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Responses of Antioxidants to Paraquat in Pea Leaves[1]

Relationships to Resistance

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Differential sensitivity to the oxidant paraquat was observed in pea (*Pisum sativum* L.) based on cultivar and leaf age. To assess contributions of inductive responses of the antioxidant enzymes in short-term resistance to oxidative damage, activities of glutathione reductase (GR), superoxide dismutase (SOD), and ascorbate peroxidase (APX) and transcript levels for plastidic GR, Cu,Zn SOD, and cytosolic APX were determined. Responses to paraquat exposure from three different leaf age classes of pea were studied. Resistance was correlated with leaf age, photosynthetic rates, enzyme activities, and pretreatment levels of plastid GR and plastid Cu,Zn SOD transcripts. In response to paraquat, small increases in activities of GR and APX were observed in the more resistant leaves. These changes were not reflected at the mRNA level for the plastidic GR or Cu,Zn SOD. Paraquat-mediated increases in cytosolic APX mRNA occurred in all leaf types, irrespective of resistance. Developmentally controlled mechanisms determining basal antioxidant enzyme activities, and not inductive responses, appear to be critical factors mediating short-term oxidative stress resistance.

The bipyridylium herbicide paraquat (1,1′-dimethyl-4,4′-bipyridylium) acts in the chloroplast in the light through the generation of superoxide in a chain reaction, producing ROS (Dodge, 1994). Chain reactions resulting in increases in ROS are terminated through the action of SOD, which dismutates the superoxide to H_2O_2 (Asada and Kiso, 1973). The H_2O_2 in turn is removed through the activity of the Asada-Halliwell scavenging cycle, which involves the oxidation and re-reduction of ascorbate and glutathione through the action of APX and GR, among other enzymes. The cycle is found in the chloroplast and the cytosol (reviews by Alscher and Hess, 1993; Foyer and Mullineaux, 1994). SOD and APX isoforms specific to the chloroplast and to the cytosol have been described (Asada, 1994; Scandalios, 1994). In the case of GR, enzyme activity was found in the chloroplast, the cytosol, and the mitochondrion, and a number of different isoforms were detected (Edwards et al., 1990; Madamanchi et al., 1992).

Much evidence exists that demonstrates that the imposition of oxidative stress results in increases in substrates and gene products associated with the scavenging cycle (reviews by Foyer and Mullineaux, 1994; Okpodu et al., 1996). Transcriptional and posttranscriptional events are implicated in these response mechanisms, in addition to increases in the pool sizes of glutathione and ascorbate. Although these adaptive responses have the potential of conferring additional antioxidant resistance on the stressed plant cell, the chain of events linking the generation of ROS with antioxidant resistance responses is not yet understood.

We have followed the responses of components of the scavenging cycle to the imposition of oxidative stress in the form of paraquat, which is thought to have its site of action in the chloroplast. The pea cvs Progress and Nugget are differentially sensitive with respect to apparent photosynthesis in a short-term exposure to 0.8 μL L^{-1} sulfur dioxide (Alscher et al., 1987). An early detectable response to exposure to sulfur dioxide was an increase in GSH in cv Progress, the resistant cultivar. Subsequent posttranscriptional processes affecting both plastid and cytosolic SOD levels were implicated as part of a resistance mechanism (Madamanchi et al., 1994b).

Amsellem et al. (1994) showed that resistance to paraquat was correlated with developmental stage and with antioxidant enzyme activities in *Conyza bonariensis*. Perl-Treves and Galun (1991) showed that levels of expression of tomato Cu,Zn SODs are altered upon exposure to oxidative stress as well as being under developmental control. Kardish et al. (1994) demonstrated that the promoter region of tomato plastid Cu,Zn SOD contained a developmentally controlled motif as well as motifs responsive to light and/or oxygen radicals arising in the chloroplast in the light. The relative importance for resistance of developmental influences on antioxidant gene expression versus stress-mediated inductive processes has, to our knowledge, not yet been addressed. We have carried out a study of the relationship between resistance to paraquat stress and antioxidant (GR, SOD, and APX) responses at the transcriptional and enzyme activity levels in cv Progress leaves of three different ages. Data were collected during a 48-h period following paraquat exposure. Although paraquat-mediated changes in steady-state mRNA levels were detected, developmentally set levels of antioxidants appear to

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Abbreviations: APX, ascorbate peroxidase; GR, glutathione reductase; ROS, reactive oxygen species; SOD, superoxide dismutase.

play a more important role in acute stress resistance than do stress-mediated inductive events.

MATERIALS AND METHODS

Two cultivars of pea (*Pisum sativum* L. cv Progress no. 9 and cv Nugget) were grown in a controlled environment chamber (Environmental Growth Systems, Chagrin Falls, OH) under a 16-h light/8-h dark regime, with a light intensity of 350 μE m^{-2} s^{-1}, RH of approximately 75%, and day/night temperatures of 23/18°C. Seedlings were grown in synthetic soil (Pro-mix, Premier Brands, Inc., Red Hill, PA) in paper pots.

Herbicide Application and Injury Assessment

For paraquat treatments leaves 1, 2, and 3 (Fig. 1) of 14-d-old plants (12–24 per treatment) were exposed in a surface application to 10^{-4} M paraquat in a 0.1% solution of the nonionic surfactant Ortho X77 (Valent USA Corp., Walnut Creek, CA) 5 h into the light period. The controls were treated with 0.1% surfactant alone. When the treatment was initiated leaf 3 was wholly or partially open in 90% of the plants. Following paraquat application plants were immediately returned to the growth chamber. Leaf injury was assessed 4, 24, 36, and 48 h after the treatment on a 0 to 4 visual scale: 0, no injury; 1, ≤25% of the leaf area injured; 2, 26 to 50% of the leaf area injured; 3, 51 to 75% of the leaf area injured; and 4, 76 to 100% of the leaf area injured. At the same time as the assessment of leaf injury, foliar samples were collected for further analysis, immediately frozen in liquid nitrogen, and processed later as indicated below. Statistical significance of paraquat injury with respect to leaf class and cultivar was determined on two sets of injury data using two-way analysis of variance with replication (Quattro Pro software, version 5.0, Borland International, Scotts Valley, CA).

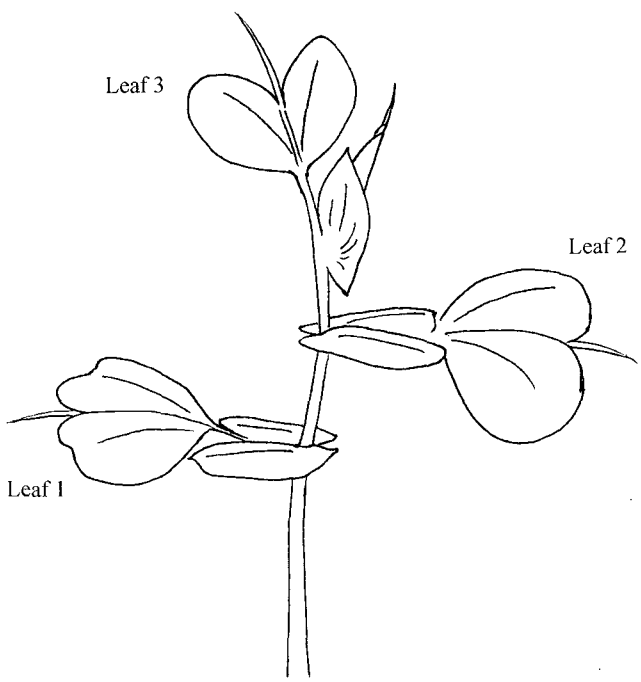

Figure 1. Diagram of a 14-d-old cv Progress plant showing leaves 1 to 3.

Apparent Photosynthesis

Rates of apparent photosynthesis were determined using a Li-Cor 6200 photosynthesis meter (Li-Cor, Lincoln, NE) on five leaf pairs in each leaf age class. Light intensity was 400 μE m^{-2} s^{-1}, CO_2 was 320 to 400 ppm, RH was 36 ± 3%, and temperature was 23 ± 0.5°C. Leaf area was determined in a Li-Cor 3000 leaf area meter.

Enzyme Assays

GR activity was determined from the rate of NADPH oxidation as measured by the decrease in A_{340} following the procedure of Foyer and Halliwell (1976) with some modifications. Leaf tissue (0.2 g) was ground to a fine powder in liquid nitrogen and then homogenized in 0.4 mL of 50 mM phosphate buffer (pH 7.0), 1 mM EDTA, 0.05% Triton, 2% polyvinylpolypyrrolidone, and 1 mM ascorbic acid using a Tissumizer (Tekmar, Cincinnati, OH). The homogenate was centrifuged at 17,000*g* for 20 min, and the supernatant was used for GR assays. The 1-mL assay mixture contained 0.1 M Tris buffer (pH 7.8), 2 mM EDTA, 50 μM NADPH, 0.5 mM GSSG, and 20 μL of the extract. The assays were initiated by the addition of NADPH and were carried out at 25°C in a temperature-controlled DU-65 spectrophotometer (Beckman). The reaction was followed over 5 min, and the activity was expressed as μmol NADPH oxidized/h. Protein was measured following the procedure of Bradford (1976).

The SOD activity assay was based on the method of Beauchamp and Fridovich (1971) as modified by Dhindsa and Matowe (1981), which measures the inhibition in the photochemical reduction of nitroblue tetrazolium. In the spectrophotometric assay the 1-mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM Met, 75 μM nitroblue tetrazolium, 2 μM riboflavin, and the same supernatant described above for the GR assay. Riboflavin was added last and the reaction was initiated by placing the tubes under two 15-W fluorescent lamps. The reaction was terminated after 10 min by removal from the light source. Nonilluminated and illuminated reactions without supernatant served as calibration standards. Reaction product was measured at 560 nm. The volume of supernatant corresponding to 50% inhibition of the reaction was assigned a value of 1 enzyme unit (Beauchamp and Fridovich, 1971). Gel activity assays were performed on samples containing 35 μg of protein separated by nondenaturing 10% PAGE at 4°C. The SOD gel activity assay method of Beauchamp and Fridovich (1971) was applied with the following modifications: the gels were preequilibrated in a solution of 0.05 M potassium phosphate, pH 7.8, and 1 mM EDTA for 30 min and then immersed in 0.24 mM nitroblue tetrazolium, 33.2 μM riboflavin, and 0.2% *N,N,N′,N′*-tetramethylethylenediamine in preequilibrating buffer for 30 min in the dark. For identifica-

tion of individual isoforms, gels containing 100 μg of foliar protein per lane were treated with either 2 mM potassium cyanide or 5 mM H_2O_2 in the pre-equilibration buffer. In some gels commercially available SOD from bovine liver (Sigma) was included to ensure that the SOD activity was in the linear range of the capacity of the gel.

APX activity was determined by following the oxidation of ascorbate to dehydroascorbate (decrease of A_{290}), which was a modification of the method of Nakano and Asada (1987). Rates were corrected for the nonenzymatic oxidation of ascorbate by the inclusion of reaction mixtures without leaf extract.

RNA Isolation and Analysis

For the isolation of total RNA, a modification of the method of Graham et al. (1994) was used. Briefly, 0.5 g of fresh leaf tissue was ground in liquid nitrogen to a fine powder and mixed with 5 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 2 mM EDTA, 1 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.6. After the sample was extracted with phenol:chloroform (1:1, v/v), aurintricarboxylic acid was added to the aqueous phase at a final concentration of 1 mM. RNA was recovered by ethanol precipitation. The phenol-chloroform extraction and ethanol precipitation steps were repeated once. RNA was quantified spectrophotometrically. Total RNA (20 μg per lane) was separated by electrophoresis through a 1% agarose gel containing formaldehyde as described by Sambrook et al. (1989). The gel was stained with ethidium bromide, destained, and photographed. Transfer of RNA to a Zeta-Probe membrane (Bio-Rad) was performed in 10× SSC according to the manufacturer's directions. The RNA was fixed to the membrane by UV cross-linking.

The following cDNAs were used as probes: a 2.03-kb *Bam*HI fragment of a cDNA encoding GR from pea (pGR201, GenBank accession no. X60373, supplied by Dr. Gary Creissen, John Innes Institute, Norwich, UK), a 1-kb *Eco*RI fragment of cytosolic APX from pea (APX, a gift of Dr. R. Allen, Texas Tech University, Lubbock), and a 200-bp *Taq*I fragment encoding the leader sequence of plastid Cu,Zn SOD plus sequence encoding the first 15 amino acids of mature protein, subcloned from pSPA2 (GenBank accession no. J04087, obtained from Dr. Barbara Zilinskas, Rutgers University, New Brunswick, NJ, as described by Madamanchi et al. [1994b]). A mitochondrial 18S rRNA probe from soybean was used as a control to monitor RNA loading (Madamanchi et al., 1994b). Genomic Southern analyses indicate that these fragments function as gene-specific probes (G. Creissen, personal communication; R. Allen, personal communication; N.R. Madamanchi and C.L. Cramer, unpublished data, respectively). Hybridization probes were generated from the gel-purified cDNA fragments by labeling with [α-^{32}P]dCTP using random priming procedures (Boehringer Mannheim).

Membranes were prewashed at 65°C for 30 min in 0.5% SDS and 0.1% SSC according to the manufacturer's directions (Bio-Rad). Hybridizations were performed overnight at 42°C in 50% formamide, 5× SSPE, 10× Denhardt's reagent, 50 μg/mL denatured salmon sperm DNA, 1% SDS, 5% dextran sulfate, and 5×10^6 cpm/mL labeled probe, after prehybridization at 42°C for 4 h in the same solution without dextran sulfate or labeled probe. Filters were washed twice for 15 min at room temperature in 2× SSC and 0.1% SDS and then twice for 30 min at the same temperature in 0.1× SSC and 0.1% SDS. The final wash was performed at 55°C (GR and APX) or 65°C (SOD) for 30 min in 0.1× SSC and 0.1% SDS. Filters were exposed to XAR-5 film (Kodak) with an intensifying screen at −70°C. The relative amounts of RNA were determined by densitometric scanning of the autoradiographs using a personal densitometer with Image Quant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Relative Susceptibility to Paraquat Injury Has a Developmental Basis

To determine whether cv Progress leaves of different ages varied in their susceptibility to paraquat, leaves 1 to 3 (Fig. 1) were exposed to the herbicide as described in "Materials and Methods." Figure 2 shows the combined mean injury data of five separate experiments carried out on cv Progress leaves during a 48-h period. There was a positive correlation between leaf age and relative susceptibility. Expansion of leaf 3 occurred during the first part of the time course of the experiment, but by 24 h into the period the size of leaf 3 was comparable to leaves 1 and 2 in all plants.

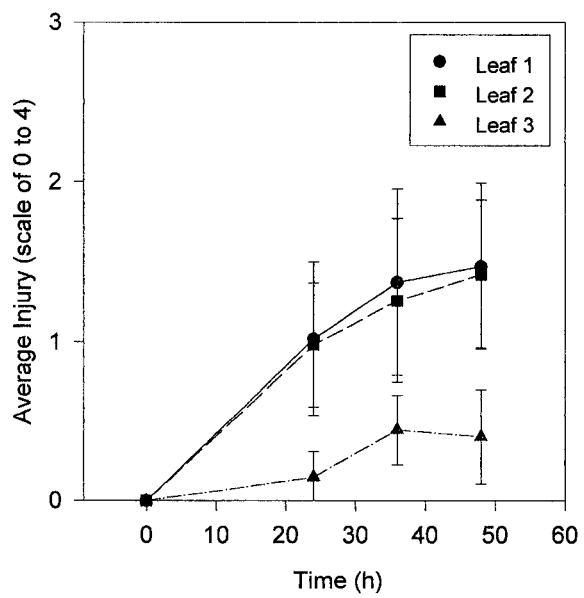

Figure 2. Effects of paraquat on leaf injury. Progress plants (14 d old) were exposed to 10^{-4} M paraquat as described in "Materials and Methods." Each leaf class was scored for injury (0, no injury; 4, >75% injury; see "Materials and Methods") at the indicated times. Means and SEs of five experiments are shown.

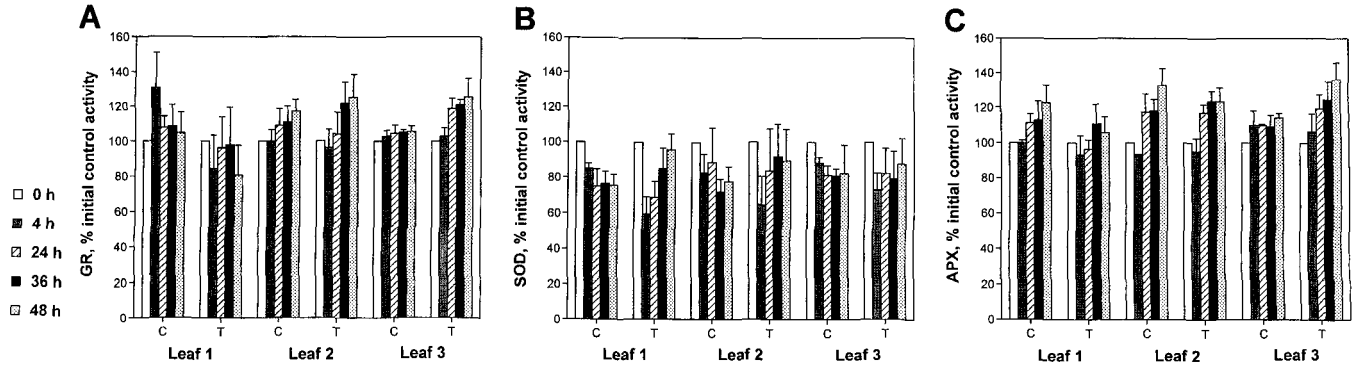

Figure 3. Effects of paraquat exposure on antioxidant enzyme activities. Three leaf age classes of control (C) and paraquat-treated (10^{-4} M) plants (T) were harvested at the indicated times and assayed for enzyme activity. Means and SEs for GR (A), SOD (B), and APX (C) activities for five, four, and three experiments, respectively, are shown as percentages of the initial values (enzyme units mg protein^{-1} h^{-1}).

Leaf 3 Is More Photosynthetically Active than Leaves 1 and 2

Apparent photosynthesis was measured on five leaf pairs of leaves 1 and 2 and three partially open and three fully open leaf pairs of leaf 3. Leaf 1 (10.3 ± 8.2 μmol CO_2 m^{-2} s^{-1}) and leaf 2 (15.8 ± 8.6 μmol CO_2 m^{-2} s^{-1}) gave lower values for apparent photosynthesis than did partially open and fully open leaf 3 (18.7 ± 2.5 and 27.9 ± 2.1 μmol CO_2 m^{-2} s^{-1}, respectively).

Response of Antioxidant Enzyme Activities to Paraquat Exposure in Three Different Age Classes of Leaves of cv Progress

The effect of exposure to paraquat on total foliar activities of GR, SOD, and APX was followed over the time course of the experiment to assess whether differential leaf resistance was correlated with changes in antioxidant enzyme activities in treated leaves. In Figure 3 results from a number of separate experiments are shown and expressed as percentages of initial control values for each leaf age class. Foliar GR, SOD, and APX activities over a 48-h time course following paraquat exposure were followed. For GR and APX (Fig. 3, A and C) in leaves 1 and 2, there were slight increases in enzyme activities in the controls over time. Exposure to paraquat resulted in little discernible change in this pattern in leaves 1 and 2. In leaf 3 the control leaves showed little change in GR and APX activities over time, although slight activity increases were evident following paraquat treatment. No trend in either control or treated leaves was detectable for SOD (Fig. 3B).

The results shown in Figure 3 were normalized on a protein basis. Specific activities, expressed on a per milligram of protein basis of control leaves, were quite similar in all three age classes. However, the different leaf age classes differed in their protein content as determined on a per gram fresh and dry weight basis. Protein content correlated with leaf age, with the youngest leaf 3 having the highest protein levels: leaf 1, 6.8 mg protein g^{-1} fresh weight; leaf 2, 10.1 mg g^{-1} fresh weight; and leaf 3, 20.3 mg g^{-1} fresh weight. Relative water content, calculated as ([fresh weight − dry weight]/[fresh weight]), differed slightly among the three leaf types (leaf 1, 89–90%; leaf 2, 87–89%; and leaf 3, 84–86%). Since protein content was so

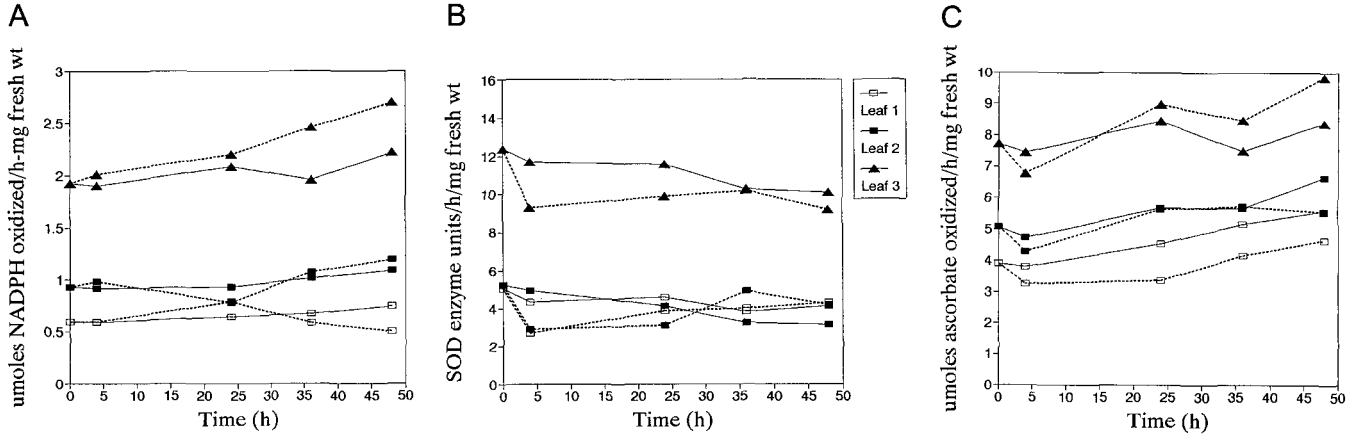

Figure 4. Effects of paraquat exposure on specific activities of GR, SOD, and APX, expressed on a per gram fresh weight basis. Leaves of the three different age classes from control (dashed lines) and 10^{-4} M paraquat-treated plants (solid lines) were harvested at the indicated times and assayed for activity. A, GR; B, SOD; and C, APX.

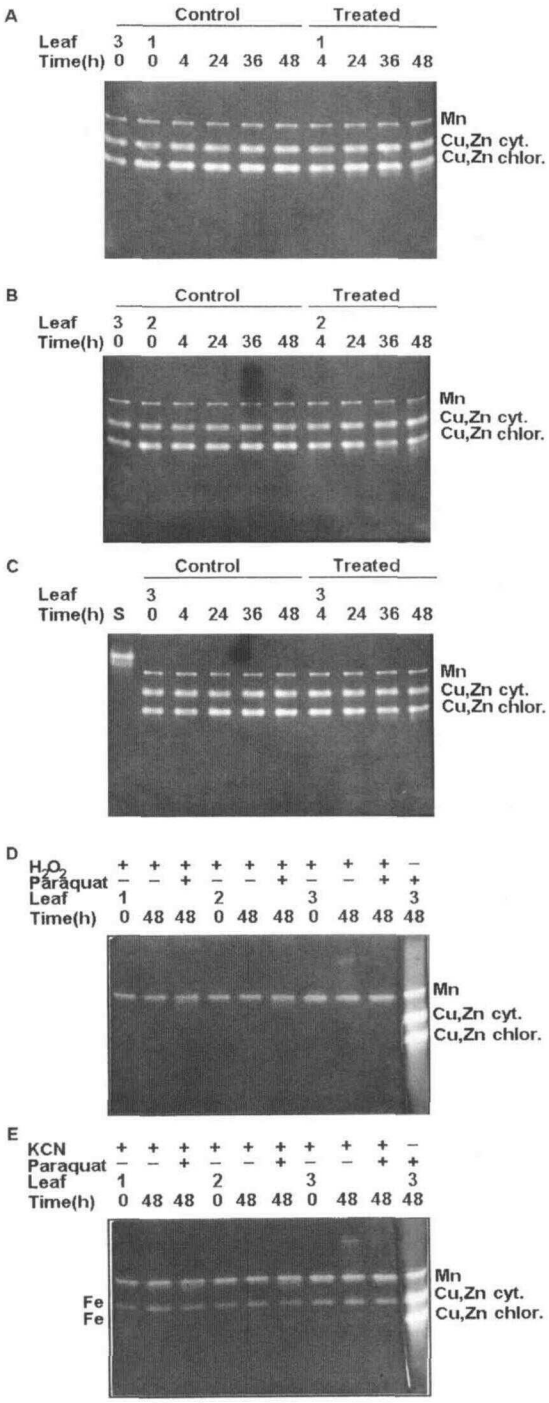

Figure 5. Effect of exposure to paraquat on the activities of individual SOD isoforms in three leaf age classes of pea. Nondenaturing activity gels were prepared and run as described in "Materials and Methods." A to C, Activities of control and treated leaves of leaf 1 (A), leaf 2 (B), and leaf 3 (C) are shown. In each case 35 μg protein per lane was loaded. For comparison, activities present at time 0 in leaf 3 are included in gels A and B. Lane S indicates 0.52 unit of activity of standard SOD (Sigma). D and E, SOD activity gels (100 μg protein per lane) were subjected to H_2O_2 (D) and KCN (E). The right lane of each gel was not treated with inhibitor. cyt., Cytosol; chlor., chloroplast.

strikingly different among the three leaf age classes, enzyme activities in these experiments were recalculated on a per gram fresh weight basis. The results of a representative experiment are shown in Figure 4. In the control, untreated plants the activities of all three enzymes were higher in leaf 3 than in leaves 1 or 2. The contrast between leaf 3 and leaves 1 and 2 was essentially the same whether activities were expressed on a fresh weight or a dry weight basis. In the case of GR and SOD activities in leaf 3 were 2.0- to 2.5-fold greater than those observed in the primary or secondary leaves. For APX the difference was less, with 1.5- to 2.0-fold more activity occurring in the younger leaves. This reflects the differences in the protein levels of the three leaf types. In no case did the amount of protein present in leaves of the three age classes change over the time course of the experiment (n = 8, data not shown).

The enzyme activity results shown in Figures 3 and 4 represent total foliar activity and not the activities of individual isoforms. To determine whether there were developmental or paraquat-mediated differences among individual SOD isoforms, SOD activity assays were also performed on control and paraquat-treated samples separated in nondenaturing gels. The three major forms of SOD that were visible on the activity gels (Fig. 5, A–C) were MnSOD, the slowest mobility band, cytosolic Cu,Zn SOD, and chloroplastic Cu,Zn SOD, the fastest moving band (Sen Gupta et al., 1993). There was no detectable difference in the activity of each of the three major forms of SOD among control or treated leaves of any of the three age classes. Table I shows the results of densitometric scans of the major bands on activity gels obtained using samples from the different leaf types of untreated plants at time 0 in three separate experiments. For purposes of comparison, the lane containing an SOD standard with 0.52 unit of enzyme activity (Fig. 5C, lane S) had 48% of the total SOD activity present in the adjacent lane, as determined by densitometric analysis.

Although there was no significant change in the amount of total SOD activity after treatment of the plants, new, minor bands of enzyme activity with greater mobility than the chloroplastic Cu,Zn SOD isoform became visible after paraquat treatment in all three leaf types (Fig. 5, A–C). Thus, a correlation between the greater resistance shown by leaf 3 and the appearance of this novel band(s) cannot be made. Inhibitor treatments were used in an effort to identify the new SOD isoforms. Analyses specific for MnSOD (H_2O_2 and KCN-resistant) and Cu,Zn SOD (H_2O_2 and KCN-sensitive) confirmed a lack of isoform-specific changes in response to paraquat (in Fig. 5 compare A–C

Table I. *SOD activity in different major isoforms of SOD of different leaf types*

Values are percentages of total SOD activity per lane. Data are the results of three experiments.

Leaf	Mn SOD	Cu,Zn SOD (Cytosolic)	Cu,Zn SOD (Plastidic)
1	14 ± 10	39 ± 3	47 ± 10
2	15 ± 2	43 ± 3	43 ± 3
3	17 ± 1	41 ± 1	42 ± 2

with D and E). The novel minor bands in question were sensitive to both KCN and H_2O_2 and thus appear to be Cu,Zn SOD isoforms.

Two new isoforms of SOD were also revealed through the inhibition of Cu,Zn SOD by KCN. Since they were insensitive to KCN and sensitive to H_2O_2, they appear to be Fe SOD isoforms (in Fig. 5 compare D and E). Fe SOD appeared to be present in both treated and untreated leaf types. Densitometric analysis of the KCN-treated activity gels indicated that the upper and lower Fe SOD isoforms have about 20 and 1 to 5% of the activity of the abundant Mn SOD isoform, respectively.

Antioxidant Transcript Abundance in Leaves of cv Nugget and cv Progress: Effects of Exposure to Oxidative Stress

To determine whether paraquat treatment resulted in changes in antioxidant transcript levels, RNA purified from leaf tissue from the paraquat-treatment experiments was analyzed by northern blot hybridization (Fig. 6). Hybridization probes specific for plastidic GR, plastidic Cu,Zn SOD, and cytosolic APX were used. In the control plants the initial abundance of transcripts encoding both plastid Cu,Zn SOD and GR was greater in RNA samples obtained from leaf type 3 than in RNA isolated from either of the other two leaf types. Steady-state levels of plastid Cu,Zn SOD mRNA decreased over the 48-h time course in leaves 2 and 3 in both control and treated samples, with paraquat-treated samples showing a greater degree of decrease than the controls. In the case of GR mRNA levels, paraquat treatment also caused a decline in transcript abundance in all three leaf types. In the case of APX, however, initial transcript levels were not consistently higher in RNA samples obtained from leaf 3 than they were in samples from leaves 1 and 2. An elevation in APX mRNA levels was apparent at the 4-h point in

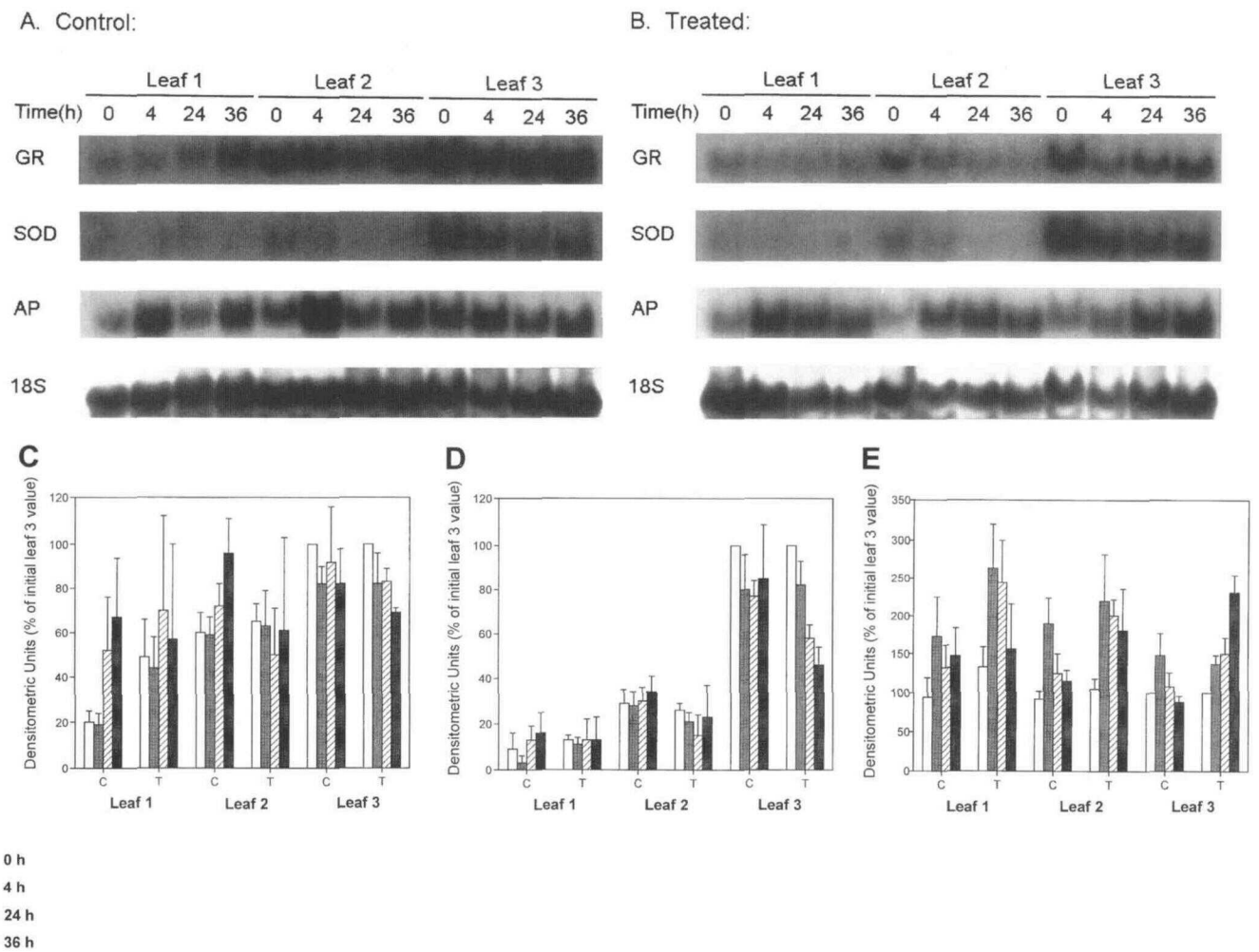

Figure 6. Effect of exposure to paraquat on plastidic GR, plastidic Cu,Zn SOD, and cytosolic APX mRNA levels in cv Progress leaves. A and B, Northern blots of RNA isolated from leaves 1 to 3 of control (A) and treated (B) plants, sampled at indicated times, were probed successively with plastidic GR, plastidic Cu,Zn SOD, cytosolic APX (AP), and mitochondrial 18S rRNA (to test for uniformity of loading). C to E, Densitometric analysis of northern blots probed with GR (C), SOD (D), and APX (E). Means and SEs from four or five experiments are shown, expressed as percentages of initial values of leaf 3, in each case for both control (C) and treated (T) plants.

control leaves 1 and 2 and presumably reflects the influence of light (Thomsen et al., 1992). A comparable elevation in APX mRNA levels at 4 h was not apparent in control leaf 3. The effect of exposure to paraquat appeared to be a net increase in APX transcript over time in all leaf age classes.

To determine whether the developmental differences in paraquat sensitivity that we observed were unique to cv Progress, we also exposed the leaves of cv Nugget to paraquat (Table II) as described in "Materials and Methods." Leaf injury was scored during the ensuing 48-h period. A two-way analysis of variance was performed on data from the three leaf types on 12 plants of each cultivar. The results of the analysis indicated highly significant differences in paraquat injury between the various leaf types of both cultivars, with the youngest leaf 3 being more resistant than the other two leaf types ($P = 3.1 \times 10^{-5}$ at 24 h, $P = 2.4 \times 10^{-8}$ at 48 h). Moreover, highly significant differences in paraquat injury between cultivars ($P = 1.1 \times 10^{-16}$ at 24 h) was indicated. In our previous results we observed a greater resistance to paraquat with cv Progress (Alscher et al., 1987; Madamanchi et al., 1994a), which is also reflected in these analyses.

DISCUSSION

The activities of three pea antioxidant proteins and mRNA levels of two plastid antioxidant proteins present when paraquat stress is imposed are correlated with resistance, in contrast to any inductive processes that occurred in response to the oxidative stress. The greater amount of steady-state transcript and enzyme activities present in the younger and more photosynthetically active leaf 3 may suffice to withstand the amount of oxidative stress imposed by paraquat. Higher rates of photosynthetic electron transport in unstressed leaves carry with them inevitably higher rates of the superoxide-generating Mehler reaction (Asada et al., 1977). The signal transduction mechanism(s) that are responsive to ROS might be expected to maintain the pool of antioxidants at a level commensurate with rates of electron transport. Plastid antioxidant levels would be expected to be correspondingly higher to maintain ROS within physiologically safe limits within the organelle. In support of this hypothesis, Gillham and Dodge (1987) showed that leaves of pea plants grown at a relatively high light intensity had higher antioxidant enzyme activities and were more tolerant of paraquat than were leaves grown at a lower light intensity.

The relative rates of generation of ROS versus speed of response of antioxidant defenses may account for the diversity of reports in the literature concerning the relation of stress-mediated changes to resistance. In some cases increases in antioxidants were correlated with resistance (Alscher et al., 1987; May and Leaver, 1993). In other cases increases appeared with the onset of visible injury (Van Camp et al., 1994; Willekens et al., 1994). On the other hand, transgenic plants altered to express constitutively higher levels of plastid GR or plastid or cytosolic Cu,Zn SOD ("artificial" preset levels) have been shown to have increased resistance to oxidative stress (Aono et al., 1993; Sen Gupta et al., 1993; Van Camp et al., 1994; Pitcher and Zilinskas, 1996).

Stress-mediated changes in the abundance of a particular transcript do not always correlate with corresponding changes in antioxidant protein level and/or enzyme activities (Perl-Treves and Galun, 1991; Williamson and Scandalios, 1992; Mittler and Zilinskas, 1994). We observed that the paraquat-mediated increases in APX mRNA levels were not reflected in correspondingly large increases in APX enzyme activities. Despite the decrease in transcript encoding a plastid GR (the major leaf GR) in all leaves upon exposure to paraquat, there was a slight increase in enzyme activity in response to paraquat exposure in the more resistant leaf 3. We previously have showed that sulfur dioxide exposure brought about a 3-fold increase in SOD activity in cv Progress with increases in cytosolic Cu,Zn SOD and plastid Cu,Zn SOD proteins (Madamanchi et al., 1994b). These changes took place in the absence of increases in the corresponding mRNAs, suggesting the existence of adaptive posttranscriptional processes and/or synthesis of additional SOD protein from the mRNA present in cv Progress at the beginning of the exposure (Madamanchi et al., 1994b). The data of Mittler and Zilin-

Table II. *The effects of exposure to 10^{-4} M paraquat on leaf injury on cvs Nugget and Progress*

Injury to 12 plants of each cultivar was scored 24 and 48 h after application of paraquat, based on a visual scale of 0 (no injury) to 4 (>75% injury, see "Materials and Methods"). Two-way analysis of variance assumes the null hypothesis that there is no significant difference in injury between leaf types and cultivars. The experiment was repeated.

Plant	24 h			48 h		
	Leaf 1	Leaf 2	Leaf 3	Leaf 1	Leaf 2	Leaf 3
cv *Progress*						
No. of leaves	24	24	24	24	24	24
Mean injury	1.125	2	0	1.7	3.0	0.46
Variance of injury	1.3	1.5	0	1.6	1.2	0.61
cv *Nugget*						
No. of leaves	24	24	24	24	24	24
Mean injury	2.5	2.5	0.42	2.9	3.3	2.4
Variance of injury	1.9	1.5	0.34	1.5	0.8	2.5
Leaf type differences	$P = 3.1 \times 10^{-5}$			$P = 2.4 \times 10^{-8}$		
Cultivar differences	$P = 1.1 \times 10^{-16}$			$P = 5.2 \times 10^{-10}$		

skas (1994) for APX responses to drought suggest the existence of stress-mediated posttranscriptional processes.

Our data do not reveal a paraquat-specific response of the novel pea Fe SOD at the protein level. In contrast, Tsang et al. (1991) showed increases in the leaves of *Nicotiana plumbaginifolia* in Fe SOD mRNA with exposure to paraquat. They did not show any data concerning protein levels. It will be informative to compare the responses of both Fe SOD mRNA and corresponding Fe SOD protein with paraquat exposure in the same experimental system.

Discrepancies between changes in transcript abundance and changes in corresponding proteins may sometimes be due to the responses of individual members of antioxidant multigene families, in which values obtained for total enzyme activities do not reflect changes in the activity of particular stress-specific isoforms. In the cases of GR and APX, only total foliar activities were measured. Stress-specific forms of the enzymes may exist in which transcripts are not recognized by the cDNA probes that we used in this work. Our previous work demonstrating the existence of a cold acclimation-specific form of GR in red spruce is consistent with this hypothesis (Hausladen and Alscher, 1994a, 1994b). Stress-mediated changes in populations of pea leaf GR isoforms on activity gels have been reported (Edwards et al., 1994). Mittler and Zilinskas (1993) have demonstrated the existence of multiple isoforms of APX on activity gels, and a second APX gene has now been described for *Arabidopsis thaliana* (Santos et al., 1996). However, the relationship of particular isoforms to individual members of putative GR or APX multigene families remains to be determined.

Our data show a paraquat-mediated increase in a cytosolic antioxidant, despite the localization of paraquat action to the chloroplast in the light. Perl-Treves and Galun (1991) reported paraquat-mediated increases (over a period analogous to the one used in this study) in both cytosolic and plastidic Cu,Zn SOD in tomato. Perl et al. (1993) demonstrated increased resistance to paraquat in transgenic potato expressing tomato Cu,Zn SOD in either the cytosol or the chloroplast. Aono et al. (1991) showed that transgenic tobacco expressing bacterial GR in their cytosol was more resistant to paraquat stress than untransformed plants. It would appear that cytosolic antioxidants respond to paraquat stress and that those responses can confer protection against ROS, which arise in the chloroplast.

The contrast that we observe between the response of plastid versus cytosolic components to oxidative stress at the mRNA level is in agreement with the results of Conklin and Last (1995) for responses of *A. thaliana* to ozone. In their study mRNAs encoding plastid antioxidant proteins decreased upon exposure, whereas cytosolic components, including APX, increased.

The transient increase in cytosolic APX mRNA levels that we observed in leaves 1 and 2 at 4 h posttreatment (9 h of illumination) in control leaves is in agreement with the results of Kubo et al. (1995), who found an elevated level of cytosolic APX mRNA levels in Arabidopsis after 13 h of light. The pattern is suggestive of a diurnal control of APX mRNA levels. Cytosolic APX has been reported to be regulated by light via phytochrome (Thomsen et al., 1992). Kubo et al. (1995) showed that the transient elevation observed in the controls was greater in ozone-exposed plants. In contrast, exposure to paraquat in pea resulted in an alteration of the pattern seen in the control leaves.

The relationship of developmental processes to fast-acting ROS-responsive mechanisms affecting the expression of antioxidant genes remains an open question. Casano et al. (1994) found that young, expanding barley leaves responded to photooxidative stress with increases in SOD transcripts and activities in various cellular compartments, in comparison to SOD decreases in the mature-senescing leaves, which indicates developmental controls on the induction process itself. Perl-Treves and Galun (1991) showed that levels of expression of tomato Cu,Zn SODs are altered upon exposure to oxidative stress as well as being under developmental control. The results of Kardish et al. (1994) suggest that the promoter sequence of tomato plastidic Cu,Zn SOD contains both motifs that are responsive to light and/or ROS arising from photosynthetic electron transport and motifs that are subject to developmental control. Their data from a GUS reporter gene study show higher expression levels of plastid Cu,Zn SOD in younger leaves. Further work will involve a detailed examination of both long-term, developmental, and faster-acting stress-mediated mechanisms affecting the expression of antioxidant genes in plants.

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