increase in the production of MDA or a decrease in the quenching of MDA via the spontaneous disproportionation and the MDA reductase-catalyzed reduction by NAD(P)H, or both. Participation of O_2^- to the production and scavenging of MDA is negligible because there is no production of O_2^- in darkness. For the following three reasons, we exclude the possibility of a decreased quenching of MDA: (i) MDA reductase activity was unchanged by MV-phototreatment for 10 min (Fig. 2). (ii) The concentration of NADPH in MV-added chloroplasts changed from 61 μM before illumination to 55 μM after 15 min illumination (averages of three runs), which were comparable to those in the chloroplasts without MV (86 and 81 μM before and after illumination), and still more than twofold higher than the K_m value of spinach MDA reductase for NADPH (22 μM) [45]. (iii) Lifetime of the MDA radical is longer if stromal pH is higher [50], but there was no enhancement of stromal alkalinization by 10-min preillumination, as judged by the nonphotochemical quenching of Chl fluorescence (data not shown). Therefore, the elongated lifetime of the MDA signal in darkness is a result of the increased production of MDA due to the H_2O_2, which has been photoproduced during 10-s actinic illumination but remained unscavenged because of the loss of chloroplast APXs. The accumulated H_2O_2 diffuses out of chloroplasts (Fig. 1) to the cytosol. Because the cytosolic APX is also inactivated (Fig. 4), the scavenging of H_2O_2 in darkness would be retarded. This caused the slow, prolonged production of MDA in the MV-photodamaged leaves. Thus, the elongated decay of the photoinduced MDA signal in darkness reflects the loss of H_2O_2-scavenging capacity of the leaf cells, the earliest biochemical event in the MV-induced oxidative stress.

3.5. Antioxidant enzymes are early targets of environmental stresses

The enhanced MDA signal in leaves has been observed at early stages of various stresses such as aminotriazole, acifluorfen [44], drought, excess phosphate [42], ultraviolet-B [27] and sulfite [40]. Such increases in the MDA level in vivo are general results of oxidative stress [51], and implying the inhibition of antioxidant enzymes. Indeed, several reports suggest inactivation of antioxidant enzymes at early stages of plant stresses, as follows.

SO_2 is absorbed through stomata, dissolved into cytosol as sulfite, diffusing into chloroplasts and is reduced by the photoproduced O_2^- to the sulfinyl radicals and produces H_2O_2 and $HO^\bullet$ via radical chain reactions [41]. Illumination of the SO_2-treated leaves inactivates APX and glutathione reductase [39]. However, SOD [52] and catalase [40] are inactivated even in darkness, so these are more specific targets of the SO_2-stress.

In chilling-sensitive species like cucumber and pumpkin, PS I is preferentially damaged by relatively low light [53,54], which damage is caused by reactive oxygen species [55]. Hull et al. [56] compared the temperature dependency of APX in *Zea mays*, a chilling-sensitive species, and in the relative *Z. diploperennis*, a chilling-tolerant species. The APX from *Z. mays* showed larger activity drop at low temperatures than that of the APX from *Z. diploperennis*. Such selective decrease in the APX activity might be the initial event to cause the chilling-induced oxidative damages in the chilling-sensitive species, as suggested previously [57].

We have recently found that under water-stress conditions chloroplastic CuZnSOD was the earliest [58] and catalase was the second targets in lettuce leaves, while APX and PS II were resistant to the stress (Y. Domae, E. Nawata, S. Kanematsu, J. Mano, unpublished results). These examples suggest that the antioxidant enzymes are early targets of various environmental stresses, but each enzyme is inactivated in a stress-specific manner. Chloroplastic APXs are inactivated when an increase in the H_2O_2 concentration and a decrease in the AsA concentration occur simultaneously. In other situations, however, different targets can be more susceptible. The loss of function of the antioxidant enzymes at early stages of environmental stress is a critical event that accelerates the development of oxidative damage of cells.

4. Concluding remarks

The primary targets of the MV-induced photooxidative stress are two chloroplastic APX isoforms, sAPX and tAPX. H_2O_2-scavenging capacity of chloroplasts is retained until 60% of the chloroplastic APX is inactivated, but further inactivation of APX allows rapid accumulation of H_2O_2 under illumination. Chloroplastic GAPDH and CuZnSOD, and the extrachloroplastic cAPX and GuPX, which are potential targets of H_2O_2, were also inactivated by the MV-phototreatment, with less sensitivity than chloroplastic APXs. The resulting loss of H_2O_2-scavenging capacity in leaf cells can be detected by in vivo ESR as a slow decay of the photoproduced MDA signal in darkness. Thus, in vivo ESR measurement of MDA is a sensitive tool to detect biochemical changes at very early stages of oxidative stress in plant leaves.

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