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Transient, oxidant-induced antioxidant transcript and enzyme levels correlate with greater oxidant-resistance in paraquat-resistant *Conyza bonariensis*

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Abstract. The elucidation of mechanisms plants use to overcome oxidative stress is facilitated where there is intra-specific genetic variability. The differential induction of higher levels of mRNAs, cytosol and chloroplast antioxidant enzyme activities, and proteins occurred after sub-lethal paraquat treatment of the oxidant-resistant biotype of *Conyza bonariensis* (L.) Cronq. By 6 h after sub-lethal paraquat treatment the activities of superoxide dismutase (EC 1.15.1.1), ascorbate peroxidase (EC 1.11.1.11), dehydroascorbate reductase (EC 1.8.5), monodehydroascorbate reductase (EC 1.6.5.4), and glutathione peroxidase (EC 1.11.1.9) had increased, peaking at 24 h and then slowly reverting back to the basal level. Similarly, the levels of mRNAs encoding these enzymes were enhanced by 12 h and peaked at 18–24 h after sub-lethal paraquat treatment. The time courses of the transient elevation of both transcript and antioxidant enzyme levels correlated with a further transient 2.5- to 3.0-fold increase of paraquat resistance, which occurred only in the constitutively resistant biotype. The individual enzymes seem to be part of a coordinately controlled oxidant tolerance in the resistant biotype, utilizing oxidant-induced, increasingly abundant transcript levels, upon which more antioxidant enzymes were synthesized.

Key words: Antioxidant enzymes – *Conyza* (oxidative stress) – Oxidative stress (tolerance) – Induction (antioxidant enzymes, mRNA) – Paraquat resistance (oxidant induced)

Introduction

Aerobic organisms must protect themselves from the toxic effects of free radicals and other reactive oxygen species that are byproducts of biological redox reactions. These reactive oxygen species can damage lipids, proteins, and nucleic acids. They rapidly react with membrane systems allowing the leakage of water, and plants wither. The increased production of these toxic reactive oxygen species is common to oxidative stress conditions such as transient drought, photoinhibition, ozone, heat and cold stresses, and some xenobiotic stresses.

Plants use a variety of antioxidant molecules such as ascorbate, glutathione (GSH), α-tocopherol, carotenoids, polyamines, flavonoids, etc., as parts of their defence systems. The enzyme systems depicted in Fig. 1 can directly scavenge reactive oxygen species as well as recycle some antioxidant molecules back to the reduced form after they scavenge oxidants. Superoxide dismutase (SOD; EC 1.15.1.1) catalyzes the disproportionation of $O_2^{\cdot-}$ to H_2O_2 and O_2. Hydrogen peroxide is mainly decontaminated by ascorbate peroxidase (APX; EC 1.11.1.11) with ascorbate as substrate, before it can generate hydroxyl radicals by the Fenton reaction. Catalase is not shown as it is mainly a housekeeping enzyme and it is typically inactivated by the levels of peroxide occurring during acute oxidative stress. Oxidized ascorbate is regenerated to ascorbate either by dehydroascorbate reductase (DHAR; EC 1.8.5.1), by monodehydroascorbate reductase (MDAR; EC 1.6.5.4), or directly by ferredoxin (Fd^-). The activity of a glutathione peroxidase (GPX; EC 1.11.1.9) that detoxifies organic peroxides has been

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Abbreviations: ANOVA = analysis of variance; APX = ascorbate peroxidase; CDNB = 1-chloro-2,4-dinitrobenzene; DHAR = dehydroascorbate reductase; DTNB = 5,5′-dithio-bis(2-nitrobenzoic acid); GPX = glutathione peroxidase; GR = glutathione reductase; GSH and GSSG = reduced and oxidized glutathione; GST = glutathione transferase; I_{50} = the concentration necessary to achieve 50% inhibition; MDAR = monodehydroascorbate reductase; SOD = superoxide dismutase

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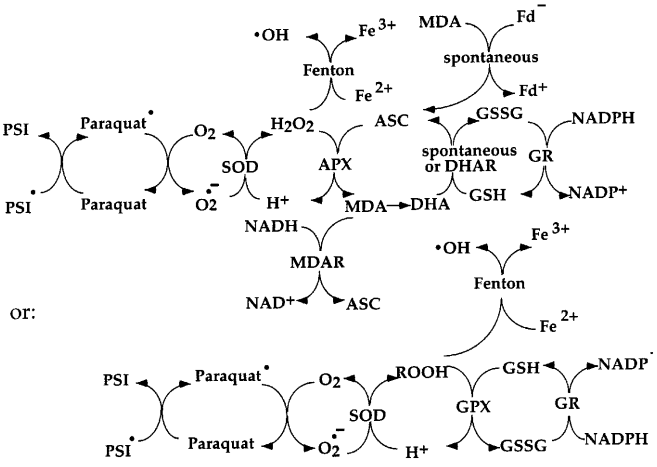

Fig. 1. Scheme for scavenging active oxygen species generated by paraquat as well as by natural sources, as found in different species and different cellular compartments. Abbreviations: *PS I* and *PS I•*, ground and activated states of photosystem I; *paraquat•*, activated paraquat; $O^{\bullet}_2$, superoxide; *•OH*, hydroxyl radicals; *ASC*, ascorbate; *DHA*, dehydroascorbate; *MDA*, monodehydroascorbate; Fd^-, reduced form of ferredoxin; Fd^+, oxidized form of ferredoxin; *ROOH*, organic peroxide. Two molecules of monodehydroascorbate can be disproportionated to one molecule each of ascorbate and dehydroascorbate

demonstrated by indirect (Eshdat et al. 1997) and by direct (Ye et al. 2000) assays in plants. Oxidized glutathione (GSSG) is reduced back to GSH by NADPH in a reaction catalyzed by glutathione reductase (GR; EC 1.6.4.2).

There is considerable evidence for the involvement of these antioxidant enzymes in tolerance to oxidative stress in many plant species (Foyer et al. 1994; Gressel and Galun 1994). Higher levels of SOD activity are often, but not always related to resistance to oxidant stress (Tepperman and Dunsmuir 1990; Perl-Treves and Galun 1991; Bowler et al. 1994). Elevated APX activities are correlated with paraquat resistance (Shaaltiel and Gressel 1986; Shaaltiel et al. 1988a; Matsunaka and Itoh 1991; Ye and Gressel 1994), drought resistance (Pastori and Trippi 1992; Mittler and Zilinskas 1994), photooxidative stress resistance (Jansen et al. 1989; Thomsen et al. 1992), and other oxidant stress resistances (Shaaltiel et al. 1988b; Mehlhorn 1990). The activity of MDAR can be induced to higher levels by photooxidative stress in wheat (Mishra et al. 1993), and was correlated to paraquat resistance in *Conyza canadensis* (Matsunaka and Itoh 1991). The activity of GR has been correlated with resistance to paraquat (Shaaltiel and Gressel 1986), photooxidative stress (Jansen et al. 1990; Amsellem et al. 1993), ozone and sulfur dioxide (Mehlhorn et al. 1987; Shaaltiel et al. 1988b) and other stresses (Malan et al. 1990; Cakmak and Marschner 1992; Edwards et al. 1994).

Transgenic plant systems have been useful for elucidating the function of each antioxidant enzyme (Foyer et al. 1994, 1995; Allen 1995). Transgenic plants with increased levels of SOD (Perl et al. 1993; McKersie et al. 1993; Pitcher and Zilinskas 1996), or GR (Strid 1993; Foyer et al. 1995) often have increased oxidant tolerance. Some attempts at engineering single SOD genes did not work (Tepperman and Dunsmuir 1990; Pitcher et al. 1991). Coordinately increased, higher levels of antioxidant enzymes were postulated to be necessary for conferring the higher levels of oxidant tolerance (Shaaltiel and Gressel 1986; Malan et al. 1990; Pastori and Trippi 1992). Indeed, in cases where transformation with a single gene did confer tolerance to oxidative stress and the expression of other gene products was measured, there were elevated levels of at least one other enzyme in the oxidant detoxification pathway (Sen Gupta et al. 1993a, b; Slooten et al. 1995). Conversely, in one case, it was only possible to obtain high-level oxidative resistance by inserting genes for two of the enzymes (Aono et al. 1995). The coordinate induction of a group of these antioxidant enzymes by low-level oxidant stress to higher levels of enzyme activity, protein and their mRNA in correlation with enhanced oxidant tolerance has not been previously shown in a single resistant biotype versus the sensitive biotype.

One dominant gene locus pleiotropically controls paraquat resistance in *Conyza* (Shaaltiel et al. 1988a). It confers cross-resistance to many other oxidant stresses such as photoinhibition; other herbicides such as atrazine and acifluorfen that act on different targets; xenobiotics causing oxidative stress, as well as sulfur dioxide (Jansen et al. 1989; Shaaltiel et al. 1988b; Vaughn et al. 1989). *Conyza* has two phases of resistance during vegetative (rosette) growth: a low level of resistance with an I_{50} value 10 times that of the sensitive wild type during most of the vegetative growth phase, and a high level of resistance at an age of 10 weeks, where it is 50–100 times more resistant to paraquat. Higher levels of the antioxidant enzymes could not be measured during the phase of low-level resistance (Amsellem et al. 1993; Norman et al. 1993; Ye and Gressel 1994), but were constitutively elevated during the stage of high-level paraquat resistance (Shaaltiel and Gressel 1986; Shaaltiel et al. 1988a,b; Amsellem et al. 1993; Ye and Gressel 1994). Similar results have been found with a paraquat-resistant biotype of *Conyza canadensis* (Matsunaka and Itoh 1991). Constitutively elevated levels of polyamines (Szigeti et al. 1996; Norman and Fuerst 1997; Ye et al. 1997b), and the putrescine-generating enzymes (Ye et al. 1997b), as well as GPX activity (Ye et al. 2000) were found in plants during the periods of both low- and high-level resistance.

In this report, we extend our own previous findings, and those of others by showing that six antioxidant enzymes are not only constitutively elevated in 10-week-old plants of the resistant biotype of *Conyza*, but that they also can be induced to higher levels by stressing with sub-lethal levels of oxidant. This occurs only in the resistant biotype, except for GR, which is also stress-elevated in the sensitive biotype. The time course of induced higher levels of these enzymes of activity, protein and mRNA correlates with a transient additional 3-fold increase of paraquat resistance in the resistant plants.

Materials and methods

Plant materials. The initial collection of seeds of wild-type *Conyza bonariensis* (L.) Cronq. was from near Alexandria, Egypt, and seeds of the resistant biotype were from the Tahrir irrigation district in Egypt, where paraquat resistance evolved. Seeds have been propagated in the greenhouse for the last 14 years, as previously described (Shaaltiel and Gressel 1986). Seeds were germinated and grown in a growth room with 10 h d^{-1} light at a photosynthetic photon flux density (PPFD) of 100 μmol m^{-2} s^{-1} at 25 ± 2 °C. This photoperiod does not induce flowering. The leaves of 10 ± 1 week-old vegetative plants were used in this study (except in one experimental series where 2-week-old plants were also used (as a control). Such plants were given a photooxidant stress by spraying with 1 or 10 μM paraquat (Zeneca, UK) in 0.2% aqueous Tween-20, under the same conditions. In some experiments the already-stressed plants were later challenged with 1.0 mM paraquat in the same carrier. Fully expanded mature leaves were harvested from the rosettes of the treated plants for all assays.

Enzyme assays. Enzymes were assayed in crude extracts of leaves of stressed and non-stressed plants (unless stated otherwise) as follows: Leaf samples (1 g FW) were put in liquid nitrogen and ground to a fine powder in a mortar and pestle. To this were added 100 mg of crystalline sodium ascorbate, 136 mg of polyvinylpolypyrrolidone, and 200 mg of acid-washed sand. Proteins were then extracted by further grinding with 3 ml of extraction buffer containing 90 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM Na_2-EDTA, 0.2 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged for 20 min at 20,000*g* at 4 °C. The clear supernatant was used for assays of SOD, GPX, GR, and MDAR activities, and for determination of soluble protein. More than 60% of the soluble protein was recovered in all crude extracts used.

Superoxide dismutase. Crude extracts were separated on 10% polyacrylamide gels by modification of the method of Laemmli (1970), i.e. without SDS on 1.5-mm-thick and 10-cm-long gels as described in Amsellem et al. (1993). The gels were run at a constant 50 mV overnight at 4 °C and stained for SOD activity with nitroblue tetrazolium (Beauchamp and Fridovich 1971). Normalized SOD levels were obtained by running four concentrations of commercial horseradish SOD (Sigma) as standards together with the samples in each gel. The gels were then photographed and the negatives were scanned with a Molecular Dynamics (Sunnyvale, Calif., USA) 3000-A computing densitometer. The peak area of each SOD isoenzyme was measured and quantitatively normalized to the SOD standards, which were linear up to 0.2 enzyme units. The assignments of cytosol and chloroplast SODs were according to the method of Shaaltiel et al. (1988a). One unit of SOD in the standards was defined by the supplier as the amount of enzyme that inhibits the rate of reduction of cytochrome *c* by 50% in a coupled system with xanthine and xanthine oxidase, at pH 7.8 at 25 °C in a 3-ml reaction volume (McCord and Fridovich 1969).

Ascorbate peroxidase. The preparation of crude extracts and measurement of APX activity were as described in Ye and Gressel (1994), based on the method of Nakano and Asada (1981). The 1.0-ml reaction mixtures contained 0.1 M phosphate-sodium buffer (pH 7.5), 5 mM EDTA, 0.4 mM sodium ascorbate, 0.2 mM H_2O_2 and 50 μg protein from the crude extract. The blank was without H_2O_2. The peroxidation of ascorbate was followed by a decrease in ascorbate absorption at 290 nm. The activities of APX on native PAGE gels were visualized by staining according to the method of Mittler and Zilinskas (1993).

Dehydroascorbate reductase. The activity of DHAR was partially purified by $(NH_4)_2SO_4$ precipitation according to Stahl et al. (1983). Briefly, the leaves were homogenized in 0.1 M potassium phosphate buffer (pH 6.3). The homogenate was centrifuged at 20,000*g* for 15 min. Solid $(NH_4)_2SO_4$ was slowly added with gentle stirring to the supernatant in an ice bath to provide 50% saturation (2.37 M) and the solution centrifuged at 20,000*g* for 15 min. Additional $(NH_4)_2SO_4$ was added to the eluted supernatant to provide 80% saturation (4.0 M). Following another centrifugation at 20,000*g* for 15 min, the pellet was dissolved in 1 ml 10 mM potassium phosphate buffer (pH 6.3). The activity of DHAR was measured according to Hossain and Asada (1984), with modifications. The reaction mixtures contained 20 mM Tricine buffer (pH 7.0), 5 mM GSH, 0.5 mM dehydroascorbate and 0.1 mM Na_2-EDTA in a final volume of 1.0 ml. The reaction was started by adding 50 μg protein to each sample. A control without enzyme was used to correct for non-enzymatic reduction. The activity was measured at 25 °C by following the increase in absorbance 265 nm due to GSH-dependent production of ascorbate.

Monodehydroascorbate reductase. The activity of MDAR was measured according to Hossain et al. (1984), with modifications. The 1.0-ml assay mixtures contained 50 mM Tris-HCl (pH 7.6), 0.125% Triton X-100, 0.2 mM NADH, 2.5 mM ascorbate, and 50 μg of protein of extract. The reaction was started by adding 0.2 units of ascorbate oxidase (EC 1.10.3.3 from *Cucurbita*, Sigma) and the decrease in absorbance at 340 nm due to NAD(P)H oxidation was followed.

Glutathione peroxidase. The activity of GPX was directly measured fluorometrically according to Kamata et al. (1994), as modified by Ye et al. (2000) using GSH purified just prior to assay, to reduce background noise. A hydrophobic organic peroxide (*tert*-butylhydroperoxide) was the preferred substrate. The excess GSH was eliminated with *N*-ethylmaleimide. The GSSG formed by the enzyme reaction was reduced back to GSH with $NaBH_4$, and the GSH was then quantified by fluorescence with *N*-(9-acridinyl)maleimide.

Glutathione reductase. The activity of GR was spectrophotometrically measured in aliquots of total cell extracts by following the increase of absorbance at 412 nm due to reaction of 5,5′-dithiobis 2-nitrobenzoic acid with GSH, as described by Smith et al. (1988). The GR activities on the native PAGE gels were determined by the specific method of Ye et al. (1997a).

Protein assays. Total cell extract protein and partially purified enzyme protein were measured by the method of Bradford (1976) with bovine serum albumin as the standard.

Western immunoblotting. For SDS-PAGE, proteins were heated at 100 °C for 5 min in 2% SDS and 5% mercaptoethanol. Electrophoresis was carried out on 12% acrylamide-SDS gels as described by Laemmli (1970). Proteins from the SDS-PAGE were electrophoretically blotted onto nitrocellulose membranes using a Bio-Rad transblot apparatus. A specific antibody against cytosol CuZn-SOD (prepared from tomato) was kindly provided by Dr. N. Ori of this department. Spinach GR polyclonal antibody (prepared by Dr. C. Malan in this laboratory) was further purified as described in Ye et al. (1997a). Both antibodies were used in 1/1000 dilution in 1× PBS buffer (0.14 M NaCl, 2.6 mM KCl, 1.7 mM KH_2PO_4, 7.7 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.3). The transferred proteins were revealed with anti-rabbit globulin conjugated to horseradish peroxidase, using the enhanced chemiluminescence western blotting detection system (protocol of Amersham). Photographs of the immunoreacted proteins were developed as positive transparencies. Relative protein quantification of cytosol SOD and GR were performed by using Molecular Analyst/Macintosh Image Analysis Software with a GS-670 Bio-Rad Densitometer.

Extraction of RNA and northern analysis. Total RNA was extracted from frozen leaves as described Logemann et al. (1987). The total RNA concentration was determined by UV_{260} absorbance. The RNA was fractionated on 13-cm long, 1.5% agarose gels containing

6% (v/v) formaldehyde, with15 μg samples loaded per lane, according to Sambrook et al. (1989). The RNA was transferred to nitrocellulose (NitroPlus) membrane (Micron Separations Inc., Boston, USA) by capillary blotting with 10× SSC (1× SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) overnight. Transferred membranes were washed with 5× SSC, air-dried for 30 min, and further dried at 80 °C in vacuo for 2 h. The membranes were pre-hybridized for 12 h at 42 °C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's reagent [1× Denhardt's reagent is 0.02% each of Ficoll (Type 400, Pharmacia), polyvinylpyrrolindone (Sigma), and bovine serum albumin (Fraction V, Sigma)], 0.5% SDS, and 0.1 mg ml^{-1} denatured salmon sperm DNA. Specific ^{32}P-labeled cDNA fragments were added for hybridization for at least 14 h. The cDNA fragments coding for cytosol SOD (*Eco*RI-*Pas* I fragment; Perl-Treves et al. 1988), chloroplast SOD (*Eco*RI-*Ava*I fragment; Perl-Treves et al. 1988), cytosol APX (*Eco*RI fragment sub-cloned in pUC19 vector; R.D. Allen, Texas A&M, College Station, Tex., USA, personal communication), chloroplast GR (*Bam*HI fragment; Creissen et al. 1992), cytosol GR (*Eco*RI fragment; Stevens et al. 1997) and GPX (*Eco*RI-*Xho*I fragment; Holland et al. 1994) were used for hybridization. Stringent washing was carried out in 1× SSC containing 0.5% (w/v) SDS at 50 °C.

Quantification of steady-state mRNA levels. The relative amounts of mRNA in different samples were determined by densitometric scanning of autoradiographs using a Bio-Rad Model GS-670 densitometer with Molecular Analyst/Macintosh image analysis software. The data were normalized by correcting against the presumably constant levels of ethidium bromide-stained 5S ribosomal RNA (for cytosol GR and GPX), or against plant tubulin mRNA (for other mRNAs), using tubulin cDNA as a hybridization probe on the same membrane blots (Liaud et al. 1992).

Plant injury measurements. Plant injury was visually evaluated separately by two people 10 h after a 1.0 mM paraquat challenge (whole plants sprayed to run-off) in continuous light of 100 μmol m^{-2} s^{-1} PPFD at 25 ± 2 °C. Damage was recorded on the following scale: 0 = no visible injury; 1 = 25% of area of leaves with injury symptoms; 2 = 50% of leaf area with injury symptoms; 3 = 75% area of leaves with injury symptoms; 4 = dead.

Measurement of $^{14}CO_2$ fixation. Fixation of CO_2 was used to measure recovery from resistance to oxidant stress, as in Amsellem et al. (1993) and Ye et al. (1997a) because it allows the simultaneous measurement of recovery in hundreds of leaves, i.e. a whole experimental series. Three detached leaves of 10-week-old plants were dipped in paraquat solutions for 30 s. The leaves were placed on wet Whatman (No. 3) filter paper in a sealed chamber and placed in light of 80 μmol m^{-2} s^{-1} PPFD at 25 ± 2 °C for a 3-h recovery period. [^{14}C]Sodium bicarbonate (5×10^5 cpm) was put in the chamber and tartaric acid was remotely dripped on to the sodium bicarbonate to release $^{14}CO_2$. Illumination was continued for 15 min. Three 7-mm-diameter discs were removed from each leaf for measurements on an equal-area basis, with three replicates per treatment. The discs were photobleached in acetone, the acetone evaporated, and the radioactivity determined as in Amsellem et al. (1993) and Ye et al. (1997a).

Statistical analyses. The experiments were organized in a random design with three replications (except where indicated). One-factor analysis of variance (ANOVA) with repeated measurements followed by a Dunnett test (Winer 1971) was used to compare the data means with the control and stressed plants. Paired *t*-tests were performed on the initial data before transformation to the ratios of R to S by using Excel 4.0 software. All experiments were repeated at least three times.

Results

Induction of antioxidant enzymes. Plants of the sensitive and resistant biotypes of *Conyza bonariensis* were treated at 2 and 10 weeks of age with two sub-lethal levels of paraquat (1 and 10 μM) for 24 h to provide different levels of photooxidative stress. Enzyme activities were measured in the treated plants and compared with those for plants that did not receive the treatment. Based on the physiological response to paraquat, no induced differences in enzyme activity were expected in the 2-week-old plants, and indeed none were found (data not shown). T_2wo types of difference were observed in the 10-week-old plants; constitutive differences between the resistant and sensitive biotypes that had not been treated, and further elevations in enzyme levels that appeared following the treatment (Fig. 2).

All the enzymes were constitutively elevated (20–80%) in the untreated resistant plants compared to the sensitive biotype ($P < 0.05$), except for MDAR (Fig. 2). The GR activity was doubled in resistant plants. These results on the elevated constitutive levels of these enzymes confirm our previous results (Shaaltiel and Gressel 1986; Amsellem et al. 1993; Ye and Gressel 1994) and extend them to DHAR and GPX.

Paraquat treatment at 1 μM induced SOD activity by 30% more than the already constitutively elevated level ($P < 0.05$), but was without effect on the sensitive biotype. The activity of APX in the resistant biotype was increased about 50% and 20% above the constitutively elevated level ($P < 0.05$) after 1 μM and 10 μM paraquat treatments, respectively, but decreased by 30% in the sensitive biotype. The activity of DHAR was about 80% higher than the constitutively elevated level ($P < 0.05$) after 1 μM paraquat treatment of the resistant biotype, but was only increased 20% after 10 μM paraquat treatment, with no changes in the sensitive biotype. Paraquat had not induced significantly higher levels of MDAR in either biotype by 24 h after treatment. In similarity with spinach MDAR (Hossain et al. 1984), *Conyza* MDAR utilizes both NADH (preferentially) and NADPH as electron donors (Ye 1998). Sub-lethal paraquat stress induced higher levels of only the NADH-dependent MDAR activity in the resistant *Conyza*. The activity of GPX increased 35% above the constitutively elevated level ($P < 0.05$) after stressing, but only in the resistant biotype. The activity of GR was induced to higher than the constitutively elevated levels in both biotypes, with 1 μM and 10 μM paraquat giving the maximum level of induction in the sensitive and resistant biotypes, respectively (Fig. 2).

Oxidant-stress-induced antioxidant enzyme activities could be due to the synthesis of new isoforms (Creissen et al. 1994; Edwards et al. 1994; Rao et al. 1995) or due to increased levels of the pre-existing isoforms. In order to ascertain whether new isoforms had been induced, crude extracts were separated on native PAGE gels and then stained for SOD, APX, and GR. No new isoforms of any of these three enzymes were found in stressed plants (data not shown). This is similar to the findings of Rao et al. (1996) who found no changes in the isoforms

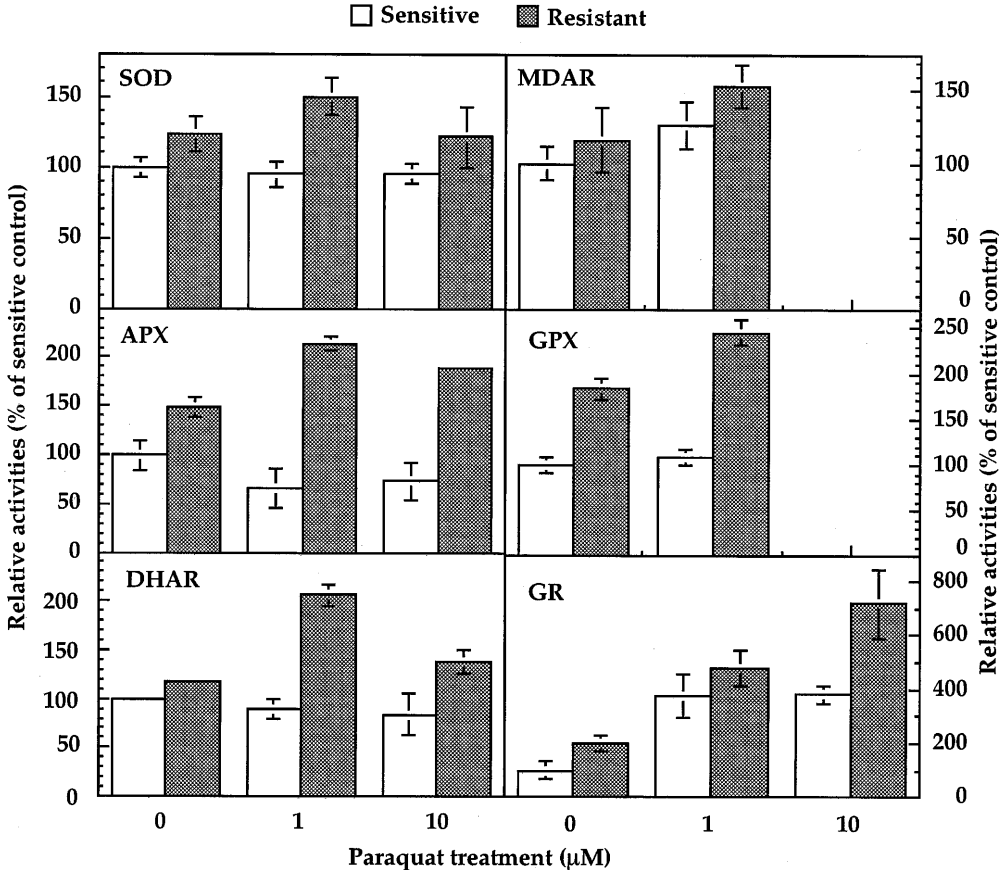

Fig. 2. Induction of higher levels of antioxidant enzyme activities by sub-lethal photooxidative stress. Ten-week-old plants of the sensitive and the resistant biotypes of *Conyza bonariensis* were sprayed with 0, 1, or 10 μM paraquat 24 h before measuring enzyme levels. The data are the averages ±SE of the means of three experiments ($n = 3$). The enzyme activities are expressed relative to the constitutive levels in the sensitive biotype: SOD, 168.4 ± 10 unit mg^{-1}; APX, DHAR, MDAR, GPX, and GR, 2300 ± 200, 8600 ± 120, 5900 ± 900, 0.07 ± 0.01 and 0.018 ± 0.002 μmol (mg protein)$^{-1}$ min^{-1}, respectively. MDAR and DHAR were not determined at 10 μM paraquat

of antioxidant enzymes in ambient and high-CO_2 growth conditions in wheat plants.

Time course of photooxidative-stress induction of antioxidant enzymes. The time courses of the increase in antioxidant enzyme activities were measured after sub-lethal photooxidative stress (Fig. 3). Both chloroplast and cytosol SOD activities of the resistant biotype increased within 6 h of stressing ($P < 0.05$), and the level peaked by 18 h after treatment. The activity of APX was significantly increased ($P < 0.05$) by 24 h in the stressed resistant biotype. The activities of MDAR, DHAR and GR increased by 6 h and peaked at 18–24 h after treatment ($P < 0.05$). The activity of GPX increased between 12 and 24 h after treatment ($P < 0.05$). Except for GR, the activities of the enzymes reverted back to basal levels within 36 h after the end of the stress treatment. All these enzymes were induced to higher levels only in the resistant biotype, except for GR, which was induced in both biotypes (Fig. 3).

Oxidant-stress-increased enzyme activity was a result of increased enzyme protein levels, at least in the cases of SOD and GR, where antibodies were available, allowing measurement. A western blot for cytosol SOD, and the summarized quantified data from three blots are shown in Fig. 4A,B. There was a 30% increase in cytosol SOD 18 h after treatment of 10-week-old resistant plants with sub-lethal levels of paraquat ($P < 0.05$). There was no increase of SOD protein in the sensitive plants after treatment. The level of GR protein increased 60% in the resistant biotype between 18 and 24 h after stressing ($P < 0.05$) (Fig. 4C,D). No stress-induced differences were found in the sensitive biotype by using this purified spinach GR antibody. There was also a constitutive difference between the sensitive and the resistant biotypes, which is consistent with the results of Shaaltiel et al. (1988b), who used a different polyclonal antibody.

The levels of mRNAs encoding antioxidant enzymes are elevated by oxidative stress. Probes were available to study the transcript levels of all the mRNAs encoding the various isoforms of the antioxidant enzymes, except for DHAR and MDAR. These mRNAs were induced to elevated levels by sub-lethal oxidative stress in the resistant biotype, but not in the sensitive biotype (Figs. 5, 6). The levels of mRNA encoding these antioxidant enzymes were about twice those of the sensitive controls by 24 h after paraquat treatment (Fig. 6). The mRNAs encoding Cu/Zn chloroplast SOD, and GR were induced to higher levels only by 10 μM paraquat, the highest concentration used. It was possible to induce higher levels of mRNA coding cytosol SOD, cytosol APX, and GPX by much less paraquat (Fig. 5). This may indicate a 10- to 100-fold greater sensitivity of the cytosol to exogenous oxidative stress. Except for the mRNAs encoding cytosol GR, there was a dose-dependent increase in mRNA abundance at the three concentrations (Fig. 5), although the magnitude of the steady-state levels varied from nearly 2- to 3-fold.

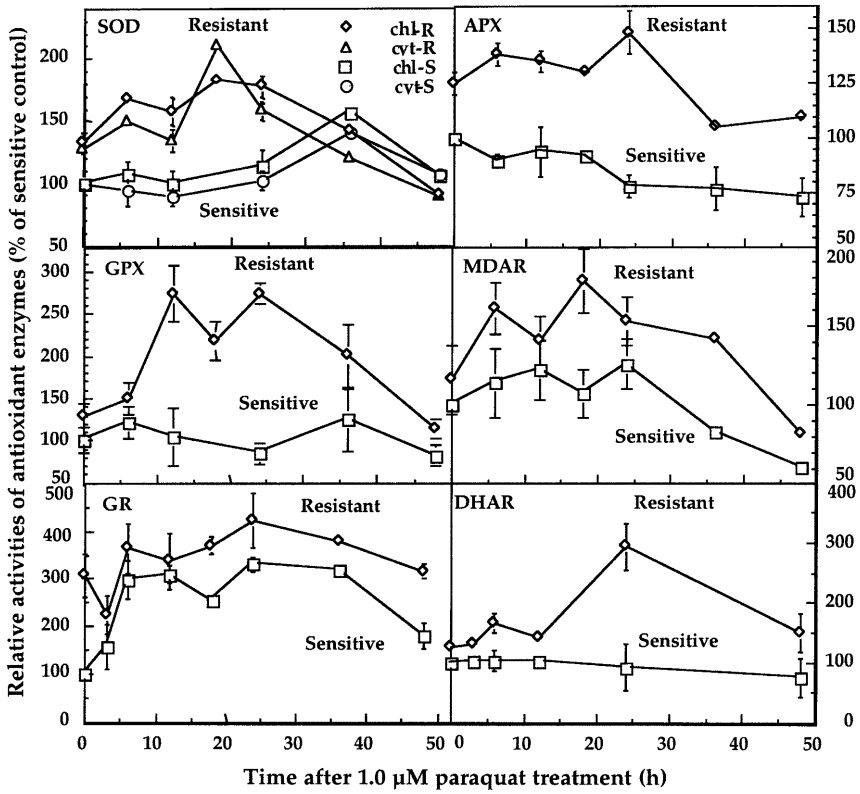

Fig. 3. Transient elevation of the levels of antioxidant enzyme activities after sub-lethal oxidant stress. Eight- to twelve-week-old plants (in different experiments) were sprayed with 1 μM paraquat and the enzyme activities were measured after different durations. The activities are expressed relative to the level in the untreated sensitive biotype (control). The chloroplast (*chl-*) and cytosol (*cyt-*) isoforms of SOD are indicated for the sensitive (*S*) and resistant (*R*) biotypes. The means and standard errors are from the averages of three separate experiments, except the points at 36 and 48 h for APX and MDAR, which are from a single experiment. Standard-error bars are not shown when they are smaller than the symbols. One-factor ANOVA with repeated measurements followed by a Dunnett test was performed. For the resistant biotype, the activities at the time points of 6, 18, and 24 h for SOD; 6 and 24 h for APX; 6, 18, and 24 h for MDAR; 6 and 24 h for DHAR; 6, 18, 24, and 36 h for GR are significantly greater than those of the untreated controls ($P < 0.05$, $n = 15$). The activities of the (non-stressed) sensitive controls are: 110 ± 10.6, 93 ± 5.9 units (mg protein)$^{-1}$ for chloroplast and cytosol SOD, respectively; 2300 ± 160, 8600 ± 120, 7400 ± 600 μmol (mg protein)$^{-1}$ min^{-1} for APX, DHAR and MDAR respectively; 65 ± 6 nmol (mg protein)$^{-1}$ for GPX. The GR activity of the sensitive control is 40.2 ± 1.7 nmol (g FW)$^{-1}$ min^{-1}

Time course of oxidative-stress induction of mRNA. As all the mRNAs probed were significantly increased at 24 h after stressing with 10 μM paraquat, the kinetics of the increase of mRNA levels were measured after a 10 μM paraquat pretreatment. The quantified data from the experimental series are summarized in Fig. 6. Discernible increases in transcript levels were apparent in most cases by 6 h and in all cases by 12 h. At no time after stressing was there a statistically significant increase in transcript levels in the sensitive biotype except for cytosol GR. The elevated levels peaked between 18 and 24 h, except for the chloroplast GR mRNA, which peaked at 36 h. The levels decayed back to near the asal constitutive levels by 48 h. The decay of the chloroplast GR mRNA was slower than that of the other mRNAs.

Time course of enhanced paraquat resistance induced by photooxidative stress. Ten-week-old plants were pre-stressed with a sub-lethal 1 μM paraquat spray treatment, kept for different durations, and then challenged with a 1 mM paraquat spray to ascertain whether the pretreatment increased the level of resistance to the high dose. The degree of plant injury was measured 10 h later (Fig. 7). It was immaterial whether plants of the sensitive biotype had been pretreated or had not been pretreated, all were nearly dead 10 h after the 1 mM paraquat challenge in the light conditions used. The resistant biotype plants that had not been pre-stressed (zero-time plants) were over 50% damaged 10 h after the challenge. The resistant plants that had been paraquat pretreated 6 h before the high-dose challenge were much less injured than the control plants. Maximal protection was found between 18 and 24 h after pretreatment (Fig. 7).

The magnitude of sub-lethal oxidant-induced photooxidant stress resistance was then further determined by assaying the recovery from paraquat inhibition. Plants were pretreated with 1 μM paraquat and incubated for various durations before a challenge with 1 mM paraquat. The plants were allowed to recover for 3 h before photosynthesis ($^{14}CO_2$ fixation) was measured (Fig. 8). Plants of the susceptible biotype did not recover any photosynthetic activity; photosynthesis remained suppressed, as expected. There was considerable protection from this nearly lethal dose of 1 mM paraquat when the resistant plants were pre-stressed and incubated at least 6 h and preferably for more than

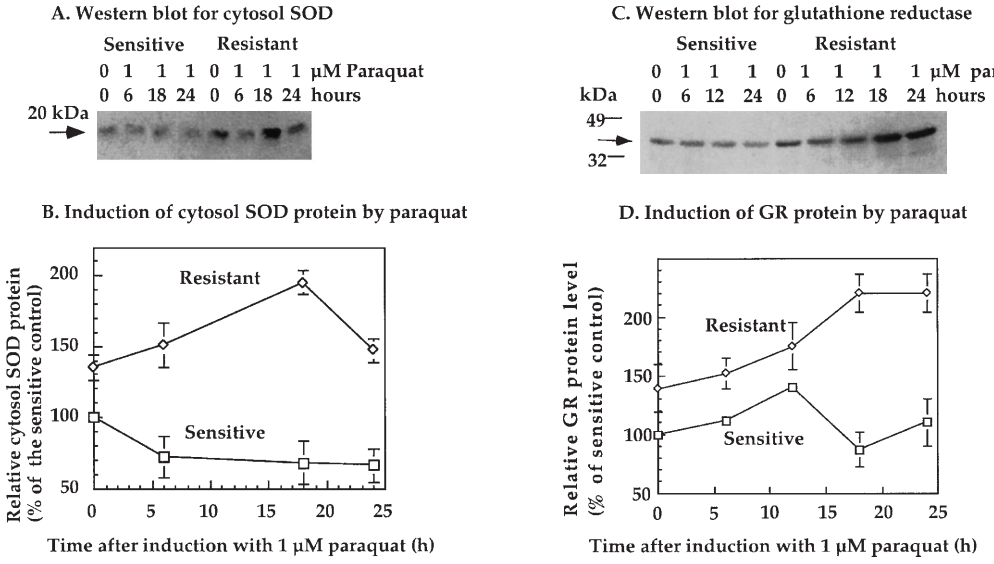

Fig. 4A–D. Transient elevation of levels of cytosol SOD and chloroplast GR protein by oxidant stressing. Plants were sprayed with 1 μM paraquat. Leaves were harvested at various times after spraying, and 10 μg of protein from each crude extract was loaded per slot for SDS-PAGE and western blotting for SOD (**A**), and 30 μg per slot for GR (**C**). The proteins were quantified by scanning the density of the specific bands after immunodetection. Immunodetected protein was quantified by scanning the density of the specific band. The relative protein levels at 10 weeks are summarized for SOD (**B**) and for GR (**D**). The means and standard errors are from the averages of three separate experiments. The data for each blot are expressed relative to the zero-time control for the sensitive biotype. The data at the zero time point are from plants that were not stressed. One-factor ANOVA with repeated measurements followed by a Dunnett test was performed. For the resistant biotype the protein levels at 18 h for cytosol SOD, and 18 h and 24 h for GR are significantly higher than the control values ($P < 0.05$, $n > 15$)

18 h before challenging with this high dose (Fig. 8). These increases in resistance to the 1 mM paraquat challenge peaked at 24 h after pretreatment. This increased ability to recover from high doses of paraquat after a sub-lethal paraquat pre-treatment was retained for 72 h.

Quantification of enhanced paraquat resistance induced by photooxidative stress. The possible correlation between increased antioxidant enzyme activities and a higher level of oxidant resistance was investigated. This was performed by pretreating 10-week-old plants of the sensitive and resistant biotypes with a sub-lethal dose of 1 μM

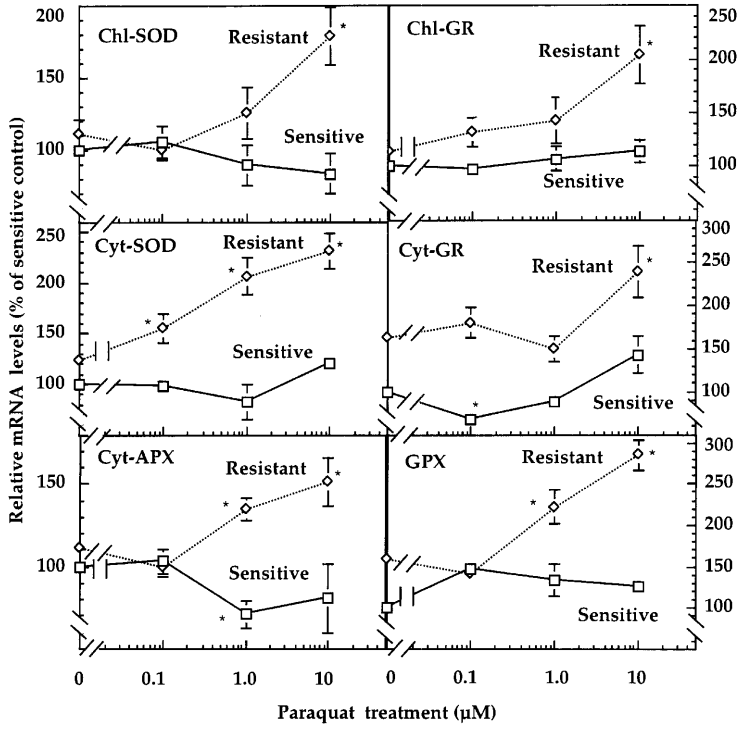

Fig. 5. Quantification of mRNAs encoding chloroplast and cytosol isoforms of antioxidant enzymes. Ten-week-old plants of the sensitive and the resistant biotypes of *Conyza bonariensis* were sprayed with water, 0.1, 1.0, or 10 μM paraquat 24 h before measuring mRNA levels. The relative mRNA levels were compared with the constitutive mRNA levels in the sensitive plants. The densitometric data are means ± SE of the averages of the replicates of three separate experiments. The equation for the slopes and r^2 for the resistant (R) and susceptible (S) biotypes (0.1, 1 and 10 μM paraquat points only) are; chloroplast SOD (*Chl-SOD*) R: y = 39 log (x) + 134, r^2 = 0.96, S: y = 11 log (x) + 92, r^2 = 0.94; chloroplast GR (*Chl-GR*) R: y = 36 log (x) + 159, r^2 = 0.85, S: y = 8.5 log (x) + 106, r^2 = 0.99; cytosol SOD (*Cyt-SOD*) R: y = 38 log (x) + 197, r^2 = 0.96, S: y = 11 log (x) + 100, r^2 = 0.33; cytosol GR (*Cyt-GR*) R: y = 29.5 log (x) + 190, r^2 = 0.42, S: y = 38 log (x) + 100, r^2 = 0.94; cytosol APX (*Cyt-APX*) R: y = 25 log (x) + 129, r^2 = 0.96, S: y = 11 log (x) + 85, r^2 = 0.45; GPX R: y = 71 log (x) + 216, r^2 = 0.99, S: y = 10.5 log (x) + 136, r^2 = 0.98. The mRNA levels at the points with an asterisk are significantly greater ($P < 0.05$, $n > 10$) than their respective sensitive and resistant controls, as ascertained by performing a one-factor analysis of variance with repeated measurements followed by a Dunnett test

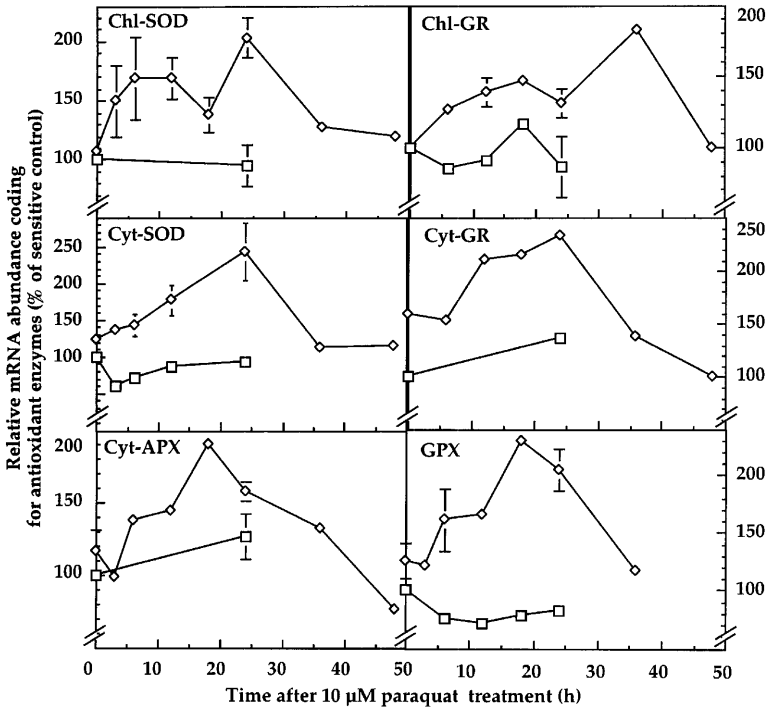

Fig. 6. Time courses of transient increase and decay of mRNA levels encoding antioxidant enzymes after oxidant stress. The levels of mRNAs coding for different antioxidant enzymes are expressed relative to the non-treated sensitive biotype (controls). The abbreviations are the same as in Fig. 5. The standard error bars (where shown) are for the bulked means of all the replicates from at least two different experiments. The data for 36 and 48 h after paraquat treatment are from one experiment

paraquat. Leaves were detached 18 h later and challenged with different concentrations of paraquat for 30 s. Leaves from resistant *Conyza* recovered from such paraquat treatments within 4 h (Shaaltiel and Gressel 1987). The recovery from paraquat was measured as the ability to fix $^{14}CO_2$ (Fig. 9). The dose-response curve of the pretreated sensitive biotype plants did not differ from the curve of the untreated controls. The I_{50} values were in the same 10–20 µM paraquat range. However, the dose-response curve of the paraquat-resistant plants was significantly shifted towards greater resistance in pretreated resistant plants, compared to the controls that had not been pretreated. The I_{50} values were 2.5 to 3 times those of the I_{50} values of the resistant control that had not been pretreated. These results indicate that the pretreatment enhances the levels of antioxidant enzymes, which correlates well with the enhanced resistance to paraquat.

Discussion

Both prokaryotic and eukaryotic cells have inducible responses that protect against oxidative damage. The antioxidant defense systems have been well characterized in *Escherichia coli* where the OxyR and SoxR transcription factors activate a set of antioxidant genes in response to H_2O_2 and superoxide-generating compounds (Storz et al. 1990; Hidalgo et al. 1997). The molecular genetic mechanisms controlling the induced antioxidant systems in plants is still not understood. Elevated levels of some of the antioxidant enzyme activities correlated with oxidant stress resistance in several plant species (Shaaltiel et al. 1988a; Matsunaka and Itoh 1991; Cakmak and Marschner 1992; Ushimaru et al. 1992; Mishra et al. 1993; Rao et al. 1996). The results herein demonstrate that the levels of antioxidant enzymes are induced to higher levels en-bloc in the resistant biotype (alone) by a sub-lethal oxidant stress, above the constitutively higher levels already found the in resistant biotype. It is unlikely that the changes in enzyme levels after oxidant stress are a function of paraquat-induced general protein degradation. This is because the levels of total soluble protein per gram fresh and dry weights and per leaf area remained constant throughout the duration of measurements (data not shown).

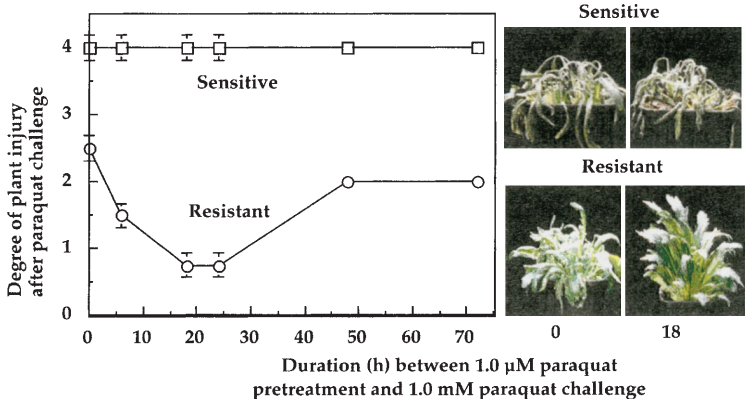

Fig. 7. Transiently enhanced protection from oxidant damage by pre-stressing. Duplicate ten-week-old sensitive (□) and resistant (○) plants were sprayed with 1 µM paraquat, and later challenged with 1.0 mM paraquat. The zero time points are for plants that were not pre-stressed but were directly challenged with 1.0 mM paraquat. The degree of plant injury was measured after 10 h in light at 100 µmol m^{-2} s^{-1} PPFD at 25 ± 2 °C and recorded separately by two people. The scale for plant injury was: 0 = no visible injury; 1 = 25% of area of leaves with injury symptoms; 2 = 50% of leaf area with injury symptoms; 3 = 75% area of leaves with injury symptoms; 4 = dead. The zero time points were without pre-treatment. The data with standard error bars are from the averages of three separate experiments. The data without error bars are from one experiment

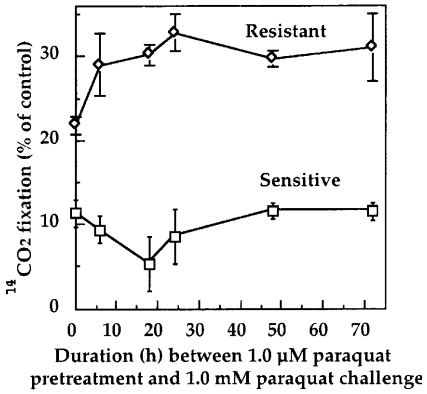

Fig. 8. Induced ability of resistant plants to recover from acute oxidant stress after a sub-lethal oxidant pre-stress. Ten-week-old sensitive (□) and resistant (◇) plants were sprayed with 1 µM paraquat. Leaves were removed after different durations and challenged with 1.0 mM paraquat. Oxidative resistance was measured as the recovery of the capacity to fix $^{14}CO_2$ 3 h after the challenge. The $^{14}CO_2$ data are normalized with respect to the sensitive control [100% = 2322 ± 143 cpm (three discs)$^{-1}$] and the resistant control [100% = 2935 ± 143 cpm (three discs)$^{-1}$]. Plants at the zero time point are without pretreatment. One-factor ANOVA with repeated measurements followed by a Dunnett test showed that all pretreated resistant plants had significantly greater photosynthetic capacity than the zero-time resistant plants ($P < 0.05$, $n > 15$). There were no differences between the pretreated and non-pretreated (at the zero time) sensitive plants ($P = 0.21$, $n > 15$)

The time course of sub-lethal stress-induced enhancement of transcript levels encoding the antioxidant enzymes parallels the increased activity and protein levels. It is possible that the changes in mRNA levels seen are due to increased stability or conversely to enhanced turnover. Such changes would be specific

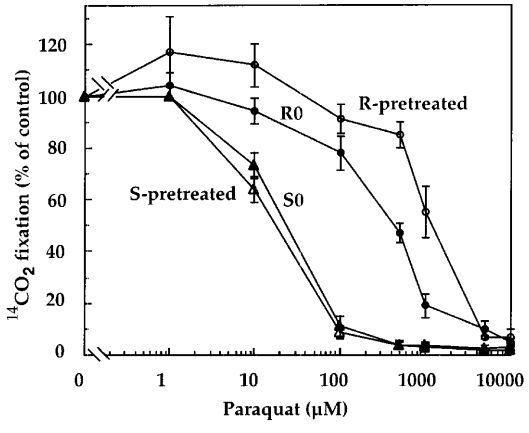

Fig. 9. Sub-lethal oxidative stress only increases photooxidative resistance in the resistant biotype. Ten-week-old plants of sensitive and resistant biotypes were sprayed with 1 µM paraquat and incubated at 100 µmol m^{-2} s^{-1} PPFD at 25 ± 2 °C for 18 h. Leaves were detached and dipped in 1 µM to 10 mM paraquat for 30 s and removed. Paraquat resistance was measured as the capacity to fix $^{14}CO_2$ (see *Materials and methods*). The data from two separate experiments are normalized as the percent of the untreated sensitive biotype [100% = 1883 cpm (three discs)$^{-1}$] and resistant biotype [100% = 2324 cpm (three discs)$^{-1}$]. S_0 and R_0 are the responses of the sensitive and the resistant plants without pretreatment

to those mRNAs, i.e. relative to the tubulin or 5S mRNAs used for normalization on the gels.

These increases in enzymes and transcripts paralleled a further transient increase of paraquat resistance in the resistant biotype (Figs. 3–4, 6–8). The individual antioxidant enzymes seem to be part of a coordinated, rather synchronous antioxidant system that confers oxidant tolerance. The ascorbate-glutathione recycling enzymes are especially needed in chloroplasts for protection against photooxidative damage, and chloroplasts are generally considered to be the primary target of paraquat. Unexplainably, there is considerable evidence correlating antioxidant enzymes in the cytosol of plant cells, with resistance to oxidative stress (Bowler et al. 1991; Perl et al. 1993; Mittler and Zilinskas 1994; Aono et al. 1995; Pitcher and Zilinskas 1996). The activities of both chloroplast and cytosol SOD were increased after sub-lethal paraquat treatment of resistant *Conyza* plants (Fig. 3). The induced activities of cytosol SOD activity correlated with increased SOD protein (Fig. 4A,B) and both peaked 18 h after pre-treatment. Overexpression of either cytosol or chloroplast SOD genes increased the level of oxidative stress (paraquat) resistance in plants (Perl et al. 1993; Sen Gupta et al. 1993a; Pitcher and Zilinskas 1996). Thus, both the chloroplast and the cytosol isoforms of antioxidant enzymes are probably involved in oxidant-stress responses.

Pre-stressing increased the level of mRNA encoding GPX and its activity level. In some cases, GPX activity is at least partially due to glutathione transferase (GST) activity (Cummins et al. 1999). The activity of *Conyza* GPX was separated from GST activity by immunoprecipitation, and the affinity-purified GPX protein has no GST activity (Ye et al. 2000). Hydrophobic organic peroxides are preferred as a substrate over H_2O_2 for GPX activity, while H_2O_2 is the preferred substrate of APX. Glutathione peroxidase may provide protection against oxidant damage to membranes caused from organic peroxides, while APX provides protection against H_2O_2 damage in plants (Asada 1992). The low specific activity measured for GPX (Figs. 2, 3) may not reflect its specific activity in situ, where the milieu is probably more appropriate for its activity.

Increases and decreases of DHAR activities parallel the changes in APX in 2-week- (data not shown) and 10-week-old plants (Fig. 3). This may indicate that DHAR is important for recycling ascorbate, which is used as a substrate for APX. The induced activity of MDAR was dependent on NADH and not NADPH (Ye 1998).

The activity (Fig. 3) and protein (Fig. 4C,D) levels of GR were induced by sub-lethal paraquat in both biotypes. This suggests GR is sensitive to oxidant stresses and the reduction of GSH is of utmost importance for protection from oxidant stress. However, the levels of mRNA coding for chloroplast GR and the protein levels of the chloroplast GR isoform were enhanced by sub-lethal oxidant stress only in the resistant biotype (Figs. 5–6). Cytosol GR mRNA levels were enhanced in both *Conyza* biotypes (Fig. 6), similar to the increase of total GR activity. These results confirm the suggestion that the regulation of GR gene

transcription, protein, and enzyme activity levels are different for different oxidant stresses, and different plant species (Edwards et al. 1994).

The present results clearly demonstrate that pre-stressing with a sub-lethal oxidant stress induces an en-bloc increase in the levels of all six enzymes assayed, whether measured as transcript levels, as activity, or measured immunologically as enzyme protein. This en-bloc increase parallels enhanced resistance to acute oxidant stress (1000 μM paraquat) in the resistant biotype. The 50–100% increases in these six antioxidant enzymes measured started at 6 h and peaked at 18–24 h after pretreatment (Figs. 3, 6), which paralleled the 2.5- to 3.0-fold transiently increased paraquat tolerance (Figs. 7–9).

The resistance to paraquat measured visually (Fig. 7), or as recovery from inhibition of photosynthetic carbon fixation (Fig. 8) is not apparent until all enzymes are elevated. Probes and antibodies were not available for detecting all transcripts encoding, and all isoforms of, antioxidant enzymes. Still, from the sampling performed herein, it would be feasible to conclude that the increased levels of antioxidant enzymes are mainly due to increased transcription. This further fits the genetic results showing that oxidant resistance and enzyme levels co-segregate under the control of a single dominant gene (Shaaltiel et al. 1988a).

The increases in the levels of mRNAs encoding the antioxidant enzymes further support the contention (Gressel and Shaaltiel 1986; Malan et al. 1990) that the elevation of a single transcript or enzyme will not necessarily confer oxidant resistance. The system must be induced nearly en-bloc, as it might be rare that single enzymes are limiting, while the full pathway is essential for resistance. Indeed, very high overexpression of SOD led to increased paraquat sensitivity (Elroy Stein et al. 1986), probably because there was insufficient peroxide detoxification capacity to prevent hydroxyl radical formation. The transformation of plants to overexpress a single component of the pathway conferred resistance when other members of the pathway simultaneously became elevated for unclear reasons (Sen Gupta et al. 1993a, b; Slooten et al. 1995), or when more than one gene was used to transform plants (Aono et al. 1995). In other cases where transforming a single gene was effective, no tests were reported measuring whether the other enzymes were also elevated.

The detailed mechanisms of this differential inducibility of the antioxidant enzymes to higher than the already constitutively elevated levels in the resistant *Conyza* biotype are still unclear. *N*-iodoacetyl-[^{125}I]-3-iodotyrosine measures thiol/dithiol-proteins, thiol/disulfide exchanges (Gitler et al. 1997). Our preliminary data (with C. Gitler) using this reagent indicated that after sub-lethal paraquat treatment six dithiol proteins in leaves of 10-week-old sensitive *Conyza* plants become more oxidized, while only one dithiol protein becomes more oxidized and three more reduced in the resistant biotype (data not shown). Perhaps paraquat differentially activates the oxidant-defense pathways by the redox regulation of thiol/dithiol-proteins, leading to new patterns of gene expression and protein activation. The redox regulation of antioxidant systems has been reported in plants (Wingate et al. 1988; Hérouart et al. 1993; Foyer et al. 1995; Wingsle and Karpinski 1996), bacteria (Storz et al. 1990), and animals (Sen and Packer 1996), but the detailed molecular mechanisms of such controls are still unclear.

The constitutively elevated polyamine system may combine together with the elevated antioxidant system to confer both the basal level and the high level of oxidant resistance (Ye et al. 1997b). It will be interesting to further identify the key factor(s) or the genes that may control the en-bloc gene expression, to ascertain whether the control mechanism is located upstream of a signal transduction that regulates the antioxidant enzyme system, polyamine systems and other unknown possible antioxidant pathways.

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