DIFFERENCES IN O_3-INDUCED SUPEROXIDE DISMUTASE AND GLUTATHIONE ANTIOXIDANT EXPRESSION IN O_3 TOLERANT AND SENSITIVE TREMBLING ASPEN *(POPULUS TREMULOIDES* MICHX.) CLONES

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ABSTRACT

Three aspen *(Populus tremuloides* Michx.) clones differing in ozone (O_3) sensitivity were exposed to O_3. The level of total glutathione, the activities of superoxide dismutase (SOD), glutathione reductase (GR) glutathione-S-transferase (GST) and mRNA levels of glutathione peroxidase (GPx), Mn SOD and cytosolic Cu/Zn SOD were simultaneously increased in the O_3-tolerant clone 216 for the long-term exposure (100 $nL.L^{-1}$ O_3 for 10 days or 2 months). For the O_3-sensitive clone 259, only GPx mRNA increased markedly. Superoxide dismutase activity and cytosolic Cu/Zn SOD mRNA levels increased less than in the O_3 intermediate and tolerant clones and total glutathione level and the activities of GR and GST were decreased relative to control following long-term O_3 exposure. Following short-term O_3 exposure (150 $nL.L^{-1}$ for 6 to 12 h), O_3-induced SOD activity increased more rapidly in the O_3-tolerant clone 216 than in the O_3-sensitive clone 259 and intermediate clone 271. These results support the hypothesis that SOD and glutathione systems play integral roles in the protection of plants from oxidative stresses.

Key words: *Populus tremuloides*, superoxide dismutase, glutathione, glutathione reductase, glutathione peroxidase, antioxidants

Abbreviations: SOD (superoxide dismutase, GR (glutathione reductase), GST (glutathione-S-transferase), mRNA (messenger ribonucleic acid), GPx (glutathione peroxidase), Mn (manganese), Cu (copper), Zn (zinc), O_3 (ozone), Fe (iron), H_2O_2 (hydrogen peroxide), GSH (reduced glutathione), GSSG (oxidized glutathione), LPI (leaf plastochron index), EDTA (ethylenediaminetetraacetic acid), PVPP (polyvinyl-polypyrrolidine), NBT (p-nitrobluetetrazolium chloride), K (potassium), NADPH (nicotinamide adenine dinucleotide phosphate-reduced), CDNB (1-chlor-2,4 dinitrobenzene), MOPS (3-N-morpholino propanesulfonic acid), SSPE (saline-sodium phosphate-EDTA), UV (ultraviolet), LiCl (lithium chloride), [a^{32}P]dCTP (dioxycytidine phosphate), SDS (sodium dodecyl sulfate), cDNA (complimentary DNA), SO_2 (sulfur dioxide), UV-B (ultraviolet-B).

Ozone (O_3), a major photochemical air pollutant, has long been recognized as phytotoxic to plants (KRUPA & MANNING 1988). This important environmental stress causes about three billion dollars in losses to agricultural crops annually (ADAMS *et al.* 1989) and is known to be an important stress of forest trees in parts of the United States (BARNARD *et al.* 1991). TRESHOW (1970) and TRESHOW and STEWART (1973) were the first to show that aspen *(Populus tremuloides* Michx.) is sensitive to O_3. The strong genetic control involved in aspen's response to O_3 was shown in clonal repeatability studies by KARNOSKY (1976, 1977). KARNOSKY and others have identified and characterized, via extensive growth and physiological studies, several aspen clones differing in O_3 sensitivity (BERRANG *et al. 1991;* COLEMAN *et al.* 1995a,b 1996; LI *et al.* 1991; KARNOSKY *et al.* 1992b; KARNOSKY *et al.* 1996). Three aspen clones differing in O_3 tolerance were chosen as plant materials to study the mechanisms responsible for O_3 stress in aspen. Ozone has been shown to trigger the production of the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and their derivatives (LEE & BENNETT 1982; TANAKA *et al.* 1985). As a protection against these toxic oxygen species, cells are equipped with antioxidants and defense enzymes (SMITH 1985; PRICE *et al.* 1990; SEN GUPTA *et al.* 1991). Superoxide dismutase (SOD), the first enzyme in the scavenging system of activated oxygen species, catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 (FRIDOVICH 1986; BOWLER *et al.* 1992). Three classes of SODs have been identified, based on the metals present at the active site: copper/zinc (Cu/Zn SOD), iron (Fe SOD) and manganese (Mn SOD). Cu/Zn SODs are generally the most abundant SODs in

plants and there are two types: cytosolic and chloroplastic (RABINOVITCH & FRIDOVICH 1983; BOWLER *et al.* 1992).

The relationship between SODs and air pollution tolerance is far from understood and several examples of contrasting data are present in the literature (BOWLER *et al.* 1992; KARPINSKI *et al.* 1992). SODs have been implicated in O_3 tolerance (LEE & BENNETT 1982) and sulfur dioxide tolerance of several species, including hybrid poplars (TANAKA & SUGAHARA 1980; TANAKA *et al.* 1988b; JAGER *et al.* 1985). The H_2O_2 that is produced by SOD is toxic. The H_2O_2 can react with O_2^- in a metal-catalyzed Haber-Weiss reaction to form the highly toxic hydroxyl radical (SEN GUPTA *et al.* 1991).

The generation of H_2O_2 is accompanied by the presence of highly active H_2O_2-scavenging systems. Glutathione synthesis has been shown to respond either directly or indirectly to H_2O_2 (SMITH 1985). Reduced glutathione (GSH) can scavenge the hydroxyl radical and possibly superoxide and is thereby oxidized to the dimmer (GSSG) (PRICE *et al.* 1990). GSH is restored by the action of glutathione reductase (GR) at the expense of oxidizing NADPH. It has been suggested that O_3 resistance in cultivars of spinach and bean are due to higher concentrations of glutathione (TANAKA *et al.* 1985) and greater GR activity (GURI 1983). Glutathione-S- transferase (GST) catalyzes the conjugation of GSH to a large variety of hydrophobic compounds containing electrophilic centers (MAUCH & DUDLER 1993). It has also been shown that GST possessed glutathione peroxidase (GPx) activity in which GSH is used to reduce hydroperoxides to the corresponding alcohol (PRICE *et al.* 1990). The important role of GPx in preventing oxidation damage in animals is well established (KIMBALL *et al.* 1976). It can catalyze the reduction of hydroperoxides such as H_2O_2 and organic hydroperoxides at the expense of the donor substrate, GSH (DROTAR *et al.* 1985). Recently, a putative GPx cDNA (6P229) was isolated from *Nicotiana sylvestris* by CRIQUI *et al.* (1992). It has a high degree of sequence similarity with animal GPx.

While considerable effort has been made to determine causes of O_3 tolerance (MADAMANCHI *et al.* 1992), mechanisms that protect plants from O_3 damage are complex and incompletely understood. The objective of this study was to examine the relationship between antioxidant production and O_3 tolerance in aspen. As a first step toward this goal, we have characterized effects of O_3 treatment on total glutathione, activities of SOD, GR and GST, as well as the expression patterns of Mn SOD, Fe SOD, chloroplastic and cytosolic Cu/Zn SOD, and GPx genes in three aspen clones. It is believed that the present study is the first report of the response of GPx transcripts to O_3 in a tree species.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Three trembling aspen *(Populus tremuloides* Michx.) clones differing in O_3 sensitivity were elected for this study: 259 (sensitive), 216 (tolerant), and 271 (intermediate) (KARNOSKY *et al.* 1992a,b 1996).

Softwood cuttings from stock plants growing in the greenhouse were taken in March, dipped in a mixture of the root-inducing compound "Hormodin 2", fungicide "Benlate 50DF" (1:3), placed in 40x20x8 cm plastic trays in a mixture of perlite and peat moss (3:1), and placed in a mist chamber for rooting. The temperature in the greenhouse was a constant 20 °C. The photoperiod was 16 hours, and average light intensity in the mist chamber was 80 $\mu mol\ m^{-2}s^{-1}$.

Rooted plants were planted in 2.5 cm diameter by 15 cm deep plastic pots in a 1:1:1 peat : perlite : topsoil supplemented with lime, superphosphate, and Peter's Hi-phos Special (15–30–15). After two months, plants were transplanted into 37.5 cm deep x 15 cm diameter plastic pots, in the above-mentioned soil mix, except the added fertilizer was 8 g of Sierra Osmocote, with micronutrients (four months formula). These plants were grown in the greenhouse under a 16 h photoperiod until two weeks before the start of the field exposure season when they were placed outdoors under 50% shade cloth to become acclimated to outdoor conditions. The plants at the start of the field exposure season were about 30 to 50 cm tall and had 10 to 15 leaves.

Table 1. The origin of the three *Populus tremuloides* clones used in this study and their sensitivity to O_3.

Clone	Origin	Foliar Response (BERRANG *et al.* 1991)	Growth Response (Potted Plants (KARNOSKY *et al.* 1992b)
216	Bayfield Co., WI	Tolerant	Tolerant
259	Porter Co., IN	Sensitive	Sensitive
271	Porter Co., IN	Intermediate	Intermediate

Ozone Treatment

Ozone treatments for the long-term study were conducted in open-top chambers (HEAGLE *et al.* 1973), modified with frustums and rain-exclusion canopies. Ozone was generated with a Griffin O_3 generator with oxygen as the air source, dispensed through microcomputer-controlled mass-flow controllers and Teflon tubing to the chambers and monitored in each chamber in a time-shared manner using a Scanivalve system and a TECO O_3 analyzer. The plants were transferred to open-top chambers in June. Five plants per clone were used as controls (charcoal-filtered air), and five plants per clone were fumigated with 100 $nL.L^{-1}$ O_3 for 6 hours per day and 4 days per week. Two replicate open-top chambers were used for each treatment. In 1992, after two months of fumigation and in 1994, after 10 days of fumigation, the recently mature leaves (leaf plastochron index 5 and 6) [ISEBRANDS & LARSEN 1973] were harvested and pooled for RNA extractions and biochemical assays. LPI 5 and 6 leaves would have developed totally under O_3 exposure in both of these regimes and were not showing any visible injury.

Total Glutathione Assay

Leaf tissues (0.5 g) were ground in liquid nitrogen and homogenized in 5 ml 5% (w/v) sulfosalicylic acid and centrifuged at 12,000 g for 10 min. 1 ml supernatant was neutralized with 1.5 ml 0.5 M K-phosphate buffer (pH 7.5). Total glutathione was measured as described by SIES & AKERBOOM (1984). Assays were performed in a 1-ml reaction mixture of 100 mM K-phosphate (pH 7.0), 1.0 mM EDTA, 75 uM DTNB, 0.25 mM NADPH, and 0.12 unit of yeast glutathione reductase. The reaction was initiated by the addition of a known amount of GSSG or extract. The reaction rate is measured as an increase in absorbance at 412 nm.

Enzyme Assays

Leaf tissues (0.5 g) were ground in liquid nitrogen and extracted in the homogenization solution containing 100 mM K-phosphate (pH7.0), 0.1 mM EDTA, and 1% (w/v) insoluble PVPP. The homogenate was centrifuged at 12,000 g for 20 min. and the supernatant obtained was used to determine enzyme activity and protein concentration (BRADFORD 1976). All extracts were prepared at 0 to 4 °C, and enzyme assays were run at 25 °C.

Superoxide dismutase activity was measured spectrophotometrically as described by DHINDSA *et al.* (1981). The 3 ml reaction mixture contained 50 mM K-phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 uM nitrobluetetrazolium (NBT), 2 uM riboflavin, and 0–50 ul enzyme extract. One unit of SOD activity is defined as the amount required to inhibit the photochemical reduction of NBT by 50% (BEAUCHAMP & FRIDOVICH 1971).

Glutathione reductase activity was assayed by monitoring the oxidation of NADPH at 340 nm in 1 ml of solution that contained 100 mM K-phosphate (pH 7.0), 1.0 mM EDTA, 0.15 mM NADPH, 0.5 mM GSSG, and enzyme extract (PRICE *et al.* 1990). Activity was calculated using the extinction coefficient for NADPH of 6.22 mM^{-1} cm^{-1}.

Glutathione S-transferase activity was determined using the method of HABIG & JAKOBY (1981). The reaction mixture contained 100 mM K-phosphate buffer (pH 6.5), 1.0 mM EDTA, 1.0 mM 1-chloro-2,4 dinitrobenzene (CDNB), 1.0 mM GSH and enzyme extract. The reaction rate was measured as an increase in absorbance at 340 nm and activity was calculated using the extinction coefficient of the conjugate, 9.6 mM^{-1} cm^{-1}. All assays were repeated at least three times from independent samples.

Total RNA Isolation and Northern Blot Analysis

An LiCl based extraction method described by PARSONS *et al.* (1989) was used for RNA preparations. Total RNA was extracted from recently mature leaves (positions 5 and 6 from top) of control or O_3-treated aspen clones 216, 259 and 271 and quantified spectrophotometrically.

Table 2. DNA probes used in this study.

Probe	Reference
Mn SOD	TSANG *et al.* (1991)
Cytosolis Cu/Zn SOD	TSANG *et al.* (1991)
Chloroplastic Cu/Zn SOD	REED SCOLI *et al.* (1988)
Fe SOD	TSANG *et al.* (1991)
GPx	CRIQUI *et al.* (1992)

Total RNA was separated by electrophoresis on a 1% agarose containing formaldehyde and 1× MOPS buffer. The RNA was transferred to nylon membrane in 10× SSPE. The membrane was subjected to UV crosslinking. The cDNA fragments of the different genes as probes: Mn SOD (TSANG *et al.* 1991), cytosolic Cu/Zn SOD (TSANG *et al.* 1991), chloroplastic Cu/Zn SOD (SCOLI *et al.* 1988), Fe SOD (TSANG *et al.* 1991) and GPx (CRIQUI *et al.* 1992). They were labeled with [a^{32}P]dCTP according to random primed DNA labelling kit protocol as specified by the manu-

facturer of the DNA labelling kit (United States Biochemical). Prehybridization was performed in 50% formamide, 6x SSPE, 5x Denhardts, 0.5% SDS, and 0.1 mg mL^{-1} denatured salmon sperm DNA at 42 °C overnight. Hybridization was performed using radiolabeled probe in 50% formamide, 6x SSPE, 0.5% SDS, and 0.1 mg mL^{-1} denatured salmon sperm DNA at 42 °C overnight. The membrane was washed twice with 2x SSPE and 0.2% SDS for 10 min at room temperature and once with 1.5x SSPE and 0.2% SDS for 30 min at 42 °C and once with 1x SSPE and 0.2% SDS for 30 min at 65 °C. Membrane was air dried for 5 min, wrapped in Cling Wrap, and used to expose to X-ray film in the presence of intensifying screens.

RESULTS

Ozone Effects of Whole Plant Responses

Visible foliar injury was present on older, mature leaves in both the long-term fumigation in 1992 and the short-term fumigation in 1994. Black, bifacial necrosis, general chlorosis, and black upper leaf surface stipple were visible on all three clones in 1992 but more severe on the O_3 sensitive clone 259. Premature leaf drop was also present in all three clones in 1992 but not in the short-term fumigation in 1994. LPI 5 and 6 leaves, which were sampled for antioxidant assays, were not injured in either year or in any of the clones. Biomass reductions in these clones caused by O_3 have been reported by KARNOSKY *et al.* 1992a,b 1996.

Ozone Induction of SOD Activity

The effect of O_3 on SOD activity is shown in Figures 1A and 2. Superoxide dismutase activities of the O_3-tolerant clone 216 and the intermediate clone 271 both increased about 2-fold compared to nonfumigated control plants after 2 months exposure to 100 $n.L.L^{-1}$ O_3. In contrast, SOD activity of the O_3-sensitive clone 259 only increased by 19.2% relative to control (Fig. 1A).

Ozone Effects on Glutathione Metabolism

The O_3 response of total glutathione is shown in Figure 1B. Fumigation with 100 $nL.L^{-1}$ O_3 for 2 months, total glutathione increased by 19% and 13% in the O_3-tolerant clone 216 and the intermediate clone 271, respectively. In comparison, total glutathione had a 17% decrease in the O_3-sensitive clone 259 (Fig. 1B).

The effect of O_3 exposure on GR activity is shown in Figure 2A. The GR activity increased by 19.2% only in the O_3-tolerant clone 216. In comparison, GR activity decreased 43.5% and 17.1% in the O_3-sensitive clone 259 and the intermediate clone 271, respectively. (Fig. 2A). The GST responded to O_3 exposure in a similar fashion to GR. The activity of GST increased by 37.5% in the O_3-tolerant clone 216. In comparison, GST activity decreased 25.8% and 11.0% in the O_3-sensitive clone 259 and the intermediate clone 271, respectively (Fig. 2B).

Ozone Effects on the Transcript Levels of SODs and GPx

We performed Northern blot analyses with various SOD gene probes to determine which particular SOD or SODs were expressed. The Mn SOD and cytosolic Cu/Zn SOD transcripts increased in the O_3-tolerant clone 216 and intermediate clone 271 compared to the O_3-sensitive clone 259, whereas all the three clones showed similar levels of transcripts from the chloroplastic Cu/Zn SOD and Fe SOD after 2 months exposure to 100 $nL.L^{-1}$ O_3 (Fig. 3).

In Northern blot analysis, the heterologous putative GPx cDNA probe hybridized specifically to a single mRNA species of about 1 kb (Fig. 4). This value is similar to the GPx mRNA of tobacco (CRIQUI *et al.* 1992). The GPx transcripts were induced in both the 216 and 259 aspen clones following long-term O_3 exposure (100 $nL.L^{-1}$ O_3 for 2 months), but not in the intermediate clone 271 (Fig. 4).

DISCUSSION

For the past several growing seasons, we have studied the effects of seasonal O_3 exposures on the growth, and biomass allocation of trembling aspen (COLEMAN *et al.* 1995a,b 1996; KARNOSKY *et al.* 1992a, 1992b, 1996; LI *et al.* 1991). From this research, we identified clones with differing O_3 responses. The present study demonstrates that 100 $nL.L^{-1}$ O_3 exposure for 2 months results in increases in total glutathione level, in activities of SOD, GR, GST, and in mRNA levels of GPx, Mn SOD and cytosolic Cu/Zn SOD in the O_3-tolerant clone 216. In the O_3-sensitive clone 259, GPx mRNA was increased markedly, SOD activity and cytosolic Cu/Zn SOD mRNA levels only slightly increased relative to control, but total glutathione and activities of GR and GST decreased relative to control. In the intermediate clone 271, only SOD activity, chloroplastic and cytosolic Cu/Zn SOD mRNA levels and total glutathione level were increased appreciably relative to control, but activities of GR and GST were decreased and GPx mRNA wasn't induced. The SOD and glutathione system, if induced simultaneously as in the tolerant clone, would represent a potent synergistic

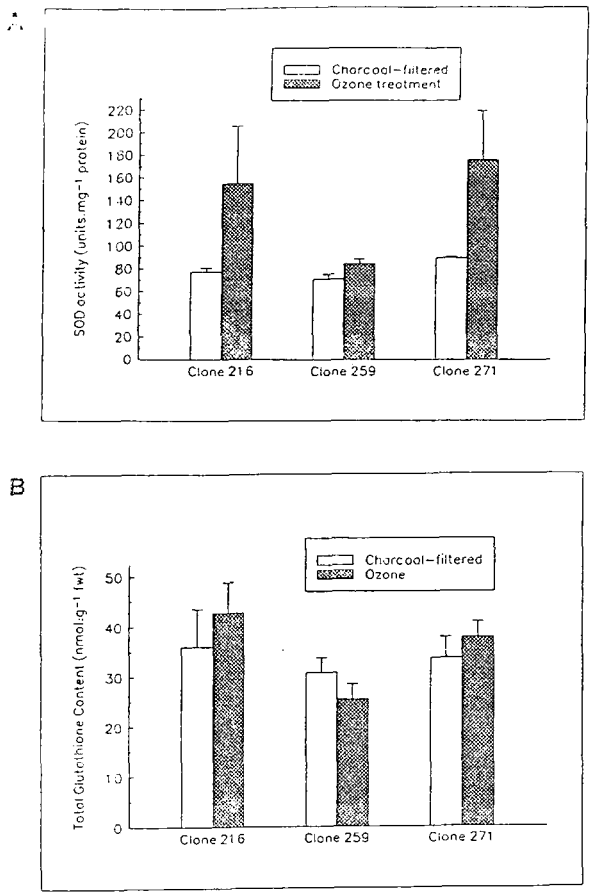

Figure 1. Effects of O_3 treatment on (A) SOD activity, (B) Total gluthathione in leaves of potted one-year-old aspen plants, exposed to either charcoal-filtered air or O_3-added at the rate of 100 nL.L^{-1} for six hours per day, four days per week, for two months. Clone 216 is O_3-tolerant, Clone 259 is O_3 sensitive and clone 271 is intermediate. Bars represent ±SE ($n = 3$).

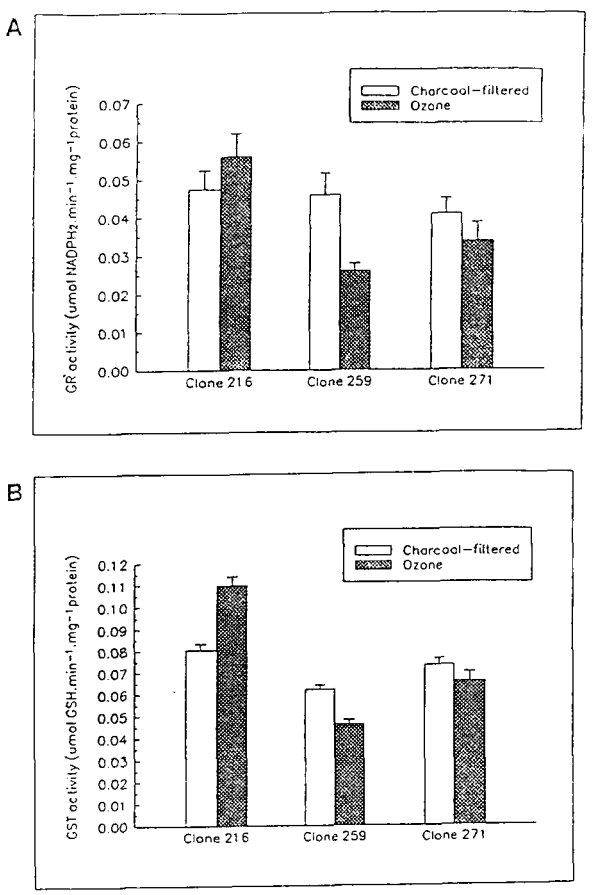

Figure 2. Effects of O_3 activities on (A) GR, (B) GST in leaves of potted one-year-old aspen plants, exposed to either charcoal-filtered air or O_3-added at the rate of 100 nL.L^{-1} for six hours per day, four days per week, for two months. Clone 216 is O_3-tolerant, clone 259 is O_3 sensitive and clone 271 is intermediate. Bars represent +SE ($n = 6$).

Table 3. Decrease of autumnal biomass for three aspen clones exposed to ozone in single-season exposures during the years of 1989-1991 (averages of the three years for ozone treatments as compared to charcoal-filtered air. All plants were grown in pots in open-top chambers (from KARNOSKY *et al.* 1996).

Clone	Ranking	Biomass Decrease (%)		
		Stem	Retained Leaf	Root
216259271	Tolerant	–8.9	–21.9	–11.8
	Sensitive	–49.7	–48.3	–45.7
	Intermediate	–23.1	–30.1	– 22.6

mechanism for diminishing O_3 effects. These antioxidant systems potentially represent important biochemical mechanisms for enhanced resistance to oxidant damage as mediated by O_2^-, H_2O_2 or lipid peroxides (KIMBALL *et al.* 1976).

The importance of SODs in conveying O_3 tolerance remains a debated issue because results have been inconsistent from study to study (BOWLER *et al.* 1992). The tolerant clone 216 and intermediate clone 271 had greater increases in SOD activity (Fig. 1A) following O_3 exposure (100 nL.L^{-1} for 2 months) than did the sensitive clone 259. From our northern blot analysis, Mn SOD and cytosolic Cu/Zn SOD mRNA levels were induced by O_3, whereas mRNA levels of

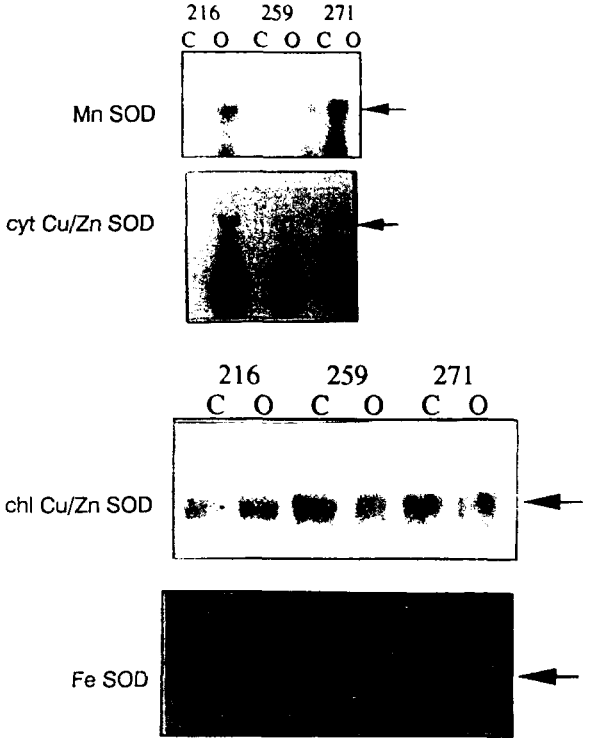

Figure 3. Effects of O_3 on SOD transcript level. Total RNA was extracted from leaves of potted, one-year-old aspen plants, exposed to either charcoal-filtered air (CF) or O_3-added (O_3) at the rate of 100 nL.L^{-1} for six hours per day four days per week, for two months in 1992. In each lane, 20 μg of total RNA was separated in an agarose-formaldehyde gel, transferred to nylon membrane, and hybridized at ^{32}P-labeled four different SOD gene probes. A RNA ladder was used as a molecular size standard. Clone 216 is O_3 tolerant, Clone 259 is O_3 sensitive and Clone 271 is intermediate.

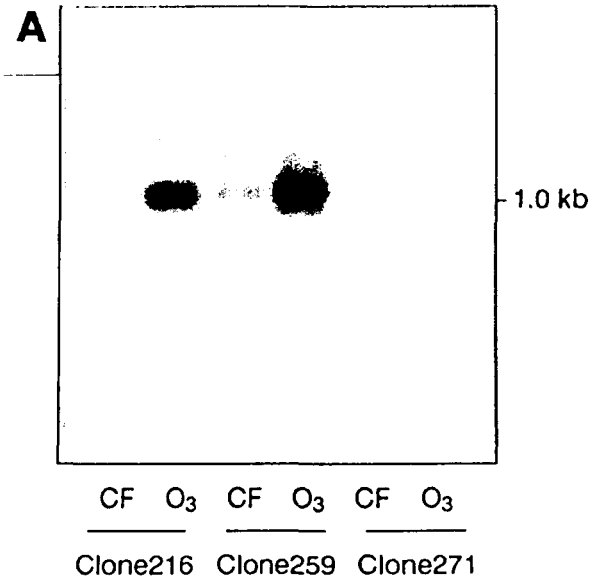

Figure 4. Effects of O_3 on Gpx transcript levels. Total RNA was extracted from leaves of potted, one-year-old aspen plants, exposed to either charcoal-filtered air (CF) or O_3-added (O_3) at the rate of 100 nL.L^{-1} for six hours per day four days per week, for two months. In each lane, 20 μg of total RNA was separated in an agarose-formaldehyde gel, transferred to nylon membrane, and hybridized to a ^{32}P-labeled Gpx cDNA probe (6P229) from *N. sylvestris*. A RNA ladder was used as a molecular size standard. Clone 216 is O_3 tolerant, Clone 259 is O_3 sensitive and Clone 271 is intermediate.

chloroplastic Cu/Zn SOD and Fe SOD were unaffected (Fig. 3). Mn SOD and cytosolic Cu/Zn SOD appeared to be most responsive of the SOD mRNAs during long-term O_3 stress. The increased SOD activities may provide one mechanism for limiting free radical chain peroxidations initiated by superoxide radicals. Increases in SOD levels in response to O_3 have also been reported in hybrid poplar (SEN GUPTA *et al.* 1991). SHARMA AND DAVIS (1994) and CONKLIN AND LAST (1995) observed a 2- to 3-fold increase in cytosolic Cu/Zn SOD mRNA level after O_3 treatment in *Arabidopsis thaliana*. MATTERS AND SCANDALIOS (1987) did not detect any changes in SOD activity in *Zea mays* after short-term exposure to high O_3 doses. Similar results were reported for *Pinus sylvestris* (WINGSLE *et al.* 1992). Decreased levels of SOD activity were reported for *Picea rubens* exposed to O_3 (HAUSLADEN *et al.* 1990). MCKERSIE *et al.* (1982) found no relationship between O_3 sensitivity and SOD production in *Phaseolus vulgaris*. The inconsistency in results may be due in part to different exposure regimes and to difference in O_3 tolerance between plant species. In this study, *P. tremuloides* clones differing in O_3 tolerance had variable SOD responses to O_3.

The tolerant clone 216 and intermediate clone 271 had greater increases in total glutathione (Fig. 1B) following O_3 exposure (100 nL.L^{-1} for 2 months) than did the sensitive clone 259. Thus, these results suggest that increases in total glutathione level may also be important in protecting the plants from O_3 stress. In an O_3-tolerant cultivar of *Phaseolus vulgaris*, GSH levels were found to be higher than in the O_3-sensitive cultivars (GURI 1983). Increases in total glutathione levels in response to O_3 have also been reported in hybrid poplar (SEN GUPTA *et al.* 1991). The reduction of H_2O_2 in higher plants can be catalyzed by a number of different enzymes located in different cellular compartments. In the absence of effective catalase (mainly in peroxisomes) in the chloroplast (BOWLER *et al.* 1992), GR occurs in the chloroplast and is involved in the maintenance of a high GSH/GSSG ratio and scavenging of H_2O_2 through the ascorbate-GSH cycle (HALLIWELL & FOYER 1978). It has been suggested that GR could play an important role in the detoxification

oxygen radicals (TANAKA *et al.* 1985). In the present studies, GR activity was increased in the O_3-tolerant clone but not in the O_3-sensitive and intermediate clones when plants were exposed to 100 $nL.L^{-1}$ O_3 for 10 days (data now shown). These results might indicate a higher capacity of H_2O_2 scavenging system in O_3-tolerant clones. The GR activity has been shown to increase in spinach plants (TANAKA *et al.* 1988a) and in pea plants (MEHLHORN *et al.* 1987) exposed to low levels of O_3, but not in spinach (TANAKA *et al.* 1985) or *Sedum album* (CASTILLO & GREPPIN 1988) exposed to higher levels of O_3.

Our northern blot analysis of total RNA samples from the O_3-treated and control plants of three clones showed larger increases in levels of GPx mRNA in O_3-treated (100 $nL.L^{-1}$ O_3 for 2 months) clones 216 and 259 than in the clone 271 when probed with the putative cDNA GPx probe from tobacco (Fig. 4). Glutathione peroxidase has rarely been described in plants (DROTAR *et al.* 1985; SABEH *et al.* 1993). Recently, induction of GPx mRNA has also been observed in stressed leaves of *N. sylvestris* (CRIQUI *et al.* 1992) and in *N. plumbaginifolia* in response to O_3 (WILLEKENS *et al.* 1994). The role of GPx might well be related to its ability to scavenge H_2O_2 in the mitochondria and cytoplasmic compartment (OVERBAUGH & FALL 1985). Alternatively, the GPx may play a role in removing lipid hydroperoxides from cellular membranes as has been suggested for mammalian GSH peroxidases (CHANCE *et al.* 1979; DROTAR *et al.* 1985).

TEPPERMAN & DUNSMUIR (1990) suggest that the antioxidant resistance mechanisms are not single enzyme or single antioxidant phenomenon, but likely the interaction of a multi-component oxygen detoxification system including SOD, ascorbate/ascorbate peroxidase and glutathione/GR. SHAALTIEL & GRESSEL (1986) reported that a paraquat-resistant biotype of *Conyza bonariensis* contained elevated levels of three enzymes involved in oxygen radical detoxification: SOD, ascorbate peroxidase and GR. BOWLER *et al.* (1992) proposed that the genetic engineering of the chloroplastic H_2O_2-detoxifying system, in addition to SOD, may be necessary to produce a resistant phenotype.

In summary, this study supports the hypothesis that SOD and glutathione systems play integral roles in the protection of plants from oxidative stress. The results also depict the variation in the response of different clones to O_3 exposure and indicate the complexity of oxidative stress resistance mechanisms in aspen. This study was facilitated by the previous identification of aspen clones differing in ozone sensitivity. These materials are extremely valuable for mechanistic research as well as to find the signal transduction mechanisms that underscore the responsiveness of aspen plants to O_3 stress.

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