SHORT COMMUNICATION

Effects of ozone on the plasma membrane proteins in
Arabidopsis thaliana (L.) leaves

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ABSTRACT

The protein pattern of leaf plasma membranes from Arabidopsis thaliana (L.) Landsberg erecta was analysed in order to detect changes induced by acute short-term ozone treatment. Plasma membranes were isolated 0, 3 and 8 h after the end of a 2 h fumigation of the plants with 500 nmol mol–1 of O3. Proteins extracted from plasma membranes were separated by high-performance two-dimensional polyacrylamide gel electrophoresis. Eight hours after the end of fumigation, one new protein appeared and the amounts of two other proteins increased significantly. The reported study is a first step towards the identification of plasmalemma proteins altered by ozone and to a more detailed characterization of structural changes occurring in the plasma membrane after ozone exposure.

Key-words: Arabidopsis thaliana; ozone; plasmalemma; two-dimensional gel electrophoresis.

INTRODUCTION

Tropospheric ozone of anthropogenic origin is the most widespread air pollutant in many industrialized regions (Krupa & Manning 1988; Kangasjärvi et al. 1994). In plants, a number of physiological and biochemical processes are affected by ozone and/or its combination with other pollutants (Darrall 1989; Treshow & Anderson 1989; Kangasjärvi et al. 1994). The main alteration, which is due to the presence of ozone-induced highly reactive oxygen derivatives, concerns the oxido-reductive equilibrium of the cell (Mehlhorn, Tabner & Wellburn 1990).

At the protein level, activities of enzymes as well as their biosynthesis were shown to be modified by ozone. As demonstrated in a number of biochemical studies (Schraudner et al. 1992; Eckey-Kaltenbach et al. 1994; Sharma & Davis 1994; for a review see Kangasjärvi et al. 1994), ozone exposure results in an increase in the activity of enzymes associated with general plant defence (pathogenesis-related proteins, as β-1,3-glucanase and chitinase) and enzymes which protect the cell from oxidative damage (peroxidases, catalases, superoxide dismutase)]. Molecular studies showed that the activity changes of these enzymes were due to induction of the corresponding genes (Eckey-Kaltenbach et al. 1994; Sharma & Davis 1994; Schlagnhaufer et al. 1995). In leaf cells of tobacco and parsley, a single subacute pulse of O3 (150 nmol mol–1 during 5 h) provoked a rapid (5–10 h after the start of ozone exposure) induction of transcripts of several pathogenesis-related proteins (Schraudner et al. 1992; Eckey-Kaltenbach et al. 1994). Other proteins were also found to be affected. Price, Lucas & Lea (1990) and Nie, Tomasevic & Baker (1993) reported that ozone exposure resulted in a reduction of the amount of total and soluble leaf proteins. In Picea abies, quantitative and qualitative changes in the protein content of needles were found after long-term exposure to ozone (Schmitt & Sandermann 1990); in wheat leaves chronic ozone treatment resulted in numerous changes in total protein pattern (Nie et al. 1993).

The plasma membrane, the outer semi-permeable barrier of the cell, is the first cellular component exposed to air pollutants. Stress-induced plasma membrane changes may strongly perturb different cellular functions, but they can also play an important role in adaptation to stress and stress defence. In the case of ozone-induced stress, Städtler & Ziegler (1993) demonstrated that variational resistance of tobacco plants to this pollutant originates mainly from membrane traits. Existing studies on the effect of ozone on plasma membrane proteins mainly concern functioning of the proteins involved in transport processes. It has been shown that ozone inhibits the activity of the membrane ATPases (Dominy & Heath 1985; Castillo & Heath 1990; Guzy & Heath 1993). This could be responsible for the loss of membrane semipermeability and disturbance in the ionic homeostasis of the cell (Heath 1988; Heath & Castillo 1988).

This work was intended to study the influence of ozone on the protein composition of plasma membranes in Arabidopsis thaliana. In a number of studies the effects of ozone on the functioning of the plasma membrane were...
observed after rather short pulses of ozone at high concentrations (Dominy & Heath 1985; Castillo & Heath 1990; Guzy & Heath 1993). A similar fumigation protocol was used in the present work. Changes in the protein pattern were analysed by high-performance two-dimensional (2D) polyacrylamide gel electrophoresis. This work is the first step in our study of membrane proteins which are a target of ozone attack. We report here on significant changes in the plasma membrane protein pattern after ozone treatment. Use of the model plant Arabidopsis should further enable identification of the proteins involved and determination of the physiological significance of the changes in their abundance.

MATERIALS AND METHODS

Plant material

Five-week-old plants of Arabidopsis thaliana (L.) $L_{\text{andsberg erecta}}$ were used for experiments. Plants (c. 50 seeds cm$^{-2}$) were grown in soil (Tarmacs Bellflower, Bachman S.A., Chevroux, Switzerland) in a growth chamber under short-day conditions (8 h light/16 h darkness) at 22 ± 2 °C and relative humidity of 80%. Light (25 W m$^{-2}$) was supplied by white fluorescent tubes (40 W, Sylvania Daylight, ref. 244 332).

Ozone treatment and sample preparation

Fumigation with ozone was carried out in special fumigation chambers. Three hours after the beginning of the light period, plants were exposed for 2 h to ozone (200, 500 and 1000 nmol mol$^{-1}$) or maintained in ambient air. One lot of plants was harvested immediately (0 h) after the end of ozone exposure, and the other plants were returned to the (ambient air) growth chamber and leaves were harvested after 3 and 8 h. Isolation of plasma membrane and protein extraction were carried out on 50 g of leaf fresh weight. During the whole experiment a minimal relative humidity of 80% was maintained.

Purification of plasma membrane

The crude membrane fraction obtained by differential centrifugation was partitioned in a dextran–polyethylene glycol aqueous two-phase system according to the method of Kjellbom & Larsson (1984) with minor modifications. The quality of the preparations was judged by morphological analysis (electron microscopy and estimation of membrane thickness) and by biochemical tests (measurements of the activities of marker enzymes for plasma membrane and possible contaminating endomembranes). The following plasmalemma markers were assayed: 1,3-β-glucan synthase II (EC 2.4.1.34; Kauss & Jeblick 1985), UDPglucose:sterol glucosyltransferase (UDPG sterol glucosyltransferase, EC 2.4.1.173; Chanson, Taiz & McNaughton 1984) and K$^+$-stimulated Mg$^{2+}$-dependent, vanadate-sensitive ATPase (EC 3.6.1.3; Hodges & Leonard 1974). Contamination by membranes of other origin was determined by quantification of the following enzymes: NADH cytochrome c oxidase (EC 1.9.3.1; Wharton & Tzagoloff 1967) for mitochondria, latent inosine diphosphatase (EC 3.6.1.6; Quail 1980) for Golgi apparatus, antimycin A-resistant NAD(P)H cytochrome c reductase (EC 1.6.2.4; Hodges & Leonard 1974) for endoplasmic reticulum, and K$^+$-stimulated Mg$^{2+}$-dependent, nitrate-sensitive ATPase (EC 3.6.1.3; Hodges & Leonard 1974) for tonoplast. Specific activities of the marker enzymes were determined in the crude membrane fraction, CM, and in the different fractions obtained during two subsequent phases partitioning procedures: L1, the lower phase after the first phase partitioning; U1, the fraction obtained by re-extraction of L1; L2, the lower phase after the second partitioning; U2, the upper phase after two partitioning procedures, i.e. the enriched plasma membrane fraction used for the analysis of protein pattern.

Electron microscopy

The aliquots of membrane preparations were fixed and embedded as described previously by Crespi et al. (1989). Ultra-thin sections were cut, stained with uranyl acetate and lead citrate and examined on a ZEISS EM 10 electron microscope. The membrane vesicles were photographed and the negatives were digitized with a computerized image analysis system (BIOCOM 200, Les Ulis, France). The measurements of membrane thickness were carried out as described by Crèvecoeur et al. (1992) with the use of IMA software.

Protein extraction

Proteins were extracted from the plasma membrane preparations by an acetone–trichloroacetic acid precipitation according to the method of Zivy (Damerval et al. 1986). Protein content was measured using the Bio-Rad reagent microassay (Glattbrugg, Switzerland) and independently by weighing dry samples. Extracted proteins were immediately run on the gel or stored at −80 °C.

Two-dimensional electrophoresis

Fifty μg of plasmalemma proteins was loaded onto each gel. The two-dimensional polyacrylamide gel electrophoresis procedure was based on the method of Hochstrasser et al. (1988) as previously described by Tacchini et al. (1995a,b). Gels were silver stained according to Oakley, Kirsh & Morris (1980) as modified by Hochstrasser et al. (1988), photographed and preserved in 5% acetic acid in sealed plastic bags. Commercially available protein markers were used for molecular mass determination.

Gel analysis and data treatment

Gels from at least three independent parallel experiments were first compared by superposition, which allowed a raw
selection of spots with marked intensity differences between control and O₃-treated plants. As numerous spots showed intensity differences, which were not reproducible in all parallel experiments, for further analysis we retained only spots with differences occurring in all repetitions. In order to better characterize the changes and take into account the typical 2D electrophoresis gel-to-gel variability, intensities of the selected spots were quantified by computer-assisted image analysis. Gels were captured by a CCD black and white camera (WV CD 50 Panasonic) and digitized in 512 × 512 pixels (256 grey levels per pixel) with the use of an image analysis system (BIOCOM 200, Les Ulis, France). The intensity of each spot was expressed as the background-corrected grey level value integrated over all pixels in the spot. In order to exclude erroneous interpretation, qualitative differences or quantitative changes of 50% or more were considered significant. The reported data are the means from at least three independent experiments ± SEM.

RESULTS AND DISCUSSION

Macroscopic effects of ozone

There is convincing evidence in the literature that short ozone pulses at high concentrations (> 200 p.p.b.) disturb quite rapidly the physiological functioning of plasma membranes and influence the pattern of protein expression (Dominy & Heath 1985; Castillo & Heath 1990; Schraudner et al. 1992; Guzy & Heath 1993; Sharma & Davis 1994). In our experiments, Arabidopsis plants were fumigated with 200, 500 and 1000 nmol mol⁻¹ of O₃ for 2 h. Ozone at concentrations of 200 and 1000 nmol mol⁻¹ resulted in no visible injury and in premature death of the plants, respectively. Plants treated with 500 nmol mol⁻¹ of O₃ showed moderate macroscopic changes and thus this concentration was chosen for the experiments on the protein pattern. Eight hours after the end of fumigation with 500 nmol mol⁻¹ O₃, leaves showed some wilting symptoms (Fig. 1) and their fresh weight was reduced by up to 5% in spite of the high humidity of air maintained throughout the experiment. This suggested that regulatory mechanisms of stomatal opening were affected. A few chlorotic spots were also observed on leaves, indicating a poisoning effect of ozone on the leaf tissue.

Purification of plasma membrane

The quality of the plasma membrane preparations used for electrophoretic analysis was assessed by measuring the activity of marker enzymes and by determining the thickness of membranes on electron micrographs. Both methods showed that our preparations were reproducible and of good purity. Figure 2 shows an electron micrograph of membrane vesicles from a fraction enriched in plasmalemma. Electron microscopy has shown that the bulk of the vesicles exhibits a clear dark-light-dark pattern, which is characteristic of the plasma membrane. Measurements of membrane thickness gave the mean value of 7.8 nm, also typical for plasmalemma (Sandellius et al. 1986; Crespi et al. 1989). No substantial differences in plasma membrane thickness or structure were detected between control and fumigated plants. However, in some cases, preparations from ozone-treated plants showed more intensively stained membrane vesicles. This could indicate changes in membrane properties.

Table 1 summarizes the specific activity of marker enzymes for plasmalemma and for different endomembranes measured in crude membranes and in fractions obtained during two subsequent phase partitioning procedures. The plasmalemma fraction used for the protein pattern analysis (U2) contained about 3% (0.011 mg g⁻¹ fresh weight) of the protein present in total membranes (0.358 mg g⁻¹ fresh weight) and showed high specific activity of the plasma membrane marker 1,3-β-glucan synthase II (2.76-fold enrichment compared to crude membranes). Activities of two other enzymes which are considered as good plasma membrane markers, UDPG sterol glucosyltransferase and K⁺-stimulated Mg²⁺-dependent, vanadate-sensitive ATPase, were also principally recovered in upper phases (U1 + U2). The ATPase activity in upper phases was inhibited by 60–100% by vanadate and was not sensitive to nitrate.

Figure 1. Macroscopic effects of ozone on Arabidopsis thaliana: (A) control plants, and (B) plants 8 h after the end of the 2 h fumigation with 500 nmol mol⁻¹ of ozone.
(KNO₃). Non-sensitivity of ATPase activity to nitrate indicated that fractions enriched in plasmalemma were free from contamination by tonoplast; this is confirmed by analysis of electron micrographs. No major contamination by either mitochondria or Golgi apparatus was found in U2 fractions, as assessed by measurements of cytochrome c oxidase and latent inosine diphosphatase activities, respectively. Antimycin A-resistant cytochrome c reductase, an endoplasmic reticulum marker, showed clear but low activity in plasma membrane-enriched fractions. This activity was constant in all phases of the partitioning and could correspond to some rough endoplasmic reticulum trapped in vesicles during the extraction procedure.

**Effect of ozone on plasma membrane proteins**

Purified plasma membrane proteins of *Arabidopsis thaliana* were resolved by high-performance 2D gel electrophoresis. About 100 protein spots could be detected by silver staining on 20 cm × 20 cm gels (Fig. 3). The global electrophoretic patterns of the proteins from the control and O₃-treated plants were similar. The patterns contained an area of high-intensity spots (relatively abundant proteins) that spanned molecular masses from 35 to 45 kDa over the whole range of pl, a few distinct spots of 75 kDa in the acidic region, and numerous less abundant polypeptides with molecular masses between 45 and 60 kDa and pl in the range of 5.0–6.5.

While the relative distribution of the protein spots was highly reproducible, the spot intensities showed some variability between independent experiments. In order to quantify this gel-to-gel variability and select only significant differences between patterns from control and O₃-treated plants, spots with visible 'by eye' intensity changes were subjected to computer-assisted image analysis with subsequent statistical treatment of intensity values (see ‘Materials and methods’). Finally, only these differences were retained which were reproduced in all parallel experiments and where the change in spot intensity was of 50% or more. According to these criteria, in leaves harvested immediately (0 h) and 3 h after the end of fumigation with 500 nmol mol⁻¹ O₃, no significant modifications of protein pattern were found. By contrast, in plants harvested 8 h after the end of the fumigation we could point out three plasmalemma proteins which were significantly different between control and O₃-treated plants (Fig. 3 & Table 2). The amounts of two proteins (proteins A and B; Fig. 3) present in control plants increased after ozone treatment by

Table 1. Specific activities of marker enzymes in the crude membrane fraction, CM, and in the different fractions obtained during two-phase partitioning procedures: L1, lower phase after the first phase partitioning; U1, fraction obtained by re-extraction of L1; L2, lower fraction after the second partitioning; U2, enriched plasma membrane fraction obtained after two partitioning procedures. The values are from one representative experiment out of three experiments performed with Arabidopsis thaliana control plants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CM</th>
<th>U1</th>
<th>U2</th>
<th>L1</th>
<th>L2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3-β-glucan synthase (dpm mg⁻¹ protein 10³)</td>
<td>208</td>
<td>490</td>
<td>573</td>
<td>437</td>
<td>52</td>
</tr>
<tr>
<td>UDPG sterol glucosyltransferase (dpm mg⁻¹ protein 10³)</td>
<td>nd</td>
<td>U1+U2</td>
<td>L1+L2</td>
<td>47</td>
<td>0.8</td>
</tr>
<tr>
<td>Vanadate-sensitive K⁺ Mg²⁺ ATPase (nM Pi min⁻¹ mg⁻¹ protein)</td>
<td>–</td>
<td>U1+U2</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c oxidase (nM cyt P₄₅₀ min⁻¹ mg⁻¹ protein)</td>
<td>104</td>
<td>54</td>
<td>14</td>
<td>31</td>
<td>11</td>
</tr>
<tr>
<td>Antimycin A-resistant cytochrome c reductase (nM cyt P₄₅₀ min⁻¹ mg⁻¹ protein)</td>
<td>34</td>
<td>59</td>
<td>65</td>
<td>57</td>
<td>42</td>
</tr>
<tr>
<td>Latent inosine diphosphatase (nM Pi min⁻¹ mg⁻¹ protein)</td>
<td>26</td>
<td>–</td>
<td>–</td>
<td>29</td>
<td>–</td>
</tr>
</tbody>
</table>

nd, not determined; -, not detected.

Figure 3. Two-dimensional gel electrophoretic separations of proteins extracted from plasma membrane of Arabidopsis thaliana leaves: (a) control, and (b) 8 h after the end of the 2 h fumigation with 500 nmol mol⁻¹ of ozone. The arrows indicate the proteins which appeared or whose abundance was significantly increased in plants treated with ozone.
rather than regulatory ones. The observation that they are up-
can suppose that these are enzymatic or structural proteins
altered polypeptides. As they were detectable on 2D gels, we
a rapid turnover.

Finally, this technique detects only changes due to the
restricted to proteins of relatively high abundance which
the most significant changes were retained for analysis.

This observation is in agreement with the generally
poisoning.

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Received 6 March 1997; received in revised form 22 May 1997; accepted for publication 22 May 1997