SHORT COMMUNICATION

RAPID RESPONSE OF ANTIOXIDANT ENZYMES TO O_3-INDUCED OXIDATIVE STRESS IN *POPULUS TREMULOIDES* CLONES VARYING IN O_3 TOLERANCE

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INTRODUCTION

The concentration of atmospheric ozone (O_3), formed at the interaction of nitrogen oxides, volatile hydrocarbons and ultraviolet radiation, is increasing globally (FOWLER *et al.* 1999) due fossil fuel consumption. While O_3 levels are increasing in the atmosphere, in fact none of it has been detected inside the leaf (LAISK *et al.* 1989). Ozone readily reacts with water giving rise to reactive oxygen species (ROS) hydrogen peroxide and superoxide anion radical (GRIMES *et al.* 1983; KANOFSKY & SIMA 1995). ROS and especially radicals can non-specifically react with lipids, proteins and nucleic acids (DAVIES 1995) thus disrupting the structural and functional integrity of the cell. While the increased rise in tropospheric O_3 and the resulting increase in ROS is the result of human activities, the active oxygen species are also produced naturally during the normal metabolism of photosynthesizing cells. All green plants as well as other aerobic organisms have evolved antioxidant defense systems to combat the inevitable ROS (ALSCHER *et al.* 1997). The antioxidant systems can be enzymatic or non-enzymatic ROS scavengers that require the activation of specific metabolic pathways and investment of energy. It has been shown that plants growing under the conditions of oxidative stress have elevated levels of antioxidants (Rao *et al.* 1996; POLLE 1997; NOCTOR & FOYER 1998). The principle of the action of the antioxidants is simple, but the mechanism of regulation of their synthesis and activity is not well established. An aspect of doubt is whether antioxidants can provide protection against brief high-O_3 episodes or are they useful for protecting plants from only long-term chronic exposure. In current experiment we subjected two aspen (*Populus tremuloides* Michx.) clones, differing in O_3 tolerance, to acute O_3 episodes of up to six hours to test the inducibility of the enzymes of the Halliwell-Asada antioxidant pathway (Fig. 1) and catalase, the major scavenger of high levels of hydrogen peroxide.

MATERIALS AND METHODS

Plant material and O_3 fumigation. Two aspen (*Populus tremuloides*) clones, differing in O_3 sensitivity (KARNOSKY *et al.* 1998) – 216 (O_3 tolerant) and 259 (O_3 sensitive) – were fumigated with O_3 (0.2 $mL \cdot L^{-1}$) in 1.0 m^3 flow–through fumigation chambers with air exchange rate of 1.2 $m^3 \cdot min^{-1}$. Control samples were collected from untreated plants. Leaf samples with LPI 6 to 9 were collected at ½, 1, 2, 4 and 6 hours after the beginning of O_3 fumigation. Samples were fast-frozen in liquid nitrogen and stored at –80°C.

Antioxidant assays. Total protein was extracted in 100mM potassium phosphate (pH 7.0), containing 0.1 mM EDTA and 1% insoluble polyvinylpoly-pyrrolidone (PVPP) and assayed by using the Bio-Rad DC Protein Assay Kit (BioRad Laboratories, Hercules, CA) using bovine serum albumin fraction V as a standard. Superoxide dismutase (SOD) assay was performed as described by (DHINDSA *et al.* 1981) with and without KCN to distinguish between Cu/Zn-SOD and Fe-SOD or Mn-SOD. Ascorbate peroxidase (APx) assay was performed as described by (CHEN & ASADA 1992). Catalase (CAT) assay was performed as described by (KATO & SHIMIZU 1987). Glutathione reductase (GR) assay was performed as described by (PRICE *et al.* 1990). Total glutathione assay was performed as described by (SMITH 1985).

Northern hybridization. Northern blot analysis was performed using standard techniques as described by (SAMBROOK *et al.* 1989). RNA samples were subjected to agarose gel electrophoresis, blotted to a nylon membrane and hybridized with radiolabeled probes for

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Figure 1. Schematic diagram of the Halliwell-Asada antioxidant pathway. Enzymes are shown in bold (SOD – superoxide dismutase; Px – peroxidases; CAT – catalase; Apx – ascorbate peroxidase; MDHAscR – monodehydroxyascorbate reductase; DHAscR – dehydroxyascorbate reductase; GR – glutathione reductase). The metabolic intermediates are ascorbic acid (Asc), monodehydroxyascorbic acid (MDHAsc), dehydroxyascorbic acid (DHAsc), reduced (GSH) and oxidized glutathione (GSSG).

SOD, APx and CAT transcripts. Equal loading of RNA samples was checked from EtBr stained gels. Probes were synthesized by using a Random Prime Labeling Kit (Ambion, Austin, TX) using following cDNA clones as templates: (i) aspen cytosolic Cu/Zn-SOD clone (AKKAPEDDI *et al.* 1999), (ii) tobacco CAT cDNA and (iii) maize APx cDNA (WILLEKENS *et al.* 1994; VAN BREUSEGEM *et al.* 1995). The X-ray films exposed to probed membranes were digitized and the band intensities were quantified by using line profile tool in ImageTool 2.0 by The University of Texas Health Science Center (San Antonio). Northern blot analysis was repeated twice to check the consistency of results.

Statistical difference between time points or treatments was detected with two sample t-test assuming equal variances.

RESULTS AND DISCUSSION

Ozone treatment increased SOD activity in both clones, while *de novo* transcription was induced only in clone 216 and altogether decreased in clone 259 (Fig. 2a). The fact that clone 216 displays slightly higher SOD activity correlates well with its higher O_3-tolerance as shown earlier (KARNOSKY *et al.* 1998). Significant increase in SOD activity could be detected within the first half an hour in clone 216 and within an hour in clone 259 and the maximum activity was recorded at 2 and 4 hours for clones 216 and 259, respectively (Fig. 2b). The lack of signal in KCN-containing SOD assays

Figure 2. The band intensities of superoxide dismuatse (SOD) northern blot (a) and SOD specific activity (b) for two trembling aspen clones exposed to O_3 for various lengths of time. The values are mean ± SE (n = 4). Dark bars – O_3 tolerant clone 216; light bars – O_3 sensitive clone 259.

Figure 3. The band intensities of catalase (CAT) northern blot (a) and CAT specific activity (b) for two trembling aspen clones exposed to O_3 for various lengths of time. Symbols are the same as in Fig. 1.

Figure 4. The band intensities of ascorbate perxidase (APx) northern blot (a) and APx specific activity (b) for two trembling aspen clones exposed to O_3 for various lengths of time. All symbols are the same as in Fig. 1.

indicates, that most of the activity comes from Cu/Zn-SOD as shown previously (SHENG *et al.* 1997). On the basis of current results it cannot be said whether the increased SOD activity was dependent on the *de novo* transcription or whether it was activation at the enzyme level.

The decrease in CAT mRNA levels occurred in both clones as a result of O_3 fumigation, but was observed earlier in clone 259 (Fig. 3a). The changes in CAT enzyme activity, however, do not follow the mRNA patterns but rather reflect the changes in SOD enzyme activity (Fig. 3b). It has been found that H_2O_2, the reaction product of SOD, acts as an activating messenger for CAT as well as for APx (ELSTNER & OSSWALD 1994), what would explain the synchronized change in activity. The increase in CAT activity during first half-hour of fumigation was statistically significant ($P<0.05$) in both clones.

The APx mRNA levels decreased as a result of O_3 treatment in both clones, more rapidly in clone 216 than in 259 (Fig. 4a). The APx enzyme activity decreased initially in both clones (Fig. 4b), and recovered by one hour in clone 216 and by 4 hours in clone 259. The activity dropped again at the end of the experiment. The enzyme activity in clone 259 followed the changes in messenger levels more closely than in clone 216 suggesting the enzyme's vulnerability to inactivating compounds and the importance of *de novo* transcription. Although the APx activity does not follow the changes in SOD activity as clearly as did CAT, its specific activity is 5 times higher than that of CAT, suggesting that APx is important in controlling the level of ROS. CAT can scavenge H_2O_2 at higher concentrations than APx since it is not (unlike APx) inactivated by H_2O_2 (NAKAYAMA *et al.* 1997). At the same time, the affinity of CAT to H_2O_2 is over 50 times lower than that of APx (DALTON *et al.* 1987), which means that CAT is not as efficient in scavenging low concentrations of H_2O_2 as is APx.

No GR activity was detected in any of the samples. This suggests that either this enzyme will be activated later or that the short version of Halliwell-Asada pathway is used in aspen, terminating with dehydroxyascorbate reductase, activity of which was not measured in current experiment. Northern hybridization analysis of GR transcript was not performed because of the absence of suitable probe. No statistically significant changes were observed in glutathione content in either of the clones – all samples fell in the range of 10–20 $nmol \cdot g^{-1}$ fresh weight (data not shown).

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Our data shows the fastest and clearest response in SOD activity, the first enzyme in the antioxidant cascade, which responds to O_3 exposure with increased activity within half an hour. The change in transcript levels could be observed within one hour of O_3 fumigation. This is faster than reported by (KOCH *et al.* 1998) who detected elevated mRNA levels of antioxidant enzymes in hybrid poplar after three hours of fumigation. In *Arabidopsis* increased transcript levels were detected after 2 hours of O_3 exposure (CONKLIN & LAST 1995). The synthesis of new gene products following a 10-hour fumigation was reported by (ECKEY-KALTENBACH *et al.* 1994). The data presented in his report show that the defense response can occur on the time-scale of minutes rather than hours. The primary increase in antioxidant activity, however, is likely to occur at enzyme level, which is then followed by *de novo* transcription and translation.

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