RAPID RESPONSE OF ANTIOXIDANT ENZYMES TO O₃-INDUCED OXIDATIVE STRESS IN POPULUS TREMULOIDES CLONES VARYING IN O₃ TOLERANCE

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Received October 5, 1999; accepted November 2, 2000

INTRODUCTION

The concentration of atmospheric ozone (O₃), formed at the interaction of nitrogen oxides, volatile hydrocarbons and ultraviolet radiation, is increasing globally (Fowler et al. 1999) due fossil fuel consumption. While O₃ 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 nonspecifically 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 O₃ 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 (Alschger 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-O₃ 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 O₃ tolerance, to acute O₃ 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 O₃ fumigation. Two aspen (Populus tremuloides) clones, differing in O₃ sensitivity (Karnosky et al. 1998) – 216 (O₃ tolerant) and 259 (O₃ sensitive) – were fumigated with O₃ (0.2 mL·L⁻¹) in 1.0 m³ flow-through fumigation chambers with air exchange rate of 1.2 m³·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 O₃ fumigation. Samples were fast-frozen in liquid nitrogen and stored at −80°C.

Antioxidant assays. Total protein was extracted in 100 mM potassium phosphate (pH 7.0), containing 0.1 mM EDTA and 1% insoluble polyvinylpyrrolidone (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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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 Image-Tool 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 (KARNOFSKY 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 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 – monodehydroascorbate reductase; DHAscR – dehydroascorbate reductase; GR – glutathione reductase). The metabolic intermediates are ascorbic acid (Asc), monodehydroascorbic acid (MDHAsc), dehydroascorbic acid (DHAsc), reduced (GSH) and oxidized glutathione (GSSG).

Figure 2. The band intensities of superoxide dismutase (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.
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 O₃ 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 H₂O₂, 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 O₃ 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 H₂O₂ at higher concentrations than APx since it is not (unlike APx) inactivated by H₂O₂ (NAKAYAMA et al. 1997). At the same time, the affinity of CAT to H₂O₂ 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 H₂O₂ 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 dehydroxy-ascorbate 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·g⁻¹ fresh weight (data not shown).
Our data shows the fastest and clearest response in SOD activity, the first enzyme in the antioxidant cascade, which responds to O₃ exposure with increased activity within half an hour. The change in transcript levels could be observed within one hour of O₃ 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 O₃ 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.

LITERATURE CITED


