Plant Physiol. (1996) 110: 125–136

Ultraviolet-B- and Ozone-Induced Biochemical Changes in Antioxidant Enzymes of *Arabidopsis thaliana*[1]

Mulpuri V. Rao*, Gopinadhan Paliyath, and Douglas P. Ormrod

Department of Horticultural Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Earlier studies with *Arabidopsis thaliana* exposed to ultraviolet B (UV-B) and ozone (O_3) have indicated the differential responses of superoxide dismutase and glutathione reductase. In this study, we have investigated whether *A. thaliana* genotype Landsberg *erecta* and its flavonoid-deficient mutant *transparent testa* (*tt5*) is capable of metabolizing UV-B- and O_3-induced activated oxygen species by invoking similar antioxidant enzymes. UV-B exposure preferentially enhanced guaiacol-peroxidases, ascorbate peroxidase, and peroxidases specific to coniferyl alcohol and modified the substrate affinity of ascorbate peroxidase. O_3 exposure enhanced superoxide dismutase, peroxidases, glutathione reductase, and ascorbate peroxidase to a similar degree and modified the substrate affinity of both glutathione reductase and ascorbate peroxidase. Both UV-B and O_3 exposure enhanced similar Cu,Zn-superoxide dismutase isoforms. New isoforms of peroxidases and ascorbate peroxidase were synthesized in *tt5* plants irradiated with UV-B. UV-B radiation, in contrast to O_3, enhanced the activated oxygen species by increasing membrane-localized NADPH-oxidase activity and decreasing catalase activities. These results collectively suggest that (a) UV-B exposure preferentially induces peroxidase-related enzymes, whereas O_3 exposure invokes the enzymes of superoxide dismutase/ascorbate-glutathione cycle, and (b) in contrast to O_3, UV-B exposure generated activated oxygen species by increasing NADPH-oxidase activity.

O_3 is the most prevalent phytotoxic air pollutant in many areas of the industrialized world (Runeckles and Chevone, 1992; Runeckles and Krupa, 1994). In North America, O_3 has caused more damage to vegetation than any other pollutant and this concern has prompted widespread attempts to evaluate plant responses to it (Runeckles and Chevone, 1992). Continuing release of chlorofluorocarbons has resulted in the depletion of stratospheric O_3, thus increasing UV-B radiation reaching the earth's surface. Recent models predict simultaneous increases in O_3 and UV-B at the earth's surface (Runeckles and Krupa, 1994). The adverse impact of UV-B and O_3 on plant growth has been shown to be either synergistic or additive, depending on the species exposed (Rao and Ormrod, 1995a).

The effect of UV-B and O_3 exposure individually has been evaluated in many plant species (reviewed by Krupa and Kickert, 1989; Tevini and Teramura, 1989; Runeckles and Krupa, 1994). Although there is a vast amount of research on UV-B- and O_3-induced changes in plant metabolism, it is not clear whether UV-B- and O_3-induced damages are brought about by similar mechanisms. This uncertainty has largely been due to the lack of an experimental approach to investigate the responses of a single species exposed to UV-B and O_3 under well-defined experimental conditions. Recently, by exposing *Arabidopsis thaliana* to UV-B and O_3 under similar experimental conditions, we have shown that the UV-B- and O_3-induced biochemical limitations of growth and photosynthesis are distinctly different (Rao et al., 1995b).

In spite of their potential differences as stress factors, both UV-B and O_3 share a common feature in generating AOS. It has been suggested that O_3 enters the mesophyll cells via the stomata, where it is converted into superoxide anion (O_2^-), hydroxyl radicals ($OH^\cdot$), and H_2O_2 (Grimes et al., 1983; Mehlhorn et al., 1990). Like O_3, UV-B exposure is believed to enhance the levels of AOS, although the mechanism of their generation is not known. Plants metabolize AOS by invoking the antioxidant defense system (Foyer et al., 1994; Kangasjarvi et al., 1994). The antioxidant defense system consists of low molecular weight antioxidants such as ascorbate, glutathione, α-tocopherol, and carotenoids (reviewed by Alscher and Hess, 1993), as well as several enzymes such as SODs, CATs, PODs, GR, and APX (reviewed by Bowler et al., 1994; Creissen et al., 1994).

SOD converts O_2^- radicals into H_2O_2 and O_2. The antioxidants like ascorbate and glutathione participate in both enzymic and nonenzymic H_2O_2 degradation (Foyer et al., 1994). CAT dismutates H_2O_2 into water and O_2, whereas POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Campa, 1991; Gaspar et al., 1991). PODs that use glutathione as a co-substrate have only rarely been identified in plants, but PODs specific for ascorbate have been frequently observed (Chen and Asada, 1989; Creissen et al., 1994). APX catalyzes the first step of the H_2O_2 scavenging pathway by oxidizing ASA. MDHAR and/or DHAR catalyzes the conversion of MDA or DHA to ASA by oxidizing glutathione.

[1] These studies were made possible by a Natural Sciences and Engineering Research Council of Canada International Fellowship to M.V.R. and a research grant to D.P.O.

* Corresponding author; e-mail vmulpuri@evbhort.uoguelph.ca; fax 1–519–767–0755.

Abbreviations: AOS, activated O_2 species; APX, ascorbate peroxidase; ASA, reduced ascorbate; CAT, catalase; CSTR, continuous stirred tank reactor; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; LER, Landsberg *erecta*; MDA, monodehydroascorbate, MDHAR, monodehydroascorbate reductase; POD, peroxidase; ppb, parts per billion; SOD, superoxide dismutase; *tt5*, *transparent testa*; UV-B, UV-B radiation (280–320 nm).

GSH is regenerated by the action in GR in a NADPH-dependent reaction (Fig. 1). In addition, PODs are believed to metabolize H_2O_2 by using phenols as a co-substrate through an ascorbate-dependent pathway (Otter and Polle, 1994).

O_3 has been reported to generate AOS directly by undergoing spontaneous dismutation (Mehlhorn et al., 1990), but the mechanism by which plants irradiated with UV-B generate AOS is not known. Although both UV-B and O_3 generate AOS such as H_2O_2 (Rao and Ormrod, 1995a, 1995b), it is not known whether plants exposed to UV-B or O_3 metabolize H_2O_2 by inducing similar antioxidant enzymes. However, the ability of plants to metabolize O_2^- and H_2O_2 has been reported to be largely dependent on the coordination of several interrelated antioxidant enzymes such as SOD, POD, GR, and APX (Fig. 1). Our preliminary studies have indicated that O_3-induced changes in SOD activities were much greater than those induced by UV-B, whereas the GR activities were enhanced only by O_3 exposure. Hence, we believed that it was imperative to investigate the parallelism of UV-B- and O_3-induced biochemical changes in the activities of several antioxidant enzymes. The main objectives of the present study were to investigate (a) whether *A. thaliana* metabolizes UV-B- and O_3-induced AOS by invoking similar antioxidant enzymes and (b) the mechanism by which plants irradiated with UV-B generate AOS.

Flavonoids, apart from possessing antioxidant properties, are believed to protect plants from impinging UV-B (Li et al., 1993). The presence of flavonoids in UV-B-irradiated leaves could alter the perception or response of other defense mechanisms. Mutants lacking flavonoids provide a valuable tool for evaluating the role of flavonoids and for identifying alternate mechanisms of protection from UV-B. Hence, we have utilized a flavonoid mutant (*tt5*) of *A. thaliana* specifically to investigate the influence of UV-B on antioxidant defense mechanisms. In this report we present results that suggest that *A. thaliana* metabolizes UV-B-induced AOS through POD-related antioxidant enzymes. By contrast, *A. thaliana* induced the activities of antioxidant enzymes of the SOD/ascorbate-glutathione cycle to metabolize O_3-induced AOS.

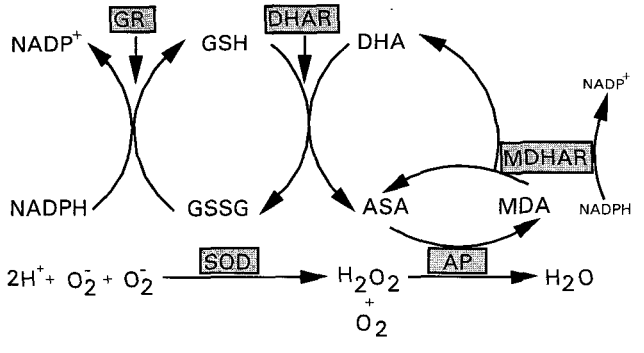

Figure 1. Schematic diagram of enzymic superoxide and H_2O_2 scavenging system. Superoxide anion radical (O_2^-) is dismutated to H_2O_2 via SOD. APX metabolizes H_2O_2 to water by oxidizing ASA to MDA and/or DHA. MDHAR reduces MDA to DHA or directly to ASA. DHAR catalyzes the conversion of DHA to ascorbate by oxidizing glutathione. GSH is regenerated by GR in an NADPH-dependent reaction.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana wild-type ecotype LER and its *tt5* mutant used in the present study are the same as described elsewhere (Rao and Ormrod, 1995a). Plants were grown in 25-cm diameter pots containing commercial rooting medium (Promix-BX; Premier Brands, Red Hill, PA) as described earlier (Rao and Ormrod, 1995a). The environmental conditions in the growth chamber were 24/18°C (day/night) temperature, 60 to 80% RH, and a PPFD of 250 μmol m^{-2} s^{-1}, with a 16-h photoperiod. Sylvania 115-W Gro-Lux fluorescent bulbs were used as the PPFD source. During growth, and prior to UV-B and O_3 exposure, both UV-C and UV-B radiation (wavelengths below 320 nm) were removed from the radiation by wrapping the lamps with Mylar polyester film (0.127 mm; Cadillac Plastic and Chemical Co., Toronto, Ontario, Canada). Eleven-day-old plants were transferred into identical chambers maintained under the same environmental conditions but provided with supplementary UV-B radiation or into CSTRs enriched with O_3. Control plants were maintained in the initial chambers or in CSTRs with no O_3. Plants were acclimated in the UV-B treatment chambers or in CSTRs overnight before the initiation of exposure.

UV-B Irradiation

The desired UV-B dose was provided by four UV-B-313 40-W fluorescent lamps (Q-Panel, Cleveland, Ohio). Plants were irradiated with UV-B for 8 h d^{-1} during the photoperiod. UV-C radiation (wavelengths below 280 nm) was removed by wrapping the UV-B lamps with cellulose diacetate (0.127 mm; Johnston Industrial Plastics, Toronto, Ontario, Canada). UV-B radiation from Sylvania Gro-Lux lamps was filtered with Mylar. Both Mylar and cellulose acetate were replaced every 2 to 3 d to maintain uniform optical properties. UV-B radiation was measured with a spectroradiometer (model OL 752; Optronics Laboratories, Orlando, FL) that was calibrated with an OL 752–150 calibration module and OL 752–10 spectral irradiance standard. The biologically effective UV-B fluence was calculated for the generalized plant response, normalized to 300 nm (Rao and Ormrod, 1995a). The average biologically effective UV-B fluences in control and UV-B growth chambers were 0.005 and 18 kJ m^{-2} d^{-1}, respectively.

O_3 Fumigation

The CSTRs used in the present study have been described elsewhere (Rao and Ormrod, 1995a). O_3 was generated by a high-voltage corona discharge generator (Aqua Air, model SL 4000; A.H. Simpson Industries Ltd., Toronto, Canada) and monitored with a Dasibi model 1008-PL analyzer (Dasibi Environmental Corp., Glendale, CA). O_3 concentrations were controlled with a programmable gas-dispensing system (Rao and Ormrod, 1995a). Plants were

exposed to 200 ppb O_3 for 6 h d^{-1} for 8 d. Environmental conditions in the CSTR were maintained at 26/19°C (day/night), 70% RH, and a PPFD of 250 μmol m^{-2} s^{-1}, with a 16-h photoperiod. One 1000-W Sylvania high-pressure sodium lamp was used as the PPFD source.

Growth Measurements

Whole plants were sampled at indicated intervals, and the rosette leaves were dried at 80°C for 48 h for dry weight measurements.

Enzyme Assay

Extraction

Rosette leaves (2 g) were homogenized with 100 mM potassium phosphate (pH 7.5) containing 2 mM EDTA and 1% PVP-40 at 4°C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000*g* for 20 min. Protein content in the supernatant was concentrated by ammonium sulfate precipitation. Ammonium sulfate fractions between 45 and 90% were pooled and extensively dialyzed against the same buffer with three changes of buffer overnight at 4°C. Dialyzed and concentrated protein extracts were stored at −80°C for further analyses. The activities of different enzymes were determined with leaf extracts equivalent to 100 μg of protein. Protein content was determined according to the method of Bradford (1976) with BSA as a standard.

SOD (EC 1.15.1.1)

SOD activity was determined essentially as described by Spychalla and Desborough (1990). The assay was performed at 25°C in a 3-mL cuvette containing 50 mM $Na_2CO_3/NaHCO_3$ buffer (pH 10.2), 0.1 mM EDTA, 0.015 mM ferricytochrome *c*, and 0.05 mM xanthine. One unit of SOD is defined as the amount of enzyme that inhibited the rate of ferricytochrome *c* reduction by 50%. Mn-SOD activity was determined as described above except that the assay mixture contained 2 mM KCN to inactivate Cu,Zn-SOD. Cu,Zn-SOD was calculated from the difference between total SOD and Mn-SOD activities.

POD (EC 1.11.1.7) and CAT (EC 1.11.1.6)

POD activities were determined specifically with guaiacol at 470 nm (extinction coefficient 25.2 mM cm^{-1}) and coniferyl alcohol at 262 nm (extinction coefficient 2.2 mM cm^{-1}) following the method of Polle et al. (1994). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 16 mM guaiacol or 50 μM coniferyl alcohol, and 10 μL of 10% H_2O_2 in a 3-mL volume. The reaction was initiated by adding plant extract and followed for 10 min. CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm for 5 min. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0) and plant extract in a 3-mL volume. The reaction was initiated by adding 10 μL of 30% (w/v) H_2O_2.

GR (EC 1.6.4.2)

GR activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM cm^{-1}) as described by Rao (1992). The 1-mL assay mixture contained 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM GSSG, and the leaf extract. The assays were initiated by the addition of NADPH at 25°C. K_m values were determined from Lineweaver-Burk plots; substrate concentrations varied between 20 and 500 μM for GSSG and 2 and 200 μM for NADPH in the presence of a saturating concentration of the other substrate (200 μM NADPH or 200 μM GSSG).

Extraction and Determination of APX (EC 1.11.1.11)

For determination of APX activity, rosette leaves were homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 5 mM ascorbate at 4°C as described earlier. Proteins were subjected to ammonium sulfate fractionation and fractions between 45 and 90% were pooled and dialyzed overnight against the same buffer containing 5 mM ascorbate as described above. APX activity was determined by following the decrease in A_{290} for 3 min (extinction coefficient 2.8 mM cm^{-1}). The 1-mL reaction volume contained 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM ascorbate, and 0.2 mM H_2O_2 at 25°C (Chen and Asada, 1989). K_m values were determined as described for GR. Substrate concentrations varied between 20 and 500 μM for ascorbate and 20 and 200 μM for H_2O_2 in the presence of a saturating concentration of the other substrate (200 μM H_2O_2 or 500 μM ascorbate).

Native PAGE and Activity Staining

Equal amounts of protein from plants exposed to different treatments were subjected to discontinuous PAGE under nondenaturing, nonreducing conditions essentially as described by Laemmli (1970), except that SDS was omitted and the gels were supported by 10% glycerol (Mittler and Zilinskas, 1993). Electrophoretic separation was performed at 4°C for 4 h with a constant current of 30 mA per gel. After completion of electrophoresis the gels were stained for the activities of SOD, POD, and GR as described by Rao et al. (1995a).

Gels were stained for SOD isoforms by incubating in a solution containing 2.5 mM nitroblue tetrazolium for 25 min, followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 mM tetramethyl ethylene diamine for 20 min in the dark. The gels were placed in distilled water and exposed on a light box for 10 to 15 min at room temperature. Visualization of cyanide-sensitive and -insensitive isozymes was achieved by incubating gels in 50 mM potassium phosphate buffer (pH 7.0) containing 3 mM KCN or 5 mM H_2O_2 for 30 min before staining for SOD activity.

Staining of POD isozymes was achieved by incubating the gels in sodium acetate buffer (pH 4.5) containing 2 mM benzidine (dissolved in DMSO). The reaction was initiated by adding 3 mM H_2O_2 and the reaction was allowed to continue for 20 min. GR activity was detected by incubat-

ing gels in 50 mL of Tris-HCl (pH 7.5) containing 10 mg of 3-(4, 5-dimethylthiazol-2–4)-2,5-diphenyl tetrazolium bromide, 10 mg of 2,6-dichlorophenolindophenol, 3.4 mM GSSG, and 0.5 mM NADPH. Duplicate gels were assayed for GR activity, one with and one without GSSG.

Electrophoresis and Detection of APX Activity

Samples were subjected to native PAGE (gels supported with 10% glycerol) as described above, except that carrier buffer contained 2 mM ascorbate. The gels were prerun for 30 min to allow ascorbate, present in the carrier buffer, to enter the gel prior to the application of samples (Mittler and Zilinskas, 1993). Following electrophoretic separation, gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min. The gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gels were washed with buffer for 1 min and submerged in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM tetramethyl ethylene diamine and 2.45 mM nitroblue tetrazolium with gentle agitation. The reaction was continued for 10 to 15 min and stopped by a brief wash in water.

In Vivo Oxidative Damage

Oxidative damage to protein was estimated as the content of carbonyl groups (Levine et al., 1990). Rosette leaves (1 g) were homogenized with 5 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA and 2.5 μg each of protease inhibitors, PMSF, leupeptin, and pepstatin A at 4°C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000*g* for 25 min at 4°C, and the foliar carbonyl groups were determined by reaction with 2,4-dinitrophenylhydrazine (Levine et al., 1990).

Preparation of Membrane-Rich Fraction

Rosette leaves were homogenized in a cold buffer (4°C) containing 100 mM potassium phosphate buffer, 1 mM EDTA, and 0.5 mM Suc (at pH 7.5). The homogenate was filtered through four layers of cheesecloth and centrifuged at 12,000*g* for 20 min. The supernatant was centrifuged at 180,000*g* for 60 min and the pellet was resuspended in the homogenizing buffer and recentrifuged at 180,000*g* for 60 min. The resulting pellet was resuspended in homogenizing buffer and stored at −80°C for further analyses.

NADPH-Oxidase

NADPH-oxidase activity was measured spectrophotometrically according to the method of Askerlund et al. (1987). The assay mixture (1 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 150 μM NADPH, 10 μM KCN, and 50 μg of membrane protein. The reaction was initiated by the addition of protein and the decrease in A_{340} was followed for 3 min.

UV-B and O_3 exposures were initiated by transferring 11-d-old plants into the growth chambers or CSTRs supplemented with UV-B or O_3. The experiment was a split-plot design with time as the main plot and genotypes as the subplots, and the experiments described were conducted at least twice in their entirety each with two replicates. The mean values presented are averages of two different experiments. Two-way analysis of variance was performed separately to test the main effects of UV-B or O_3 exposures, time, and their interactions. Mean separation was based on the calculation of 95% confidence limits from the appropriate experiment error mean square and tabled *t* value (Rao et al., 1995b). All enzyme assays were performed with ammonium sulfate-precipitated and -dialyzed extracts (except NADPH-oxidase). Native PAGE was conducted with crude extracts. For comparisons between treatments, gels were loaded with equal amounts of protein found to be within a linear activity range (data not shown). Activity staining was repeated twice with as many extracts from each experiment, and the photographs presented are from one representative experiment.

RESULTS

Growth Responses

Growth under UV-B decreased the dry matter production of *tt5* within 3 d ($P < 0.05$), whereas there were no major changes in the dry matter production of LER plants during the 5-d exposure period (Fig. 2a). O_3 exposure reduced dry matter production of LER plants within the first 4 d of exposure ($P < 0.05$) (Fig. 2b). In general, UV-B and O_3 exposure for more than 5 and 8 d, respectively, resulted in the appearance of visible injury symptoms (except in LER irradiated with UV-B) (data not shown).

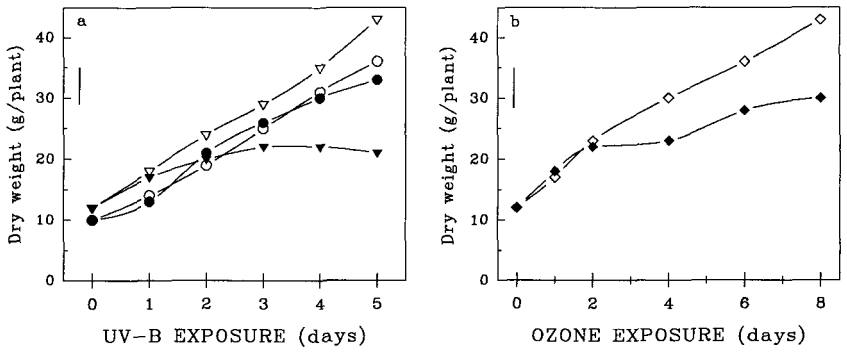

Figure 2. Growth responses of *A. thaliana* genotypes LER and *tt5* exposed to UV-B for 5 d (a) and of LER exposed to O_3 for 8 d (b). UV-B and O_3 exposure was initiated when plants were 11 d old. ○, LER-control-UV-B; ●, LER-UV-B; ▽, *tt5*-control; ▼, *tt5*-UV-B; ◇, LER-control-O_3; ◆, LER-O_3. Bars denote LSD ($P < 0.05$).

SOD

In comparison to control plants, UV-B radiation enhanced total SOD activity of *tt5* plants within the first 2 d of exposure ($P < 0.05$). However, a similar enhancement was observed in LER plants only after 4 to 5 d of UV-B exposure ($P < 0.05$) (Fig. 3a). Total SOD activity represents the combined action of Cu,Zn-, Mn-, and Fe-SOD, which can be distinguished by their differential sensitivity to cyanide and H_2O_2. No major Fe-SOD activity was observed (see "Native PAGE and Activity Staining"). UV-B exposure caused significant increases in Cu,Zn-SOD ($P < 0.05$) but had no effect on Mn-SOD in either genotype (Fig. 3, b and c).

O_3 exposure for 8 d enhanced total SOD activity of LER by 83% ($P < 0.01$) as compared to control plants (Fig. 3d). Similar to UV-B exposure, O_3 enhanced total SOD activity of LER plants and this appeared to be largely due to enhanced Cu,Zn-SOD activity (Fig. 3e). No significant changes were observed in the Mn-SOD activity of LER plants exposed to O_3 (Fig. 3f).

CAT and POD

UV-B and O_3 enhanced guaiacol-PODs in both genotypes. However, the magnitude of increase differed significantly between the two treatments (Fig. 4, a and b). UV-B exposure for 5 d enhanced total guaiacol-PODs by about 4- and 11-fold ($P < 0.01$) in LER and *tt5* plants, respectively (Fig. 4a). On the other hand, O_3 exposure for 8 d enhanced total guaiacol-PODs of LER by 90% ($P < 0.01$) compared to those of control plants (Fig. 4b). PODs are known to utilize different substrates to metabolize H_2O_2, and one such substrate is coniferyl alcohol (Polle et al., 1994). When coniferyl alcohol was used as a substrate, POD activities were enhanced by 2- and 3-fold ($P < 0.01$) in UV-B-irradiated LER and *tt5* plants, whereas there were no major changes in LER plants exposed to O_3 (Table I).

UV-B radiation for 5 d decreased CAT activities of *tt5* plants ($P < 0.05$) but had no significant effect on CAT activity of LER plants (Fig. 4c). O_3 exposure for 8 d had no major effect on CAT activity of LER plants compared to that of control plants (Fig. 4d).

GR

UV-B exposure for 5 d had no effect on GR activities of LER, whereas GR activity was enhanced by 36% ($P < 0.05$) in *tt5* plants irradiated with UV-B for 5 d compared to those of control plants (Fig. 5a). On the other hand, O_3 exposure for 8 d enhanced foliar GR activities of LER by 98% ($P < 0.01$) compared to that of control plants (Fig. 5b). Oxidative stress is known to modify the properties

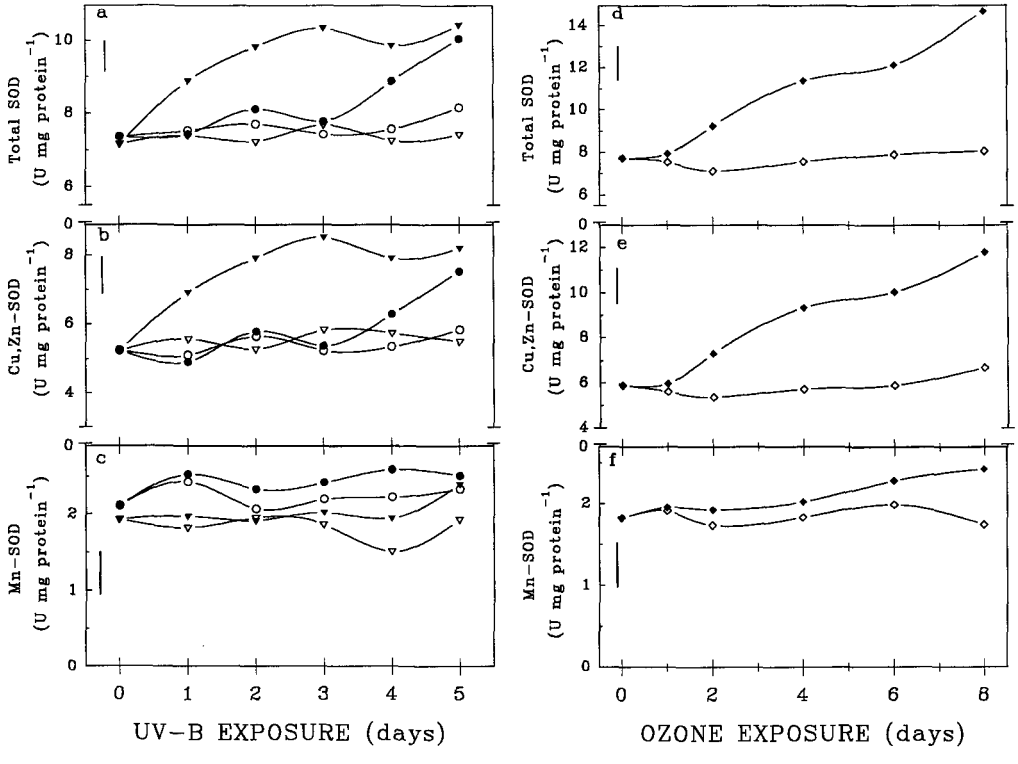

Figure 3. *Responses of total, Cu,Zn-, and Mn-SOD of A. thaliana genotypes irradiated with UV-B of 18 kJ m^{-2} d^{-1} (a, b, and c) for 5 d or exposed to O_3 of 200 ppb for 8 d (d, e, and f). UV-B exposure was initiated when plants were 11 d old. ○, LER-control; ●, LER-UV-B; ▽, tt5-control; ▼, tt5-UV-B; ◇, LER-control-O_3; ◆, LER-O_3. Mn-SOD activities were determined as for total SOD except that the assay mixture contained 2 mM KCN. Preliminary studies have indicated that 2 mM KCN completely inactivates Cu,Zn-SOD (data not shown). Cu,Zn-SOD was obtained by subtracting Mn-SOD from total SOD. Bars denote LSD ($P < 0.05$). U, Units.*

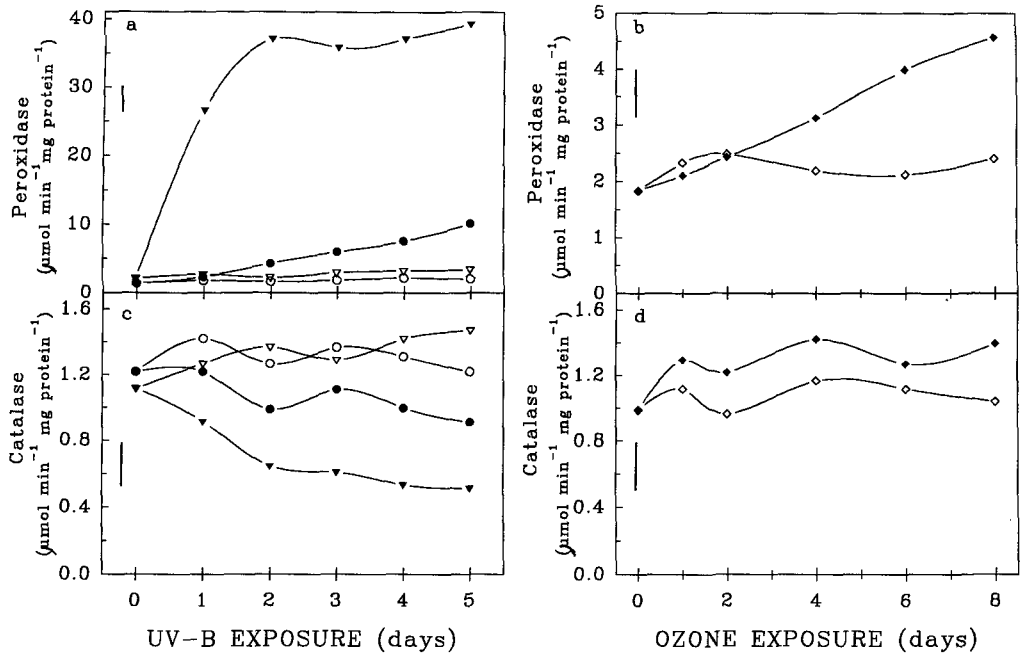

Figure 4. Total guaiacol-POD and CAT activities of *A. thaliana* genotypes irradiated with UV-B of 18 kJ m^{-2} d^{-1} for 5 d (a and c) or exposed to O_3 of 200 ppb for 8 d (b and d). UV-B and O_3 exposure was initiated when plants were 11 d old. ○, LER-control-UV-B; ●, LER-UV-B; ▽, *tt5*-Control; ▼, *tt5*-UV-B; ◇, LER-control-O_3; ◆, LER-O_3. Bars denote LSD ($P < 0.05$).

of GR in several species (Guy and Carter, 1984; Edwards et al., 1994; Hausladen and Alscher, 1994). Since both UV-B and O_3 induce oxidative stress, we have evaluated the changes in the kinetic properties of GR. UV-B exposure for 5 d had no major influence on the K_ms of GR toward GSSG and NADPH in either genotype (Table II). However, O_3 exposure for 8 d decreased the K_m of GR for GSSG and NADPH by 35 and 47% ($P < 0.05$), respectively (Table II).

APX

In LER and *tt5* plants, UV-B exposure for 5 d enhanced APX activity by 2- and 3.6-fold ($P < 0.01$), respectively, compared to that of control plants (Fig. 5c). Similarly, O_3 exposure for 8 d enhanced APX activity of LER by 87% ($P < 0.01$) (Fig. 5d). Under oxidative stress, the kinetic properties of antioxidant enzymes change so as to make them more efficient and help alleviate stress more efficiently (Creissen et al., 1994). The affinity of APX toward ascorbate and H_2O_2 was determined in plants exposed to UV-B and O_3. In both LER and *tt5* plants, growth under UV-B for 5 d caused a significant decrease in the K_m for ascorbate by 38 and 33%, respectively ($P < 0.05$). The K_m values for H_2O_2 also decreased by 54 and 61% ($P < 0.01$), respectively (Table III). O_3 exposure for 8 d decreased the K_m for both ascorbate and H_2O_2 by 27 and 29% ($P < 0.05$), respectively, in LER plants (Table III).

Plants possess different isoforms of SOD, POD, GR, and APX, localized in different organelles (Bowler et al., 1994; Creissen et al., 1994). UV-B- and O_3-induced changes in the activities of SOD, POD, GR, and APX represent total activities and do not reflect the changes in their isoform composition. Under oxidative stress, plants are believed to synthesize new isoforms with altered kinetic properties (Creissen et al., 1994; Edwards et al., 1994). Since both UV-B and O_3 appeared to have altered the kinetic properties of several enzymes studied, we determined whether the changes in kinetic properties are

Table I. *POD activity with coniferyl alcohol as a substrate (CA-POD) in A. thaliana genotypes irradiated with UV-B of 18 kJ m^{-2} d^{-1} for 5 d or exposed to O_3 of 200 ppb for 8 d*

Values in parentheses are relative to control plants.

Genotype	Treatment		
	Control	UV-B	O_3
		μmol min^{-1} mg^{-1} protein	
LER	1.82 ± 0.162 (100)	3.64 ± 0.212 (200)[a]	–[b]
LER	2.27 ± 0.242 (100)	–[b]	2.73 ± 0.226 (120)
tt5	2.27 ± 0.198 (100)	7.13 ± 0.623 (313)[a]	–[b]

[a] Significantly different values compared to control ($P < 0.05$) ± SE. [b] –, Not determined.

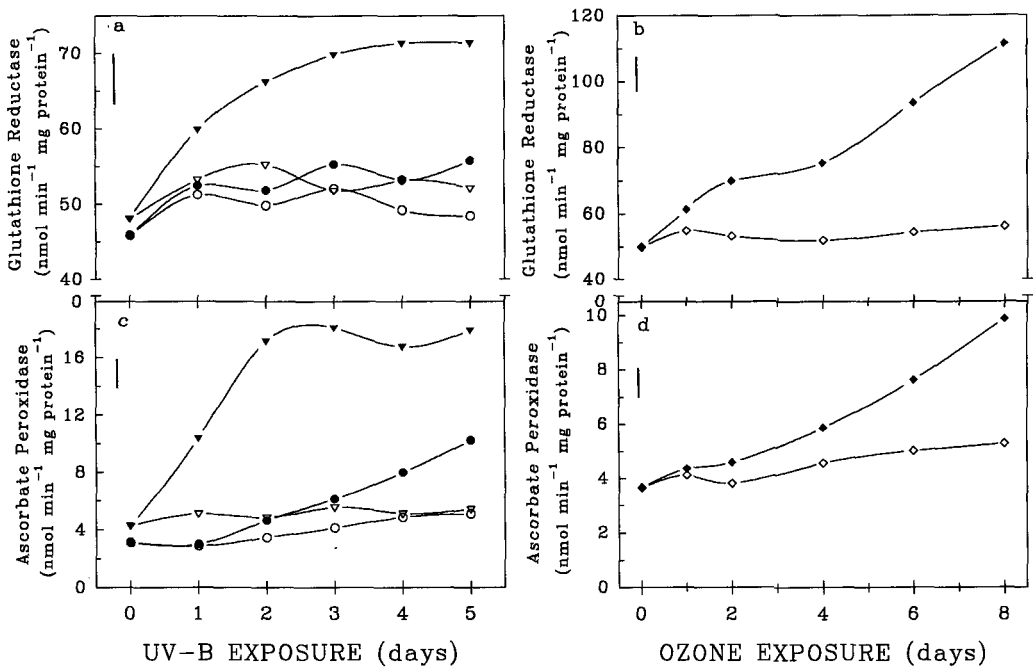

Figure 5. GR and APX activities of *A. thaliana* genotypes irradiated with UV-B of 18 kJ m^{-2} d^{-1} for 5 d (a and c) or exposed to O_3 of 200 ppb for 8 d (b and d). UV-B and O_3 exposure was initiated when plants were 11 d old. ○, LER-control- UV-B; ●, LER-UV-B; ▽, *tt5*-control; ▼, *tt5*-UV-B; ◇, LER-control-O_3; ◆, LER-O_3. Bars denote LSD ($P < 0.05$).

in fact due to changes in the isoform composition of SOD, POD, GR, and APX.

Activity Staining of SOD

When foliar extracts were subjected to native PAGE and monitored for SOD activity, five different SOD isoforms were observed in control plants (Fig. 6). Incubation of gels in 2 mM KCN or 5 mM H_2O_2 before staining for SOD activity indicated isoform 1 to be Mn-SOD, and the other four isoforms were identified as Cu,Zn-SOD (data not shown; Fig. 6). No Fe-SOD isoform was observed in either genotype. Under the influence of UV-B, the intensities of Cu,Zn-SOD-2, -3, and -4 were preferentially enhanced in both genotypes, whereas the intensity of Mn-SOD (SOD-1) increased slightly (Fig. 6, lanes b and d). However, under the influence of O_3, Cu,Zn-SOD-1 disappeared but the intensities of other Cu,Zn-SOD isoforms were preferentially enhanced (Fig. 6, lane f). Similar to UV-B exposure, the intensity of Mn-SOD increased slightly in plants exposed to O_3 (Fig. 6, lane f).

Activity Staining of POD

Gels stained for POD activity revealed only one isoform in the control plants of both genotypes (Fig. 7). UV-B exposure enhanced the intensity of the existing isoform and caused the synthesis of a new isoform of POD in LER plants (Fig. 7, lane b). However, four new isoforms were synthesized in *tt5* plants irradiated with UV-B (Fig. 7, lane

Table II. *K_m of GR toward GSSG and NADPH in A. thaliana genotypes irradiated with UV-B of 18 kJ m^{-2} d^{-1} for 5 d or exposed to O_3 of 200 ppb for 8 d*

Values in parentheses are relative to control plants.

Genotype	Treatment		
	Control	UV-B	O_3
		K_m (μM GSSG)	
LER	41 ± 5 (100)	39 ± 5 (95)	–[a]
LER	45 ± 5 (100)	–[a]	29 ± 4 (65)[b]
tt5	44 ± 5 (100)	35 ± 6 (80)	–[a]
		K_m (μM NADPH)	
LER	14 ± 2 (100)	13 ± 3 (93)	–[a]
LER	15 ± 2 (100)	–[a]	8 ± 1 (53)[b]
tt5	15 ± 2 (100)	14 ± 3 (93)	–[a]

[a] –, Not determined. [b] Significantly different values compared to control ($P < 0.05$) ± SE.

Table III. *K_m of APX toward ascorbate and H_2O_2 in A. thaliana genotypes irradiated with UV-B of 18 kJ m^{-2} d^{-1} for 5 d or exposed to O_3 of 200 ppb for 8 d*

Values in parentheses are relative to control plants.

Genotype	Treatment		
	Control	UV-B	O_3
		K_m (μM ascorbate)	
LER	139 ± 15 (100)	86 ± 10 (62)[b]	–[a]
LER	145 ± 13 (100)	–[a]	106 ± 12 (73)[b]
tt5	142 ± 12 (100)	66 ± 7 (46)[b]	–[a]
		K_m (μM H_2O_2)	
LER	51 ± 6 (100)	34 ± 5 (67)[b]	–[a]
LER	49 ± 5 (100)	–[a]	35 ± 4 (71)[b]
tt5	47 ± 5 (100)	18 ± 2 (38)	–[a]

[a] –, Not determined. [b] Significantly different values compared to control ($P < 0.05$) ± SE.

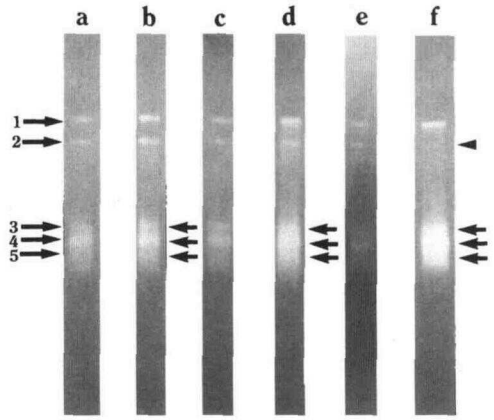

Figure 6. Native gels stained for the activity of SOD of *A. thaliana* genotypes exposed to UV-B of 18 kJ m^{-2} d^{-1} for 5 d or to O_3 of 200 ppb for 8 d. Equal amounts of protein (200 μg) from plants exposed to UV-B and O_3 were loaded on the gel. Lane a, LER-control-UV-B; lane b, LER-UV-B; lane c, *tt5* control; lane d, *tt5*-UV-B; lane e, LER-control-O_3; lane f, LER-O_3. Large arrows indicate different isoforms in control plants. Incubation of gels with 3 mM KCN before staining for SOD activity indicated one isoform corresponding to SOD-1 (data not shown). Note that isoform 1 is Mn-SOD and the other four isoforms are Cu,Zn-SOD. Small arrows indicate the isoforms whose staining intensities are preferentially enhanced by either UV-B or O_3 exposure. Arrowhead indicates the absence of Cu,Zn-SOD-1 in plants exposed to O_3.

d). Under the influence of O_3, no new isoforms were synthesized but the intensity of the existing isoforms was enhanced (Fig. 7, lane f).

Activity Staining of GR

Three GSSG-specific GR isoforms were present in *A. thaliana* along with two GSSG-nonspecific isoforms (Fig. 8). The intensities of all three isoforms remained unaffected by UV-B exposure in LER plants (Fig. 8, lane b), whereas the intensity of the GR-3 isoform was enhanced slightly in *tt5* plants irradiated with UV-B (Fig. 8, lane d). Similarly, the intensity of the GR-3 isoform was significantly enhanced compared to other isoforms in LER plants exposed to O_3 (Fig. 8, lane f).

Activity Staining of APX

Leaves of *A. thaliana* have two isoforms of APX. Exposure of LER to either UV-B or O_3 enhanced the intensity of both of the isoforms to a similar extent (Fig. 9, lanes b and f). However, irradiation of *tt5* plants with UV-B resulted in the synthesis of a new isoform, in addition to enhancing the intensities of the existing isoforms (Fig. 9, lane d). Our attempts to detect APX in a purified chloroplastic preparation in the presence of ascorbate yielded no activity. Since we could not observe any chloroplastic APX, we assumed that the isoforms detected on gels were of cytosolic origin. Similar attempts to detect chloroplastic APX on native gels have also been unsuccessful (Mittler and Zilinskas, 1993).

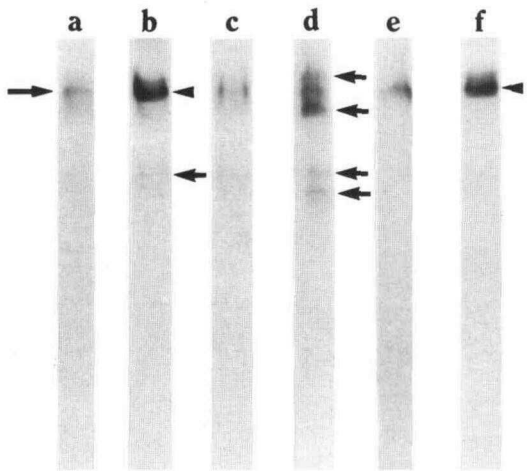

Figure 7. Native gels stained for the activity of POD of *A. thaliana* genotypes exposed to UV-B of 18 kJ m^{-2} d^{-1} for 5 d or to O_3 of 200 ppb for 8 d. Equal amounts of protein (200 μg) from plants exposed to UV-B and O_3 were loaded on the gel. Lane a, LER-control-UV-B; lane b, LER-UV-B; lane c, *tt5* control; lane d, *tt5*-UV-B; lane e, LER-control-O_3; lane f, LER-O_3. Large arrow indicates the isoform in control plants. Small arrows indicate the newly synthesized isoforms. Arrowheads indicate the isoform whose staining intensity was preferentially enhanced by different treatments.

Oxidative Damage to Proteins

AOS generated by UV-B and O_3 are believed to cause oxidative damage to proteins. Oxidative damage to proteins can be monitored by assaying foliar carbonyl content (Levine et al., 1990). In vivo carbonyl groups remained unaltered in LER plants irradiated with UV-B for 5 d, whereas those in *tt5* increased 2-fold ($P < 0.01$) (Table IV).

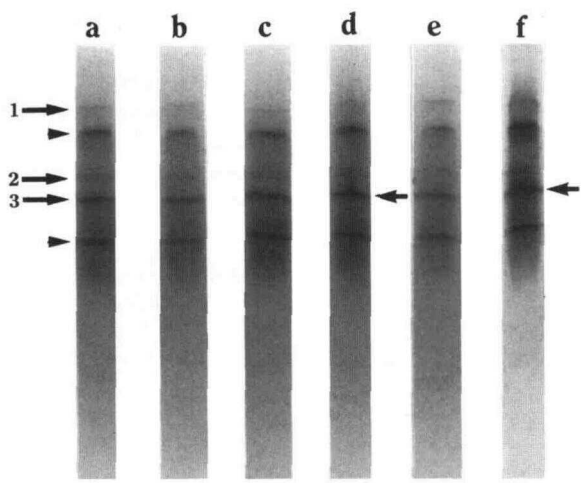

Figure 8. Native gels stained for GR of *A. thaliana* genotypes exposed to UV-B of 18 kJ m^{-2} d^{-1} for 5 d or to O_3 of 200 ppb for 8 d. Equal amounts of protein (100 μg) from plants exposed to UV-B and O_3 were loaded on the gel. Lane a, LER-control-UV-B; lane b, LER-UV-B; lane c, *tt5* control; lane d, *tt5*-UV-B; lane e, LER-control-O_3; lane f, LER-O_3. Large arrows indicate different isoforms in control plants. Small arrows indicate the isoforms whose staining intensities were preferentially enhanced by UV-B or O_3 exposures. Arrowheads indicate GSSG-nonspecific isoforms.

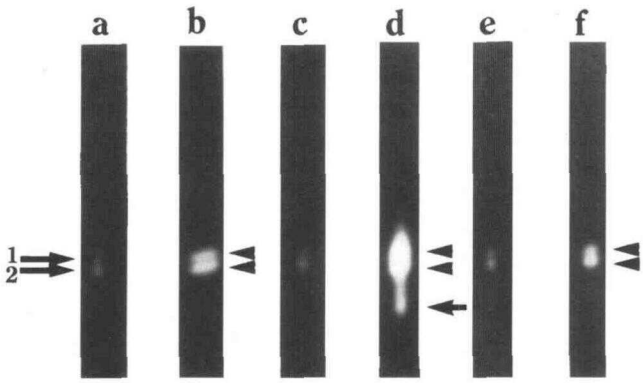

Figure 9. Native gels stained for the activity of APX of *A. thaliana* genotypes exposed to UV-B of 18 kJ m^{-2} d^{-1} for 5 d or to O_3 of 200 ppb for 8 d. Equal amounts of protein (150 μg) from plants exposed to UV-B and O_3 were loaded on the gel. Lane a, LER-control-UV-B; lane b, LER-UV-B; lane c, *tt5* control; lane d, *tt5*-UV-B; lane e, LER-control-O_3; lane f, LER-O_3. Large arrows indicate different isoforms in control plants. Small arrow indicates newly synthesized isoform. Arrowheads indicate isoforms whose staining intensities were significantly enhanced compared to control plants.

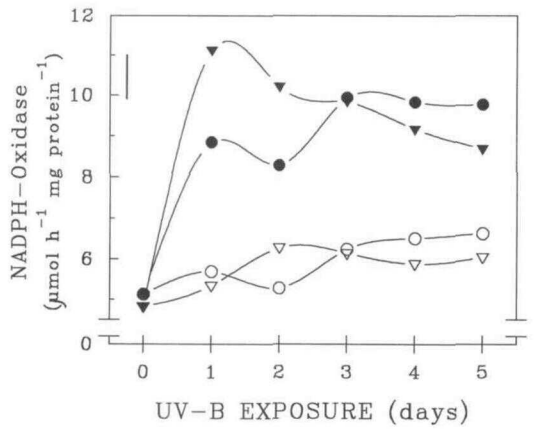

Figure 10. Membrane-localized NADPH-oxidase of *A. thaliana* genotypes irradiated with UV-B of 18 kJ m^{-2} d^{-1} for 5 d. UV-B and O_3 exposure was initiated when plants were 11 d old. ○, LER-control-UV-B; ●, LER-UV-B; ▽, *tt5*-control; ▼, *tt5*-UV-B. Bars denote LSD ($P < 0.05$). No significant changes were observed in the NADPH-oxidase activities of LER plants exposed to O_3 of 200 ppb for 8 d (data not shown).

O_3 exposure for 8 d enhanced foliar carbonyl groups by 3-fold ($P < 0.01$) compared to those of unexposed plants (Table IV). Carbonyl groups were significantly higher in plants exposed to O_3 when compared to carbonyl groups of plants irradiated with UV-B.

NADPH-Oxidase

The origin of AOS, particularly O_2^- and H_2O_2, has been linked to redox changes at the plasma membrane (Rubinstein and Luster, 1993). Although growth under UV-B enhanced the oxidative damage to proteins, it is not known how UV-B generates AOS. On the other hand, O_3 is believed to generate AOS directly by undergoing spontaneous dismutation (Grimes et al., 1983; Mehlhorn et al., 1990). The involvement of NADPH-oxidase in the generation of H_2O_2 has long been established in various plant species (Moller and Lim, 1986). Microsomal membrane fractions were isolated from control and UV-B- and O_3-exposed plants and analyzed for NADPH-oxidase activities. There was a dramatic and significant increase ($P < 0.01$) in the membrane-localized NADPH-oxidase in both genotypes after 1 d of UV-B treatment (Fig. 10). However, the initial increase in NADPH-oxidase activity declined during further exposure of plants to UV-B. By the end of UV-B exposure, NADPH-oxidase activity was significantly higher by 48 and 46% ($P < 0.01$) in LER and *tt5* plants, respectively (Fig. 10). No significant changes were observed in NADPH-oxidase of LER plants exposed to O_3 (data not shown).

DISCUSSION

The metabolism of AOS is dependent on several functionally interrelated antioxidant enzymes such as SOD, POD, CAT, GR, and APX. Although both UV-B and O_3 have been shown to induce one or more antioxidant enzyme(s) (Strid, 1993; Willekens et al., 1994), to date there has been no detailed report concerning the responses of different antioxidant enzymes in a single species exposed to UV-B and O_3 under similar experimental conditions. Earlier studies in our laboratory showed that the exposure of *tt5* plants to UV-B for 6 to 7 d and LER to O_3 for 9 to 10 d results in visible injury symptoms (Rao and Ormrod, 1995a, 1995b; Rao et al., 1995b). Hence, we terminated these experiments after 5 d of UV-B and 8 d of O_3 exposure to evaluate plant responses under physiologically relevant conditions (i.e. before the onset of visible injury symptoms). These studies also suggested that the changes in

Table IV. *Foliar carbonyl content of A. thaliana genotypes irradiated with UV-B of 18 kJ m^{-2} d^{-1} for 5 d or exposed to O_3 of 200 ppb for 8 d*

Values in parentheses are relative to control plants.

Genotype	Treatment		
	Control	UV-B	O_3
		nmol C = O mg^1 protein	
LER	12.12 ± 1.86 (100)	13.87 ± 1.52 (114)	–[a]
LER	13.07 ± 1.44 (100)	–[a]	40.13 ± 2.12 (307)[b]
tt5	16.30 ± 1.42 (100)	35.86 ± 2.12 (220)[b]	–[a]

[a] –, Not determined. [b] Significantly different values compared to control ($P < 0.05$) ± SE.

AOS scavenging systems were similar in LER and *tt5* plants exposed to O_3 (Rao and Ormrod, 1995a, 1995b). Hence, only the responses of the antioxidant enzymes of LER were investigated in detail.

Both UV-B and O_3 are widely believed to generate AOS. Although O_3 has been shown to generate AOS directly by undergoing spontaneous dismutation (Grimes et al., 1983; Mehlhorn et al., 1990), it is not known how plants irradiated with UV-B generate AOS. Plants respond to oxidative stress by inducing the activities and/or the mRNA transcripts of several antioxidative enzymes (Foyer and Mullineaux, 1994; Kangasjarvi et al., 1994; Madamanchi et al., 1994; Sharma and Davis, 1994; Willekens et al., 1994). Furthermore, it has been shown that plants are capable of synthesizing new isoforms of antioxidant enzymes with altered kinetic properties (Guy and Carter, 1984; Edwards et al., 1994). However, there has been no consensus about whether different stresses that generate AOS would cause similar activation of AOS scavenging systems.

SODs catalyze the dismutation reaction of superoxide anion (O_2^-) into H_2O_2 and O_2 and can be distinguished into three classes according to their metal co-factor: Cu,Zn-, Mn-, or Fe-SOD (Bowler et al., 1994; Van Camp et al., 1994). Despite the fact that these SODs can easily be differentiated on the basis of mRNA, as well as activity levels with in situ staining technique on gel, to date only a few studies have been conducted to study the expression of different SODs individually (Perl-Treves and Galun, 1991; Tsang et al., 1991; Willekens et al., 1994). Drought stress has been shown to preferentially induce cytosolic Cu,Zn-SOD (Perl-Treves and Galun, 1991), and both UV-B and O_3 have been reported to have similar effects on chloroplastic and cytosolic Cu,Zn-SOD (Willekens et al., 1994). However, in the present study UV-B- and O_3-induced SOD activities (Fig. 3) appeared to be due to preferential expression of Cu,Zn-SOD-3, -4, and -5 isoforms (Fig. 6). Neither UV-B nor O_3 exposure appeared to have a significant effect on Mn-SOD (Figs. 3 and 6).

PODs usually occur as multiple molecular forms (isozymes) and have a number of potential roles in plant growth, development, and differentiation (Gaspar et al., 1991). PODs require H_2O_2 as an essential substrate and, therefore, POD metabolizes H_2O_2 to water. UV-B-induced POD activities were significantly higher in both genotypes compared to LER plants exposed to O_3 (Fig. 4, c and d). Anionic PODs are believed to utilize phenolic compounds such as coniferyl alcohol and H_2O_2 to initiate the chain reaction that leads to lignification (Otter and Polle, 1994; Polle et al., 1994). Enhanced POD activity specific to coniferyl alcohol (Table I) and the synthesis of several new isoforms (Fig. 7) suggests that UV-B radiation may have enhanced the synthesis of secondary metabolites such as lignin in *A. thaliana*, whereas O_3 exposure appeared to have no role in lignin formation.

GR and APX are believed to act in conjunction to metabolize H_2O_2 to H_2O through a metabolic cycle widely known as the ascorbate-glutathione cycle or Halliwell-Asada pathway. Both UV-B and O_3 have been shown to enhance the expression of GR (Strid, 1993; Willekens et al., 1994), whereas O_3 exposure has been shown to have no effect on GR protein in peas (Edwards et al., 1994). Results presented in Figures 5a and 8 and Table II suggest that UV-B exposure may have only a minimal effect on GR activity in both genotypes. However, O_3 exposure significantly enhanced the GR activity and modified the K_m of GR for GSSG and NADPH, which could be related to the preferential induction of the GR-3 isoform (Figs. 5 and 8; Table II). Both UV-B and O_3 have been shown to induce cytosolic APX in tobacco (Willekens et al., 1994). UV-B-induced changes in the APX activity (Fig. 5, c and d) and the K_m of APX toward ascorbate and H_2O_2 (Table II) were significantly higher in *tt5* plants compared to those of LER plants exposed to O_3, which could be related to the synthesis of a new isoform (Fig. 9).

Although irradiation of both genotypes with UV-B modified the activities of various antioxidant enzymes such as SOD, POD, CAT, GR, and APX (Figs. 3–5), the responses of these antioxidant enzymes attained maximum levels in *tt5* plants within 1 to 2 d of UV-B exposure. In contrast, UV-B-induced changes in antioxidant enzymes of LER appeared to be dependent on the duration of exposure. These responses could be related to the inability of *tt5* plants to synthesize flavonoids. Irradiation of *tt5* plants, blocked in flavonoid biosynthesis, with UV-B could have resulted in increased flux of UV-B reaching mesophyll cells, as compared to LER plants capable of synthesizing flavonoids. This, in turn, would have generated substantially higher AOS, thus necessitating more efficient antioxidant defense mechanisms in *tt5* plants. However, with the increase in duration of UV-B exposure, LER plants appeared to have enhanced AOS production, thus inducing antioxidant enzymes during later stages of UV-B exposure. Just as with UV-B exposure, O_3 exposure of LER plants appeared to have generated AOS and induced antioxidant enzymes in an exposure-dependent manner.

The small increases observed in the SOD and GR activity and unaltered K_m of GR toward GSSG and NADPH in LER and *tt5* plants suggest that UV-B exposure may not have a strong role in inducing SOD and GR in *A. thaliana*. UV-B-induced changes in the activities and isoform composition of POD, coniferyl alcohol-POD, and APX and modified substrate affinity of APX toward ascorbate and H_2O_2 suggests that UV-B may favor POD-related scavenging systems. It is not clear why UV-B exposure enhanced PODs preferentially over other antioxidant enzymes like SOD and GR. It may be possible that *A. thaliana* utilizes UV-B-generated H_2O_2 for the synthesis of secondary metabolites such as lignin, similar to what happens during pathogen infection. On the other hand, O_3 exposure enhanced SOD, POD, GR, and APX (ranging between 80 and 100%) and modified the K_ms of both GR and APX, suggesting that O_3 exposure may prompt an overall efficient SOD/ascorbate-glutathione cycle. The ability of a plant to maintain a high redox state of ascorbate and glutathione has been attributed to the coordination between SOD that generates H_2O_2 and GR-APX that metabolizes H_2O_2 (Foyer et al., 1994). High redox state of the ascorbate-glutathione cycle is believed to develop plant tolerance to oxidative stress (Creissen et al., 1994; Edwards et al., 1994; Foyer et al., 1994).

Foliar carbonyl content serves as an indicator of oxidative damage to proteins (Levine et al., 1990; Pacifici and Davies, 1990). Although both UV-B and O_3 are capable of enhancing the levels of carbonyl groups in vivo, O_3 exposure appears to have resulted in greater oxidative damage (Table IV). UV-B exposure enhanced NADPH-oxidase, whereas O_3 exposure had no major effect on NADPH-oxidase activities (Fig. 10). It is not clear why plants irradiated with UV-B would enhance NADPH-oxidase activities generating H_2O_2 and overwhelming the plant antioxidant defense system. It may be possible that the plant recognizes UV-B radiation through mechanisms identical with those used to detect pathogen infection. Upon pathogen infection, plants are reported to activate membrane-localized NADPH-oxidase and generate AOS (Vianello and Marci, 1989; Vera-Estrella et al., 1994), which, in turn, are believed to transduce the signal (Apostol et al., 1989). Recently, it has been shown that UV-B- and pathogen-induced signal transduction pathways are similar and require AOS (Grene and Fluhr, 1995). Recognition of O_3 appears to be independent of the NADPH-oxidase system since it has been shown to generate AOS directly (Grimes et al., 1983; Mehlhorn et al., 1990).

From these results it is clear that both UV-B and O_3 are capable of generating AOS and inflicting oxidative damage on proteins. However, there are differences in the mechanisms by which *A. thaliana* recognizes UV-B and O_3 and the pathways by which it metabolizes H_2O_2 generated by UV-B or O_3 exposures. The reasons for such preferential utilization of AOS scavenging systems are not known and merit further study. Currently, experiments are being conducted to help us understand the possible role of signal-transducing mechanisms in determining the differential induction of antioxidant enzymes in UV-B- and O_3-exposed *A. thaliana*.

ACKNOWLEDGMENT

The authors acknowledge Radhika for assisting with the maintenance of plants.

Received July 7, 1995; accepted October 12, 1995.

LITERATURE CITED

Alscher RG, Hess JL, eds (1993) Antioxidants in Higher Plants. CRC Press, Boca Raton, FL

Apostol AJ, Heinstein P, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol **90:** 109–116

Askerlund P, Larsson C, Widell S, Moller IM (1987) NAD(P)H oxidase and peroxidase activities in purified plasma membranes from cauliflower inflorescences. Physiol Plant **71:** 9–19

Bowler C, Van Camp W, Van Montagu M, Inze D (1994) Superoxide dismutase in plants. CRC Crit Rev Plant Sci **13:** 199–218

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Campa A (1991) Biological roles of plant peroxidases: known and potential function. *In* J Everse, K Everse, MB Grisham, eds, Peroxidases in Chemistry and Biology, Vol II. CRC Press, Boca Raton, FL, pp 25–50

Chen G-X, Asada K (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol **30:** 987–998

Creissen GP, Edwards EA, Mullineaux PM (1994) Glutathione reductase and ascorbate peroxidase. *In* CH Foyer, PM Mullineaux, eds, Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press, Boca Raton, FL, pp 343–364

Edwards EA, Enard C, Creissen GP, Mullineaux PM (1994) Synthesis and properties of glutathione reductase in stressed peas. Planta **192:** 137–143

Foyer CH, Descourvieres P, Kunert KJ (1994) Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. Plant Cell Environ **17:** 507–523

Foyer CH, Mullineaux PM, eds (1994) Causes of Photooxidative Stress and Amelioration of Defense System in Plants. CRC Press, Boca Raton, FL

Gaspar TH, Penel C, Hagega D, Greppin H (1991) Peroxidases in plant growth, differentiation and development processes. *In* J Lobarzewski, H Greppin, C Penel, TH Gaspar, eds, Biochemical, Molecular and Physiological Aspects of Plant Peroxidases. University de Geneve, Switzerland, pp 249–280

Grene R, Fluhr R (1995) UV-B-induced PR-1 accumulation is mediated by active oxygen species. Plant Cell **7:** 203–212

Grimes HD, Perkins KK, Boss WF (1983) Ozone degrades into hydroxyradical under physiological conditions. A spin trapping study. Plant Physiol **72:** 1016–1020

Guy CL, Carter JV (1984) Characterization of partially purified glutathione reductase from cold-hardened and non-hardened spinach leaf tissues. Cryobiology **21:** 454–464

Hausladen A, Alscher RG (1994) Purification and characterization of glutathione reductase isozymes specific for the state of cold hardiness of red spruce. Plant Physiol **105:** 205–213

Kangasjarvi J, Talvinen J, Utriainen M, Karjalainen K (1994) Plant defence systems induced by ozone. Plant Cell Environ **17:** 783–794

Krupa SV, Kickert RN (1989) The greenhouse effect: the impact of carbon dioxide (CO_2), ultraviolet-B radiation (UV-B) and ozone (O_3) on vegetation. Environ Pollut **61:** 263–392

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtmann ER (1990) Determination of carbonyl content in oxidatively modified protein. Methods Enzymol **186:** 464–478

Li J, Ou-Lee T-M, Raba R, Amundson RG, Last RL (1993) *Arabidopsis* flavonoid mutants are hypersensitive to ultraviolet-B radiation. Plant Cell **5:** 171–179

Madamanchi NR, Donahue JL, Cramer CL, Alscher RG, Pedersen K (1994) Differential response of Cu,Zn-superoxide dismutase in two pea cultivars during a short-term exposure to SO_2. Plant Mol Biol **26:** 95–103

Mehlhorn H, Tabner B, Wellburn AR (1990) Electron spin resonance evidence for the formation of free radicals in plants exposed to O_3. Physiol Plant **79:** 377–383

Mittler R, Zilinskas BA (1993) Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate dependent reduction of nitroblue tetrazolium. Anal Biochem **212:** 540–546

Moller JM, Lim W (1986) Membrane bound NAD(P)H dehydrogenase in higher plants. Annu Rev Plant Physiol **37:** 309–334

Otter T, Polle A (1994) The influence of apoplastic ascorbate on the activities of cell-wall associated peroxidases and NADH-oxidases in needles of Norway spruce (*Picea abies* L.). Plant Cell Physiol **35:** 1231–1238

Pacifici RE, Davies KJA (1990) Protein degradation as an index of oxidative stress. Methods Enzymol **186:** 485–502

Perl-Treves R, Galun E (1991) The tomato Cu,Zn superoxide dismutase genes are developmentally regulated and respond to light and stress. Plant Mol Biol **17:** 745–760

Polle A, Otter T, Seifert F (1994) Apoplastic peroxidases and lignification in needles of Norway spruce (*Picea abies* L.). Plant Physiol **106:** 53–60

Rao MV (1992) Cellular detoxifying mechanisms determine age dependent injury in tropical plants exposed to SO_2. J Plant Physiol **140:** 733–740

Rao MV, Hale B, Ormrod DP (1995a) Amelioration of Ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. Role of antioxidant enzymes. Plant Physiol **109:** 421–432

Rao MV, Ormrod DP (1995a) Ozone pre-exposure decreases UV-B sensitivity in a UV-B sensitive flavonoid mutant of *Arabidopsis*. Photochem Photobiol **61:** 71–78

Rao MV, Ormrod DP (1995b) Impact of UV-B and ozone on oxygen free radical scavenging system in *Arabidopsis thaliana* genotypes differing in flavonoid biosynthesis. Photochem Photobiol **62:** 719–726

Rao MV, Paliyath G, Ormrod DP (1995b) Responses of photosynthetic pigments, Rubisco activity and Rubisco protein of *Arabidopsis* exposed to UV-B and ozone. Photochem Photobiol **62:** 727–735

Rubinstein B, Luster DG (1993) Plasma membrane redox activity: components and role in plant processes. Annu Rev Plant Physiol Plant Mol Biol **44:** 131–155

Runeckles VC, Chevone BI (1992) Crop responses to ozone. *In* AS Lefohn, ed, Surface Level Ozone Exposures and Their Effects on Vegetation. CRC Press, Boca Raton, FL, pp 185–266

Runeckles VC, Krupa SV (1994) The impact of UV-B radiation and ozone on terrestrial vegetation. Environ Pollut **83:** 191–213

Sharma Y, Davis KR (1994) Ozone-induced expression of stress-related genes in *Arabidopsis thaliana*. Plant Physiol **105:** 1089–1096

Spychalla JP, Desborough SL (1990) Superoxide dismutase, catalase and alpha tocopherol content of stored potato tubers. Plant Physiol **94:** 1214–1218

Strid A (1993) Alteration in expression of defence genes in *Pisum sativum* after exposure to supplementary ultraviolet-B radiation. Plant Cell Physiol **34:** 949–953

Tevini M, Teramura AH (1989) UV-B effects on terrestrial plants. Photochem Photobiol **50:** 479–487

Tsang EWT, Bowler C, Herouart D, Van Camp W, Villaroel R, Genetello C, Van Montagu M, Inze D (1991) Differential regulation of superoxide dismutase in plants exposed to environmental stress. Plant Cell **3:** 783–792

Van Camp W, Van Montagu M, Inze D (1994) Superoxide dismutase. *In* CH Foyer, PM Mullineaux, eds, Causes of Photooxidative Stress and Amelioration of Defense System in Plants. CRC Press, Boca Raton, FL, pp 318–341

Vera-Estrella R, Higgins VJ, Blumwald E (1994) Plant defense response to fungal pathogens. II. G-Protein-mediated changes in host plasma membrane redox reactions. Plant Physiol **106:** 97–102

Vianello A, Marci F (1989) NAD(P)H oxidation elicits anion superoxide formation in radish plasmalemma vesicles. Biochim Biophys Acta **980:** 202–208

Willekens H, Van Camp W, Van Montagu M, Inze D, Langerbelts C, Sandermann H Jr (1994) Ozone, sulfur dioxide and UV-B radiation have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* L. Plant Physiol **106:** 1007–1014