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Monodehydroascorbate Radical Detected by Electron Paramagnetic Resonance Spectrometry Is a Sensitive Probe of Oxidative Stress in Intact Leaves

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Monodehydroascorbate radical (MDA) was determined in leaf segments of several plant species using electron paramagnetic resonance spectrometry. When the leaves were young and healthy, MDA was often below detection level in both the light and dark. However, in senescent leaves, higher levels of MDA were observed in the light than in the dark. After removal of ascorbate by infiltration of such leaves with ascorbate oxidase, dark-signals of MDA did not disappear; thus the apoplast was not a major source of MDA in the dark. Methylviologen induced high levels of light-dependent MDA, indicating the participation of the photoproduced superoxide and the hydrogen peroxide derived from the superoxide in the production of MDA in chloroplasts. The photoproduction of MDA was greatly enhanced by intense light, water stress, and suppression of the photosynthetic reactions due to either infiltration or cyanide. Thus, MDA is a sensitive endogeneous probe of oxidative stress in leaf tissues in the sense that increased MDA levels indicate either increased oxidation of ascorbate or decreased efficiency of ascorbate regeneration, or a combination of both.

Key words: Ascorbate — Chloroplasts — Monodehydroascorbate radical — Photooxidative stress — Radicals — Reactive species of oxygen.

Ascorbate (AsA) occupies a central role in protecting plant cells against the action of the reactive oxygen species. Generally, its content is high in leaf tissues, but AsA has also been found in non-photosynthetic tissues such as root tubers. When AsA functions as an antioxidant in cells, it is univalently oxidized to the monodehydroascorbate radical (MDA). In chloroplasts, the thylakoid-bound and stromal AsA peroxidases (Miyake and Asada 1992a) generate MDA (Hossain et al. 1984) in scavenging the hydrogen peroxide produced via the superoxide dismutase-catalyzed disproportionation of the photogenerated superoxide in PSI (Nakano and Asada 1981, Asada and Badger 1984, Ogawa et al. 1995). Further, AsA peroxidase scavenges hydrogen peroxide in peroxisomes (Yamaguchi et al. 1995, Bunkelmann and Trelease 1996) and other compartments. In addition to AsA peroxidase, guaiacol peroxidase, as represented by horseradish peroxidase, also produces MDA when AsA is the sole electron donor, although the production rate is low (Yamazaki and Piette 1961). In cucurbitaceous plants, AsA oxidase generates MDA during oxidation of AsA (Yamazaki and Piette 1961). When leaves are illuminated by bright light, violaxanthin is deepoxidized to zeaxanthin in thylakoid lumen. AsA is required for this reaction (Yamamoto 1979), and MDA is generated in the deepoxidation (Miyake, C. et al. unpublished). AsA in the lumen is also oxidized to MDA by PSII when the water-oxidizing enzyme is inhibited (Mano, J. et al. unpublished). In addition to the enzymatic production, MDA has been shown to be generated by the interaction of AsA with oxidative radicals such as tocopherol chromanoxy radical, carbon-centered, aminoxy, peroxy, phenoxy, and thiyl radicals (Schuler 1977, Bielski 1982), which are all produced when cells suffer from oxidative stress. Furthermore, superoxide radical and hydroxyl radical oxidize AsA to MDA at a rapid rate when superoxide and hydrogen peroxide are not properly scavenged. Finally, MDA is also produced during the autooxidation of AsA (Scarpa et al. 1983). Thus, MDA is produced by enzymatic and non-enzymatic reactions; its generation in plant cells is inevitable and can be considered as an indicator of oxidative stress.

Plants have two direct systems for reducing MDA to regenerate AsA. One is MDA reductase, which catalyzes the reduction of MDA by NAD(P)H at a diffusion-controlled rate of over $10^8\ M^{-1}\ s^{-1}$ (Hossain and Asada 1984, Sano et al. 1995, Kobayashi et al. 1995). The other is the reduction of MDA by reduced ferredoxin, which serves to regenerate AsA on chloroplast thylakoids for the scavenging of hydrogen peroxide photoproduced in PSI (Miyake and Asada 1992b, 1994, Grace et al. 1995). This reduction also proceeds at a high rate ($>10^7\ M^{-1}\ s^{-1}$), close to PSI in which superoxide and hydrogen peroxide are generated (Asada 1994). MDA reductase is localized not only in the chloroplast stroma, but also in mitochondria (Leonardis et

Abbreviations: AsA, ascorbate; DHA, dehydroascorbate; EPR, electron paramagnetic resonance; MDA, monodehydroascorbate radical; PPFD, photosynthetic photon flux density.

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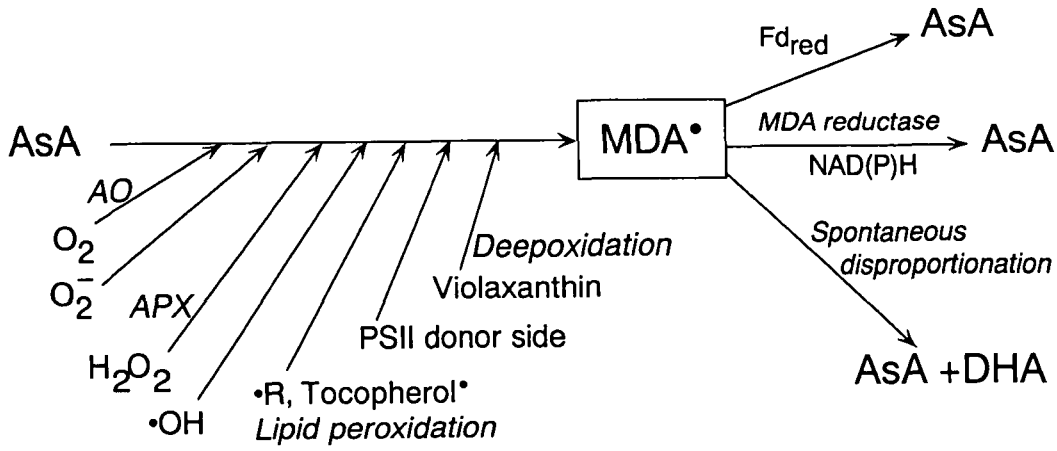

Fig. 1 Production and scavenging of MDA in leaf cells. AO, AsA oxidase; APX, AsA peroxidase.

al. 1995), peroxisomes (Bunkelmann and Trelease 1996), and apoplast (Dalton et al. 1993). When MDA fails to be reduced directly to AsA by either pathway, it disproportionates spontaneously to AsA and DHA at a rate constant of about 10^5 M^{-1} s^{-1} at pH 7 (Bielski 1982). DHA thus produced is reduced to AsA by GSH catalyzed by DHA reductase (Hossain and Asada 1984).

The cellular level of MDA represents a balance of oxidative stress-induced production of MDA and its reduction to AsA either directly or via DHA, as schematically shown in Fig. 1. When cells suffer from photooxidative stress, the production of oxidative radicals and reactive oxygens increases; and all of the oxidative radicals are trapped by AsA generating MDA. Thus, MDA can be regarded as a "sink" for oxidative radicals. MDA is a comparatively stable radical which can be detected by EPR at room temperature as an endogeneous probe for assessment of oxidative stress in cells. Cases of overproduction of oxidative radicals and of lowered capacity of AsA regeneration from MDA may both be revealed by EPR analysis. The present communication describes a survey of MDA in intact leaves in response to various stresses.

Materials and Methods

Plant materials—Leaves from different plant species (*Allium cepa, Brassica oleracea, Erigeron annuus, Hedera helix* cv. argenteo-variegata, *Rumex obtusifolius, Solidago altissima, Stellaria media, Spinacia oleracea* and *Trifolium repens*) were freshly obtained from field locations close to the laboratory or from a greenhouse (*B. oleracea*).

Determination of MDA in intact leaves—Each leaf segment (4 × 20 mm, or smaller, 14–40 mg in fresh weight) was attached to a open flat cell without a lid, and the cell was inserted to a EPR cavity of a JES-RE2X ESR spectrometer (JEOL, Tokyo, Japan). Covering of the cell blocked observation of the EPR signal of MDA for undetermined reasons. EPR signals from leaves were recorded at room temperature at a frequency of 9.4 GHz close to 337.5 mT. Instrument settings were as follows: microwave power 3 mW, receiver gain 4,000 or lower, time constant 1 s or 0.3 s, modulation amplitude 0.032 mT, modulation frequency 100 kHz, magnetic field centered at 337.5 mT, and sweeping rate 1.25 or 2.5 mT min^{-1}. The EPR signals observed in leaves were identified as MDA by comparing them with the EPR spectrum of MDA, which was generated in a reaction mixture containing 4 mM AsA and AsA oxidase (Yamazaki and Piette 1961) in a flat cell (4 × 45 mm, 0.4 mm in thickness). The hyperfine splitting constant was 0.23 mT. The leaf segment in the EPR cavity was illuminated with white light from a halogen lamp that contained little UV-B. The light was guided to the cell through fiber glass optics.

The MDA levels in leaves are estimated from the difference between the signal heights at 337.46 and 337.52 mT, as marked in Fig. 2B. The sensitivity of the EPR signal of MDA is lowered due to dielectric loss of microwave by increasing of leaf thickness (water contents per unit area) over 0.4 mm; therefore, the MDA levels shown here are approximate and could not compare among differnt species of plant, because of their differences in leaf thickness. For *S. altissima* leaves, the molar ratio of MDA and $P700^+$ was estimated by determination of the spin concentration of MDA radical under white light and that of $P700^+$ under far-red light, respectively. The detailed method for this estimation will be reported elsewhere. Chl was determined spectrophotometrically (Arnon 1949).

Results and Discussion

MDA in darkened and illuminated leaves—Low EPR signals which, however, were clearly above noise levels as ascertained by repeated scanning across a range of 0.5 mT, were seen close to 337.5 mT in darkened leaves of several, but not all, examined plant species. The observed signal was assigned to MDA by its *g* value, hyperfine splitting constant, and identity to the EPR spectrum produced in the AsA-AsA oxidase system. Fig. 2 shows EPR signals from a leaf of *A. cepa* in the dark (A) and light (B). The signal obtained with a darkened leaf was increased on illumination and depended on its intensity (Fig. 5 control). The light-dependent increase in MDA concentration would be largely restricted to the chloroplasts, since a red light (600 nm <) was effective and the hydrogen peroxide photoproduced in

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chloroplasts gives rise to MDA via the AsA peroxidase reaction (Asada 1994).

It was of interest to know whether the MDA signal observed in darkened leaves originated from the apoplast or not, since AsA in apoplast detoxifies exogeneous oxidants such as ozone (Luwe et al. 1993). A leaf segment of *A. cepa* was infiltrated with buffer containing AsA oxidase to remove AsA in the apoplastic compartments and subsequently subjected to EPR analysis. Although infiltration per se affected the leaf so that it induced higher MDA signals (Fig. 5, discussed later), no appreciable difference in MDA level was observed in the dark between the leaf segment infiltrated with AsA oxidase and a control infiltrated with water. Additionally, a leaf segment stripped of its lower epidermis and subsequently infiltrated in vacuo with water and washed several times with water still displayed the EPR signal of MDA in the dark. Thus, the removal of AsA from the apoplastic region by either AsA oxidase or washing did not affect the dark MDA level, indicating that the major generation site of MDA in the dark was either cytoplasm or vacuole.

The results in Fig. 2 illustrate the higher response of MDA signal in illuminated, but otherwise unstressed, leaves from different species under physiological conditions. Even in *A. cepa*, light-dependent MDA signals of EPR were often only 10 or 20% of the maximum value observed in Fig. 2. In young leaves of *S. oleracea* and *T. repens*, in mature leaves of *E. annuus* and *S. altissima* grown outdoors, and in leaves of *B. oleracea* grown in nutrient solution in a greenhouse, the EPR signals from MDA were usually so low, both in the dark and in the light, that they could not be distinguished from background noise. Thus, healthy leaves under little environmental stresses show very low levels of MDA, because the MDA generated would be very rapidly reduced to ascorbate coupled to MDA reductase and reduced ferredoxin (Fig. 1).

Unexpectedly, non-green, white leaf segments from a variegated *H. helix* showed a small increase in MDA with illumination (Fig. 2C, D). For this small photoproduction of MDA, red light was ineffective; bright white light (760 μmol m^{-2} s^{-1}) was required. Therefore, the most likely mechanism for the photoproduction of MDA in the non-green segments is not a chloroplastic reactions, but the interaction of AsA with reactive oxygens generated via photosensitized reactions by a light below 600 nm. However, the pigment participating in the photosensitizing reaction was not identified, and the cellular compartment where MDA is generated was not located.

Effect of methylviologen on the steady state concentration of MDA—A leaf of *S. altissima* that had been fed with methylviologen displayed low but distinct signals in the dark (Fig. 3). A control leaf left to stand in water did not show this characteristic MDA signal. Illumination with white light increased MDA to high steady state levels with a spin concentration ratio of MDA to $P700^+$ of about 3 at saturating intensity of light (Fig. 3). Similar methylviologen-induced production of MDA has been observed in maize, spinach and pea (Stegmann et al. 1993). Although the control leaf did not show any light-dependent accumulation of MDA, the methylviologen-fed leaf showed a steep increase in MDA at low light intensities and then a gradual

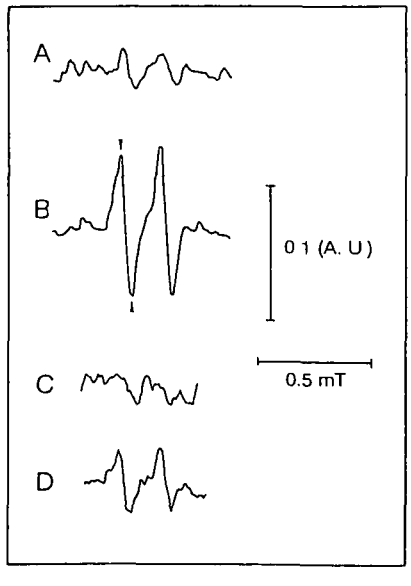

Fig. 2 MDA signals from leaf segments of *A. cepa* (A, B) and of a white region of *Hedera helix* (C, D) in the dark (A, C) and in the light (B, D). The leaf segment was illuminated by a white light (PPFD 760 μmol m^{-2} s^{-1}), and its EPR spectrum was recorded 10 s after starting illumination. Arrows in (B) show 337.46 mT (left) and 337.52 mT (right).

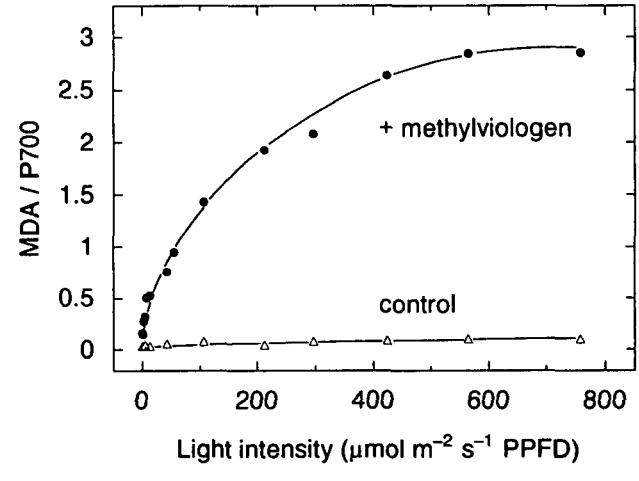

Fig. 3 MDA in methylviologen-fed *S. altissima* leaf as a function of light intensity expressed on its spin concentration ratio to $P700^+$. Upper curve; MDA in the leaf after feeding overnight in the dark via the petiole with 1 mM methylviologen. Lower curve; MDA in the leaf fed with water. Data points were measured using different leaf segments 5 s after the illumination.

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increase towards saturation. The same response to light was also observed in methylviologen-administered *Erigeron* leaves. Saturation of low light intensities is reminiscent of photoreduction of dioxygen in thylakoids which are saturated at low light intensities (Heber and French 1968, Asada and Nakano 1977).

Methylviologen is photoreduced to its cationic radical in thylakoids, competing effectively with ferredoxin for electrons from PSI, and the methylviologen radical is rapidly oxidized by dioxygen yielding O_2^- ($7.7 \times 10^8\ M^{-1}\ s^{-1}$, Farrington et al. 1973). MDA is generated either by the direct interaction of AsA with O_2^-, or by the H_2O_2 derived from O_2^- catalyzed with AsA peroxidase. A large increase in the level of MDA by administration of methylviologen should be due partly to an increased photoproduction of superoxide radicals. As shown in Fig. 1, the ferredoxin-mediated photoreduction of MDA and MDA reductase-mediated one are responsible for suppressing the light-dependent accumulation of MDA in chloroplasts. In the presence of methylviologen, however, little photoreduced ferredoxin and NAD(P)H in PSI are available for the reduction of MDA; therefore, this also should cause a large increase in MDA concentration in the light.

During illumination of the methylviologen-fed *S. altissima* leaf with high light intensity, MDA levels were kept at maximum for about 1 min and then decreased to about 20% of the initial maximal level within the following few minutes (Fig. 4). In the subsequent dark period, the leaf recovered its capacity to photogenerate large MDA signals. The time course of this recovery in the dark was determined by short light pulses lasting 5 s after various dark intervals. A half-time of the recovery was about 3–5 min. When the duration of the light pulses was prolonged, the MDA-levels declined much more rapidly during illumination than during the first illumination (data not shown). Observations very similar to those made with *S. altissima* were also made with *E. annuus, S. media*, and *B. oleracea* after feeding the leaves with methylviologen.

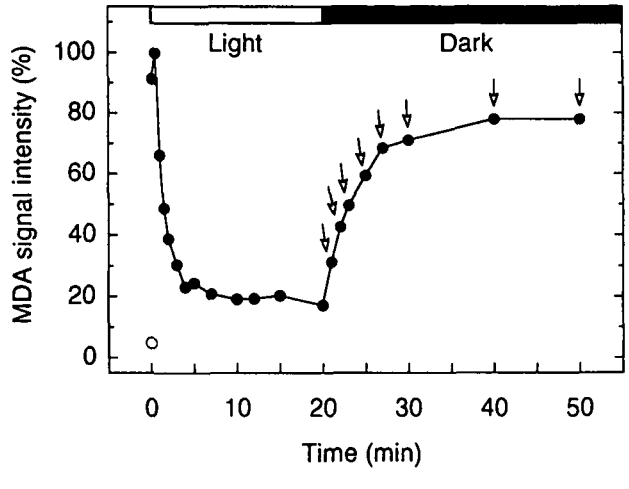

Fig. 4 Changes in intensity of light-induced EPR signal of MDA during illumination and subsequent dark period in a methylviologen-fed leaf segment of *S. altissima*. A leaf segment fixed in the EPR cavity was illuminated with white actinic light at saturated intensity (440 μmol $m^{-2}\ s^{-1}$) for 20 min, followed by a dark period. At each datum point, the EPR spectrum was recorded, and the intensity of MDA signal was determined. Open circle at 0 min represents the level of MDA signal before illumination. MDA level during the dark period was determined by 5-s pulse of a saturating light (open arrows). Signal intensity represents a ratio to the maximum intensity.

In the presence of methylviologen, the decrease in MDA-level in the leaves during prolonged illumination scarcely represents a decrease in the production potential of MDA. An increase in MDA-reducing potential is not likely either. We suggest that the decrease in MDA under prolonged illumination indicates the decreased concentration of AsA at the site where MDA is photogenerated (PSI) due to little photoreduction of MDA, resulting in the accumulation of DHA (Fig. 1). In the dark, AsA is only slowly regenerated via the reduction of DHA, which is produced through spontaneous disproportionation of MDA, catalyzed by DHA reductase using GSH as the electron donor. The rate of dark regeneration of AsA is limited by the availability of GSH, which is regenerated from GSSG by NADPH, and the reduction of $NADP^+$ is slow in the darkened chloroplasts (Hossain and Asada 1984). Diffusion of AsA into chloroplasts from the cytosol (Beck et al. 1983) may also participate in the dark recovery of MDA photoproduction. Thus, even early events ranging from several tens of seconds to minutes in photooxidative damage in leaves could be monitored as changes in MDA.

Photogeneration of MDA under other environmental conditions

Anaerobiosis—In none of the leaves tested were MDA signals observed in both the dark and the light when the atmosphere in the EPR cavity was replaced by argon. This strongly suggests that the generation of MDA in either dark or light is oxygen-dependent and likely to be caused by reactive species of oxygen. Even when MDA is generated by interaction of AsA with organic radicals, the present results indicate that the radicals responsible for MDA generation are generated by reactive species of oxygen.

Infiltration effect—Decreasing the gas exchange of *A. cepa* leaves by replacing air in their intercellular spaces with water or buffer increased the steady state MDA signal in the light (Fig. 5). Infiltration of water to apoplastic space limits access of CO_2 to chloroplasts in mesophyll cells (Evans and Caemmerer 1996), whereas oxygen remains available, at least in the light, owing to persisting photooxidation of water supported by endogeneous electron acceptors. The kinetics of MDA accumulation showed different characteristics, depending on whether effective gas exchange was possible or not. Illumination of a briefly

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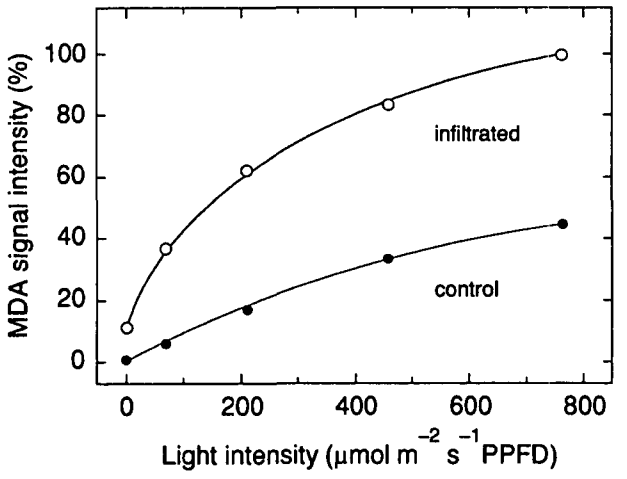

Fig. 5 Effect of infiltration on the intensity of MDA signal in leaf segments of *A. cepa* as a function of light intensity. Upper curve: the leaf segment was infiltrated with 50 mM HEPES, pH 7.6, and MDA was measured after the initial light-induced MDA maximum had declined towards a steady state (see the kinetics in Fig. 6). Lower curve: Control leaf, not infiltrated.

predarkened control leaf of *A. cepa* (Fig. 6A) resulted in only a small increase in MDA, followed by a smaller subsequent decline until finally a slightly higher steady state level was reached. In infiltrated leaf segments, the light-dependent increase in MDA was fast and large, followed by a fast decline to a lower plateau (Fig. 6B).

Cyanide—When a leaf segment of *A. cepa* was exposed for 5 min to 1% HCN in air and subsequently exposed to light, the kinetics of the increase in MDA were very similar to that shown in Fig. 6B for an infiltrated leaf segment. When steady state levels of MDA in the HCN-poisoned leaf segment were plotted against light intensity, the obtained curve (not shown) resembled that of the infiltrated leaf in Fig. 5. Cyanide inhibits CuZn-superoxide dismutase, AsA peroxidase, and catalase, thereby increases intracellular concentrations of O_2^- and H_2O_2. Furthermore, it inhibits the photosynthetic carbon reduction cycle, but permits photoreduction of oxygen in PSI. An increase in MDA level by cyanide would be caused by suppression of CO_2 fixation, similarly to infiltration treatment (Fig. 5).

Light stress—A *T. repens* leaf which initially did not exhibit MDA signals either in the light or in the dark was exposed for 60 s to very bright irradiation with a xenon source (36,000 μmol m^{-2} s^{-1}) which caused no visible damage to the leaf. Nevertheless, the radical scavenging systems of the leaf were overtaxed as shown by the appearance of several EPR signals in addition to the typical signals of MDA even in the dark (Fig. 7A). These MDA signals were enhanced in the light (Fig. 7B).

Water stress—When watering of potted cabbage was interrupted and considerable wilting, particularly of the older leaves, was permitted to occur, distinct MDA signals could be observed by EPR even in the dark. They increased

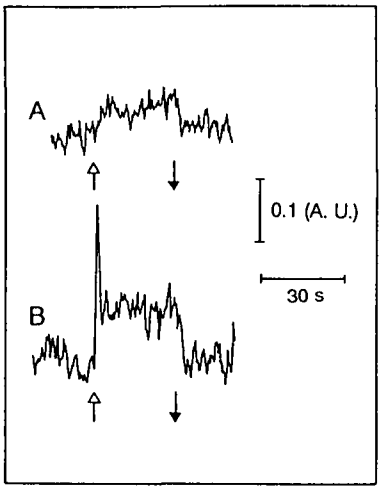

Fig. 6 Kinetics of light-induced MDA level in leaf segments of *A. cepa*. (A) Untreated leaf segment. (B) Leaf segment infiltrated in vacuo with water. The magnetic field was kept at 337.46 mT while the intensity of EPR signal was followed. Time constant of the recordings, 0.3 s. PPFD=760 μmol m^{-2} s^{-1}. White and black arrows indicate the turning on and off of light, respectively.

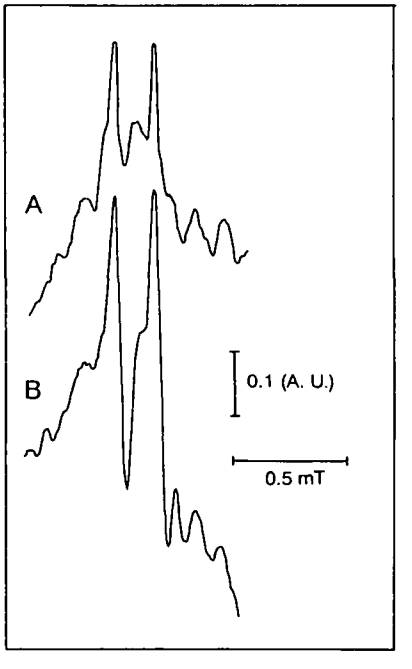

Fig. 7 EPR signals from a leaf segment of *T. repens* after a 30-s irradiation with a Xenon lamp. UV from the light source was filtered out. Considerable MDA was produced in the dark (A) and in the light (B) after 30-s exposure to the Xenon source at 36,000 μmol m^{-2} s^{-1}. Before illumination with the Xenon source, EPR did not show accumulation of MDA in the leaf in the dark or during illumination with 760 μmol m^{-2} s^{-1} white light.

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in the light by a factor of usually less than two (data not shown). Well-watered controls showed only indistinct MDA signals in both the dark and the light.

Leaf age—When leaves were old enough to exhibit yellowing, distinct MDA signals were observed at least in the light, but also often in the dark, even in plants where mature leaves showed no signs of MDA accumulation. Whereas young and unstressed spinach and cabbage leaves did not appear to accumulate MDA either in the dark or in the light, a yellow spinach leaf which was almost devoid of Chl and showed no MDA in the dark accumulated considerable MDA in the light. Very similar observations were made with yellow cotyledons of cabbage seedlings and a yellow leaf of *R. obtusifolius*. Fig. 8 shows the light-dependent accumulation of MDA in green (0.9 mg Chl (g fresh weight)$^{-1}$) and yellow (0.2 mg Chl (g fresh weight)$^{-1}$) *A. cepa* leaves as a function of light intensity. At the highest light intensity, the yellow leaf showed, on a basis of leaf area, twice as much MDA as the green leaf, but the differences were more pronounced on a Chl basis.

Concluding remarks—Levels of MDA in leaf tissue as observed by EPR spectrophotometry reflect the balance between the rate of univalent oxidation of AsA and the combined rates of ferredoxin- and MDA reductase-dependent reductions of MDA and spontaneous disproportionation of MDA to AsA and DHA (Fig. 1). Increased levels of MDA represent stronger oxidative stress, as long as AsA regeneration remains unchanged. Conversely, when the regenerating systems of AsA decline, constant radical production must result in increased levels of MDA. However, in methylviologen-fed leaves, the prolonged illumination caused lowering of the MDA levels, probably due to a decrease in the pool size of AsA (Fig. 4). Therefore, for assessment of oxidative stress, the determination of MDA by illumination for several seconds is recommended. The present experiments with methylviologen-, cyanide-, light- and water-stressed plants and the effects of leaf age on MDA accumulation indicate that MDA is a useful endogenous probe for stress effects within the limitations outlined above.

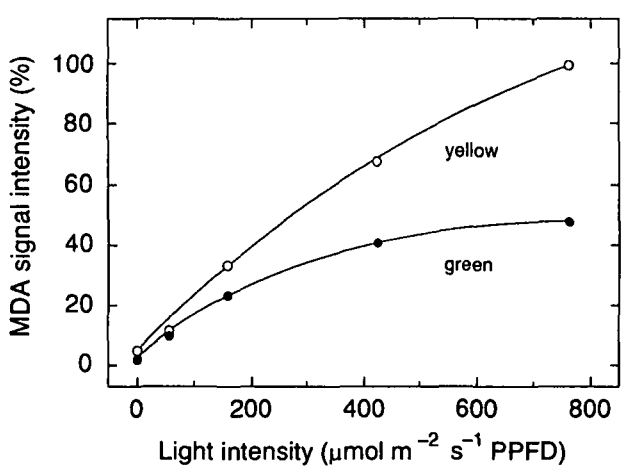

Fig. 8 Light-dependent accumulation of MDA as a function of light intensity in green and yellow segments from the leaf of *A. cepa*. The green segment, was taken from the middle of the leaf; the yellow segment, from the tip of the leaf.

The photooxidative stress of leaf cells has usually been assessed by following oxidative decomposition of cell membranes (lipid peroxide or ion leak) or of Chl. However, these events represent results of the degradation of cellular components by reactive oxygens and occur only after prolonged illumination. The redox state of AsA or glutathione also has been used to assess oxidative stresses, but its assay requires destruction of the cells. The high sensitivity and quick response of the MDA signals in leaves enable an in situ estimation of the oxidative stress being suffered by the cells prior to the decomposition of cellular components, and the measurement of the MDA radical provides information about initial biochemical events in the process of photooxidative damage in leaves.

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