



RESEARCH PAPER

The apoplastic antioxidant system in *Prunus*: response to long-term plum pox virus infection

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Abstract

This work describes, for the first time, the changes taking place in the antioxidative system of the leaf apoplast in response to plum pox virus (PPV) in different *Prunus* species showing different susceptibilities to PPV. The presence of *p*-hydroxymercuribenzoic acid (*p*HMB)-sensitive ascorbate peroxidase (APX) (class I APX) and *p*HMB-insensitive APX (class III APX), superoxide dismutase (SOD), peroxidase (POX), NADH-POX, and polyphenoloxidase (PPO) was described in the apoplast from both peach and apricot leaves. PPV infection produced different changes in the antioxidant system of the leaf apoplast from the *Prunus* species, depending on their susceptibility to the virus. In leaves of the very susceptible peach cultivar GF305, PPV brought about an increase in class I APX, POX, NADH-POX, and PPO activities. In the susceptible apricot cultivar Real Fino, PPV infection produced a decrease in apoplastic POX and SOD activities, whereas a strong increase in PPO was observed. However, in the resistant apricot cultivar Stark Early Orange, a rise in class I APX as well as a strong increase in POX and SOD activities was noticed in the apoplastic compartment. Long-term PPV infection produced an oxidative stress in the apoplastic space from apricot and peach plants, as observed by the increase in H₂O₂ contents in this compartment. However, this increase was much higher

in the PPV-susceptible plants than in the resistant apricot cultivar. Only in the PPV-susceptible apricot and peach plants was the increase in apoplastic H₂O₂ levels accompanied by an increase in electrolyte leakage. No changes in the electrolyte leakage were observed in the PPV-inoculated resistant apricot leaves, although a 42% increase in the apoplastic H₂O₂ levels was produced. Two-dimensional electrophoresis analyses revealed that the majority of the polypeptides in the apoplastic fluid had isoelectric points in the range of pI 4–6. The identification of proteins using MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) and peptide mass fingerprinting analyses showed the induction of a thaumatin-like protein as well as the decrease of mandelonitrile lyase in peach apoplast due to PPV infection. However, most of the selected polypeptides showed no homology with known proteins. This fact emphasizes that, at least in *Prunus*, most of the functions of the apoplastic space remain unknown. It is concluded that long-term PPV infection produced an oxidative stress in the leaf apoplast, contributing to the deleterious effects produced by PPV infection in leaves of inoculated, susceptible *Prunus* plants.

Key words: Apoplast, apricot, breeding, 2D electrophoresis, germplasm, oxidative stress, peach, resistance, sharka.

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Abbreviations: APX, ascorbate peroxidase; ASC–GSH cycle, ascorbate–glutathione cycle; DHAR, dehydroascorbate reductase; DPI, diphenylene iodonium; GR, glutathione reductase; G6DPH, glucose-6-phosphate dehydrogenase; IEF, isoelectric focusing; MALDI, matrix-assisted laser desorption/ionization; MBTH, 3-methyl-2-benzo-thiazolidine hydrazone hydrochloride hydrate; MDHAR, monodehydroascorbate reductase; MDL, mandelonitrile lyase; 4MN, 4-methoxy- α -naphthol; MS, mass spectrometry; *p*HMB, *p*-hydroxymercuribenzoic acid; POX, peroxidase; PPO, polyphenoloxidase; PPV, plum pox virus; O₂⁻, superoxide radical; ROS, reactive oxygen species; SOD, superoxide dismutase; TOF, time of flight.

Introduction

The plant cell apoplast, which includes the cell wall matrix, consists of a complex mixture of carbohydrates, proteins, lignin, water, metabolites, and inorganic compounds (Dietz, 1996). It has a primary role in cell nutrition, because cells import ions and metabolites from the apoplast, and in plant development, by determining the capacity and the rate of cell wall elongation (Dani *et al.*, 2005). The constituents of the apoplastic fluid co-ordinate events at the cell wall and the external face of the plasmalemma, playing a central role in the metabolism and signal transduction co-ordinating physiological processes such as cell expansion, differentiation, repair, and resistance to pathogen attack (Dietz, 1996). The apoplast is considered to have a lower soluble protein content and enzyme complement than most of the intracellular compartments, and to have a rather low pH (pH 5–6) (Dietz, 1996). The apoplast is involved in a variety of functions during normal growth and under stress conditions, including biotic (Vanacker *et al.*, 1998*a, b*; Bolwell *et al.*, 2002; Patykowski and Urbanek, 2003) and abiotic stresses (Hernández *et al.*, 2001; Dani *et al.*, 2005). The protein composition of the apoplast is influenced by several kinds of stress, such as wilting, salinity, heavy metals, and pathogens (Dietz, 1996; Hernández *et al.*, 2001; Dani *et al.*, 2005).

There is scarce information about the nature of the proteins present in the apoplastic space, and their responsiveness to environmental constraints. In a recent paper, Haslam *et al.* (2003), by using two-dimensional electrophoresis, identified some proteins expressed in the apoplastic space of *Arabidopsis thaliana*, *Triticum aestivum*, and *Oryza sativa*. The principal proteins identified in the aqueous matrix of these apoplasts were involved in defence against pathogen infections (germin-like proteins and glucanases), cell expansion (ascorbate oxidase and β -D-glucan glucohydrolases), signalling (serine-specific protein kinase and serine carboxypeptidase), and nutrient assimilation (nitrate reductase) (Haslam *et al.*, 2003). In the apoplastic space from *Nicotiana tabacum*, salt stress led to a significant increase in two chitinases and a germin-like protein, whereas two lipid transfer proteins were expressed entirely *de novo* (Dani *et al.*, 2005). However, some proteins decreased upon salt treatment; for example, cell wall peroxidases (POXs; Dani *et al.*, 2005). Nevertheless, relatively little information is available regarding the presence of the antioxidant enzymes in the apoplast of plant tissues, and the results found are often contradictory (Vanacker *et al.*, 1998*a*; Hernández *et al.*, 2001).

Sharka, a common disease caused by plum pox virus (PPV), is the most important viral disease affecting European trees, resulting in severe economic losses in *Prunus* species including apricot and peach (Dicenta *et al.*, 2000). Obtaining *Prunus* cultivars resistant to sharka is one of the main objectives of breeders. In these breeding

programmes, the evaluation of PPV resistance is the most time-consuming and expensive task. Therefore, biochemical and molecular markers associated with resistance would be of great interest. These markers will improve the selection process regarding the evaluation of a higher number of individuals. In recent work, carried out under field conditions, it has been reported that PPV-resistant apricot cultivars showed higher activities of catalase, ascorbate peroxidase (APX), and dehydroascorbate reductase (DHAR) than PPV-susceptible cultivars, suggesting that these activities could be used as potential biochemical markers for sharka (Hernández *et al.*, 2006*a*).

Most studies on the effect of biotic stress on the activity of antioxidant enzymes have been conducted with herbaceous plants, whereas studies with woody plants are less common. Moreover, most of the studies with PPV have been carried out at the levels of detection and molecular characterization, identification of the pathogenicity determinants, and study of their replication and mapping for PPV resistance (Sáenz *et al.*, 2000; Hurtado *et al.*, 2002; Chen *et al.*, 2005). The effect of PPV infection on the nutrient status of apricot, as well as the application of green fluorescent protein (GFP)-tagged PPV to track PPV invasion in susceptible peach and apricot plants, has been carried out recently (Lansac *et al.*, 2005; Stylianidis *et al.*, 2005). However, information about the effect of PPV infection on the antioxidant systems in *Prunus* is very scarce, being non-existent at the apoplastic level. In this work, the response of the apoplastic antioxidant system in PPV-inoculated susceptible (peach cv. GF305 and apricot cv. Real Fino) and resistant [apricot cv. Stark Early Orange (SEO)] *Prunus* plants was studied, in order to deepen knowledge of the physiological and biochemical processes of the *Prunus*–PPV interaction.

Materials and methods

Plant material

The North American apricot (*Prunus armeniaca* L.) cultivar SEO, characterized as resistant to PPV, and the Spanish cultivar Real Fino, described as susceptible to this virus (Martínez-Gómez and Dicenta, 2000), were used in the present work. In addition, peach [*Prunus persica* (L.) Batsch] cultivar GF305, characterized by its high susceptibility to fruit viruses including PPV (Pelet and Bovey, 1968) and commonly used as a rootstock in PPV resistance tests on *Prunus* (Martínez-Gómez and Dicenta, 2000), was also included. Plants were grown as described previously (Hernández *et al.*, 2004*a*, 2006*b*).

PPV isolate

The PPV isolate used was RB3.30, a Dideron type isolate obtained from the Red Beut plum cultivar (Instituto Valenciano de Investigaciones Agrarias, IVIA, Valencia, Spain). This isolate produces strong sharka symptoms in young leaves, consisting of veinal chlorosis in peach cv. GF305 and veinal chlorosis and rings in susceptible apricot leaves (Pelet and Bovey, 1968). Plants were

inoculated and evaluated as described previously (Hernández *et al.*, 2004a, 2006b).

Apoplast extraction

The apoplastic fraction was isolated by vacuum infiltration in the presence of 50 mM TRIS-acetate buffer pH 6.0 (infiltration buffer). Briefly, leaves (5–10 g), previously washed with cold deionized water, were cut into pieces (1–2 cm²) and washed with the infiltration buffer. Subsequently, the leaf pieces were infiltrated for 3 min, at 1.0 kPa and 4 °C, with the infiltration buffer containing 2 mM CaCl₂ and 0.2 M KCl (peach) or 0.5 M KCl (apricot). For APX activity, 5 mM sodium ascorbate was added to the infiltration buffer. The leaves were then dried quickly and centrifuged at 1000 g for 5 min, at 4 °C, in a 25 ml syringe barrel placed in a centrifuge tube. To assay enzymatic activities, the apoplastic fraction was concentrated ~2-fold using Centricon YM-10 filter devices (Amicon, Millipore) and pre-purified by chromatography on Sephadex G-25 NAP columns (Amersham, BioSciences) equilibrated with the infiltration buffer, with or without 5 mM sodium ascorbate. The samples were again concentrated ~2-fold using the same procedure mentioned above. To determine H₂O₂, the apoplastic fraction obtained after centrifugation at 1000 g was used directly, but, in this case, the leaf pieces were infiltrated with MilliQ water.

Contamination by cytoplasmic constituents was assessed by measuring the levels of glucose-6-phosphate dehydrogenase (G6PDH) (Hernández *et al.*, 2000). G6PDH is a cytoplasmic enzyme used as a specific marker for any plasma membrane damage that may occur during apoplast extraction by the vacuum infiltration method (Vanacker *et al.*, 1998b).

Leaf enzyme extraction

All operations were performed at 4 °C. Leaf residues (2 g), which resulted from the apoplastic extraction, were homogenized with a mortar and pestle in 4 ml of ice-cold 50 mM TRIS-acetate buffer pH 6.0, containing 0.1 mM EDTA, 3 mM cysteine, 2% (w/v) polyvinylpyrrolidone (PVP), 2% (w/v) polyvinylpolypyrrolidone (PVPP), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM ascorbate, and 0.2% (v/v) Triton X-100. For APX activity, 20 mM sodium ascorbate was added. The 'symplastic' homogenate was centrifuged at 14 000 g for 20 min and the supernatant fraction was filtered through Sephadex G-25 NAP columns equilibrated with the same buffer used for the homogenization, with or without 2 mM sodium ascorbate.

Determination of H₂O₂ and electrolyte leakage

The H₂O₂ concentration in the apoplastic fraction was determined immediately after isolation by a POX-coupled assay, using 4-aminoantipyrine and phenol as donor substrates (Frew *et al.*, 1983). For electrolyte leakage measurements, leaves (2 g) were cut into pieces (~2 cm²) and incubated in 8 ml of MilliQ water in sealed tubes, for 2 h at room temperature. After incubation, the conductivity of the bathing solution was measured with an Ion Check 30 conductivity meter (Radiometer Analytical, France). This value was referred to as value A. The bathing solutions were returned to the sealed tubes, containing the pieces of leaves, which were then incubated in a water bath at 95 °C for 25 min. After cooling to room temperature, the conductivity of the bathing solution was measured again. This is referred to as value B. For each measurement, electrolyte leakage was expressed as percentage leakage: [(value A/value B) × 100] (Mittler *et al.*, 1999).

Enzymatic analyses

APX, DHAR, monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), and superoxide reductase (SOD) activities

were assayed as described in Hernández *et al.* (2000, 2004a). APX was measured in the presence and absence of the specific inhibitor *p*-hydroxymercuribenzoic acid (*p*HMB) (0.5 mM final concentration). The *p*HMB-sensitive APX activity was considered as being due to class I APX (EC 1.11.1.11), while *p*HMB-insensitive APX activity was considered as due to class III APX (EC 1.11.1.7) (Ros-Barceló *et al.*, 2006). Total POX was analysed according to Ros-Barceló (1998).

Polyphenoloxidase (PPO, EC 1.14.18.1) activity was measured at 410 nm by monitoring the oxidation of tert-butylcatechol according to Espín and Wichers (1999). The reaction contained 50 mM K-phosphate buffer, pH 7, 3 mM tert-butylcatechol, and the enzymatic extract. The PPO activity was calculated from the molar extinction coefficient of 1850 M⁻¹ cm⁻¹ for tert-butylcatechol. In order to calculate latent PPO activity, the reaction was also carried out in the presence of 0.02% (v/v) SDS.

NADH oxidase/peroxidase was measured by monitoring the decrease in absorbance at 340 nm due to NADH oxidation. The reaction mixture contained 50 mM TRIS-acetate buffer pH 6.0, 0.2 mM NADH, the peroxidase activators *p*-coumaric acid (25 µM) and MnCl₂ (5 mM), and the enzymatic extract (Patykowski and Urbanek, 2003). The activities were calculated from the molar extinction coefficient of 6.3 M⁻¹ cm⁻¹ for NADH.

Electrophoretic analyses

To separate POX and PPO isozymes, non-denaturing PAGE and isoelectric focusing (IEF) were performed on 10% and 6.5% acrylamide gels, respectively, using a Bio-Rad Mini-protean III dual slab cell. The range of ampholytes (Pharmacia) used was pH 3.5–10. Samples were prefocused at 200 V for 90 min, and then focused at 400 V for 30–60 min. Staining of POX isoenzymes with 4-methoxy- α -naphthol (4MN) was performed as described by Ros Barceló (1998). Staining of PPO isoenzymes was carried out with 10 mM catechol and 5 mM 3-methyl-2-benzo-thiazolidine hydrazone hydrochloride hydrate (MBTH) (Cantos *et al.*, 2002).

One-dimensional SDS-PAGE was carried out using 10% (w/v) polyacrylamide gels in a Bio-Rad Mini-protean III dual slab cell. Protein samples (7 µg or 4 µg of protein for peach and apricot, respectively) were resuspended in a denaturing buffer [0.5 M TRIS-HCl pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol] and heated at 95 °C for 5 min. SDS-PAGE molecular weight standards (Bio-Rad) were used as molecular size markers on the gels. Gels were silver stained according to Blum *et al.* (1987). The protein fingerprint resolved by SDS-PAGE can also be used to evaluate the levels of apoplastic contamination by symplastic constituents.

For 2D electrophoresis, proteins were initially separated by IEF in the first dimension. IEF was carried out using 25 µg samples of apoplastic or symplastic proteins in gel strips with an immobilized linear pH gradient of 4–7 (Bio-Rad). Prior to loading on the second dimension, gel strips were equilibrated using two sequential washes (15 min each) in two different equilibration solutions. Equilibration buffer 1 contained 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 0.375 M TRIS-HCl pH 8.8, and 0.13 M dithiothreitol (DTT). Equilibration buffer 2 was the same as buffer 1, except that DTT was omitted and it was supplemented with 0.13 M iodoacetamide. Equilibrated gel strips were placed on top of vertical 10% (w/v) polyacrylamide gels, containing 10% SDS (w/v). A commercial 1% (w/v) solution of low-melting point agarose (Bio-Rad) was loaded on the gel strips. Electrophoresis was performed at room temperature, in a buffer containing 0.1 M TRIS, 0.1 M glycine, and 0.1% SDS, for 15 min at 100 V and 1 h at 150 V. Gels were silver stained according to Blum *et al.* (1987).

Protein spots were excised manually and then digested automatically using a Proteiner DP protein digestion station (Bruker-Daltonics,

Bremen, Germany). The digestion protocol used was that of Schevchenko *et al.* (1996) with minor variations. For peptide mass fingerprinting and LIFT time of flight (TOF)/TOF (Suckau *et al.*, 2003) spectra acquisition, an aliquot of α -cyano-4-hydroxycinnamic acid in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid was mixed with an aliquot of the above digestion solution and the mixture was deposited onto an AnchorChip MALDI probe (Bruker-Daltonics).

Peptide mass fingerprint spectra were measured on a Bruker Ultraflex TOF/TOF matrix-assisted laser desorption (MALDI) mass spectrometer (Bruker-Daltonics) (Suckau *et al.*, 2003) in positive ion reflector mode. Mass measurements were performed automatically, through fuzzy logic-based software, or manually. Each spectrum was calibrated internally with mass signals of trypsin autolysis ions to reach a typical mass measurement accuracy of ± 25 ppm. The measured tryptic peptide masses were transferred through the MS BioTools programme (Bruker-Daltonics) as inputs to search the NCBI database using Mascot software (Matrix Science, London, UK). When available, MS/MS (tandem mass spectrometry) data from LIFT TOF/TOF spectra were combined with MS peptide mass fingerprint data for database searching.

Results

Antioxidant enzymatic activities

The cytoplasmic contamination of the apoplastic extracts from peach and apricot was very low. In peach, the value was ~ 0.15 – 0.30% when calculated on a fresh weight basis. In apricot, the cytoplasmic contamination of the apoplastic fraction was lower than in peach, the values always being $< 0.2\%$. Further confirmation of the absence of noticeable symplastic contamination in the apoplastic fractions was

obtained by protein fingerprint analysis. SDS-PAGE analyses bands of the major proteins in the symplastic fractions of both peach and apricot were almost absent in the respective apoplastic fractions. After SDS-PAGE, the peach symplast showed a major band of ~ 50 kDa, probably corresponding to the Rubisco large subunit, but this was only a minor protein in the apoplastic fraction (Fig. 5A). These data indicate the very low intracellular contamination of the apoplastic fraction, in agreement with data obtained from G6PDH measurements. However, this 50 kDa band was not visible in the apricot symplast. Nonetheless, the method used for apoplast isolation was the same as in peach and, likewise, the G6PDH activity was very low in this fraction. Therefore, it can be assumed that contamination by intracellular constituents was also very low in the apoplastic fraction from apricot leaves.

After corrections for cytosolic contamination, the presence of *p*HMB-sensitive APX (class I APX) and *p*HMB-insensitive APX (class III APX), SOD, POX, NADH-POX, and PPO was described in the apoplastic space from peach and apricot leaves. However, other enzymes from the ascorbate–glutathione (ASC–GSH) cycle (MDHAR, DHAR, and GR) seemed to be absent in this compartment from both peach and apricot leaves.

In the apoplastic space of peach leaves, PPV infection brought about an increase in class I APX (60%), POX (80%), and NADH-POX (100%) as well as a slight increase in PPO activity (21%) (Fig. 1). A rise in class III APX was also produced, although in this case the increase was not

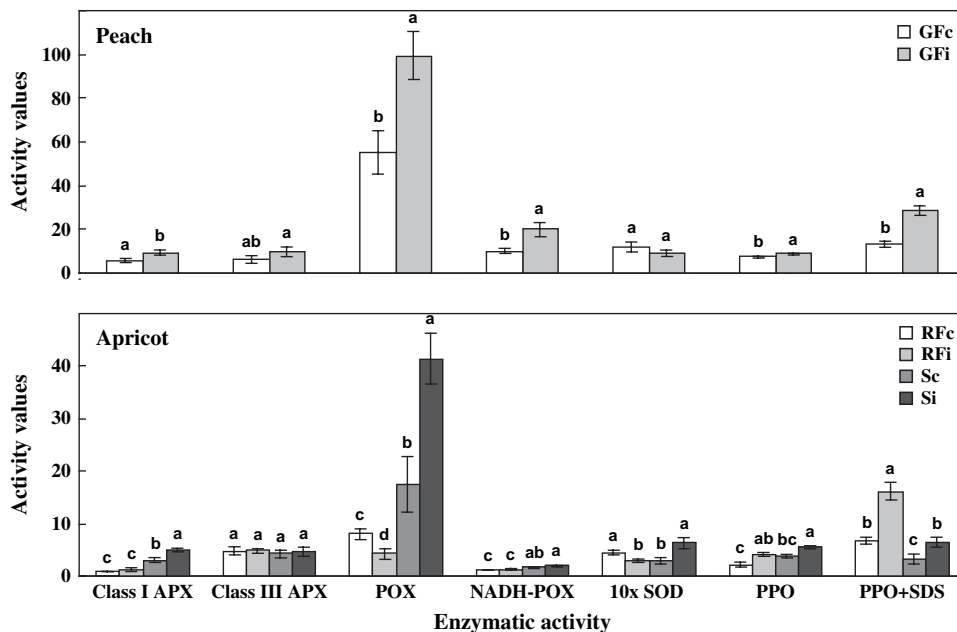


Fig. 1. Effect of PPV infection on the levels of ROS-related enzymes in the apoplastic space of peach and apricot leaves. APX activities are expressed as $\text{nmol min}^{-1} \text{g}^{-1} \text{FW}$. SOD is expressed as $\text{U g}^{-1} \text{FW}$. POX, NADH-POX, and PPO are expressed as $\mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$. GFC, control plants cv. GF305; GFI, infected plants cv. GF305; RfC, control plants cv. Real Fino; RfI, infected plants cv. Real Fino; Sc, control plants cv. SEO; Si, infected plants cv. SEO. Values for the same enzymatic activity with the same letter are not significantly different (LSD, $P < 0.05$).

significant (Fig. 1). PPV infection also produced some changes in the apoplastic space of apricot leaves. In the susceptible apricot cultivar, PPV infection brought about a decrease in apoplastic POX and SOD activities, whereas a strong increase in PPO was observed (93%) (Fig. 1). However, in the resistant cultivar, a rise in class I APX, as well as a strong increase in POX and SOD activities (nearly 100%), was noticed in the apoplastic compartment. In this case, a rise in PPO activity (45%) was also observed, although to a lesser extent than in the susceptible apricot cultivar (Fig. 1).

The effect of PPV infection on the symplastic enzymes from peach and apricot was studied using the leaf residue resulting from the apoplastic extraction. In the peach symplast, PPV infection also produced a strong increase in class I APX, POX (62%), NADH-POX (59%), and PPO activity (45%) (Table 1). In the symplastic compartment from apricot, a differential behaviour was observed depending on the cultivar. In the susceptible apricot cultivar, a decrease in APX and POX, as well as an increase in PPO occurred, whereas, in the resistant apricot cultivar, a strong increase in both class I APX and class III APX was observed with PPV inoculation, whilst no changes were detected in the other enzymes studied (Table 1).

In the PPV-susceptible varieties, under both control and stress conditions, higher PPO activity values were obtained in the presence of SDS (0.02% final concentration). However, in the resistant apricot cultivar, no latent apoplastic PPO activity was observed, in control or PPV-infected plants (Table 1).

The specific inhibitor of NADPH oxidase, diphenylene iodonium (DPI), had only small effects on NADH oxidation, whereas it was inhibited almost completely by 1 mM KCN. In the case of peach plants, DPI did not affect NADH oxidation. However, in non-inoculated apricot plants, DPI inhibited NADH oxidation by nearly 10%, the percentage inhibition being lower in inoculated apricot plants (data not shown). These results suggest that most of the NADH oxidation in the apoplastic space of *Prunus* species is due to an NADH-POX.

PPV infection produced an oxidative stress in the apoplastic space of apricot and peach plants, as observed by the increase in H₂O₂ contents in this compartment (Fig. 2A). However, this increase was much higher in the PPV-susceptible plants (nearly 100%) than in the resistant apricot cultivar (an increase of up to 42%) (Fig. 2A). On the other hand, only in the PPV-susceptible plants was the increase in apoplastic H₂O₂ accompanied by an increase in leaf electrolyte leakage (Fig. 2B). Since electrolyte leakage is caused by lipid hydroperoxidation of the cell membrane (Yoshimura *et al.*, 2004), this parameter is used as a marker of cell damage. However, no changes in the leaf electrolyte leakage were observed in the PPV-inoculated, resistant apricot plants, although a 42% increase in the apoplastic H₂O₂ level was produced (Fig. 2).

Table 1. Levels of ROS-related enzymes in the symplast space of peach and apricot leaves

Treatment	Class I APX	Class III APX	POX	NADH-POX	SOD	PPO	PPO+SDS
Peach cv. GF305							
Control symplast	388±38 b (1.5)	726±145 ab (0.9)	518±30 b (9.6)	14.1±0.5 b (41)	46.4±9.2 ab (2.5)	234±25 b (3.1)	944±18 b (5.5)
PPV-infected symplast	1064±194 a (0.9)	1203±241 a (0.7)	839±52 a (10.6)	22.5±0.3 a (47)	67.4±12.5 a (1.3)	340.7±16.0 a (2.5)	1301±124 a (8.0)
Apricot cv. Real Fino							
Control symplast	35.0±2.5 b (2.4)	146±12 a (3.1)	569±53 a (1.4)	26±3 a (4.2)	53.4±4.1 a (0.8)	61.9±0.5 c (3.3)	95.2±5.1 c (6.6)
PPV-infected symplast	12.3±1.5 d (9.0)	138.2±16.2 a (3.4)	257.3±17.2 c (1.6)	20.6±1.3 a (5.9)	44.2±2.4 b (0.7)	112.2±16.8 ab (3.5)	176.6±30.0 b (8.4)
Apricot cv. SEO							
Control symplast	20.3±4.4 c (4.4)	29.8±7.1 c (7.1)	363.7±8.6 b (4.6)	18.8±1.3 a (8.2)	52.7±2.2 a (0.6)	125.2±6.3 a (2.6)	275.2±8.6 a (1.18)
PPV-infected symplast	69.9±17 a (6.7)	115.3±23.1 b (3.8)	385.0±57.6 b (9.7)	18.4±4.0 a (9.3)	65.17±6.09 a (1.0)	107.7±1.2 b (4.9)	111.2±2.2 c (5.38)

APX activities are expressed as nmol min⁻¹ g⁻¹ FW. SOD is expressed as U g⁻¹ FW. POX, NADH-POX, and PPO are expressed as μmol min⁻¹ g⁻¹ FW. The percentage of activity in the apoplast (with respect to the total present in the whole leaf) is given in parentheses. Values in columns followed by the same letter are not significantly different (LSD, *P* < 0.05).

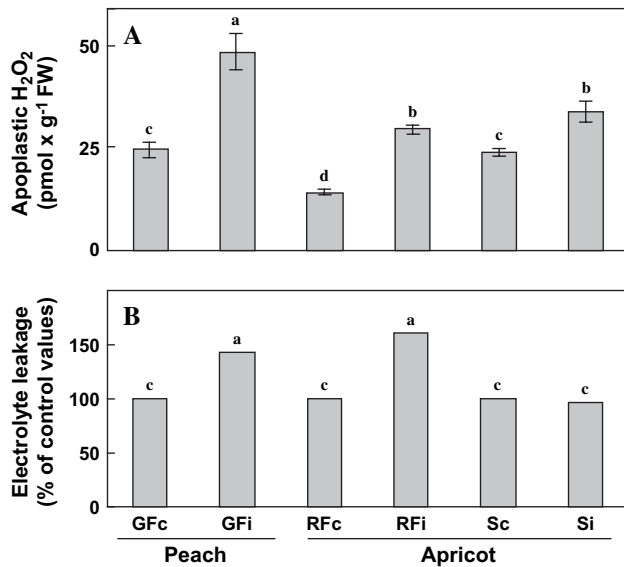


Fig. 2. Effects of long-term PPV infection on leaf electrolyte leakage and leaf apoplastic H₂O₂ contents from peach and apricot plants. Differences from control values were significant at $P < 0.05$ (a), $P < 0.01$ (b), or $P < 0.001$ (c), according to Duncan's multiple range test. GFc, control plants cv. GF305; GFi, infected plants cv. GF305; RFc, control plants cv. Real Fino; RFi, infected plants cv. Real Fino; Sc, control plants cv. SEO; Si, infected plants cv. SEO. Values with the same letter are not significantly different (LSD, $P < 0.05$).

Protein analyses

PAGE and staining of total proteins, POX, and PPO were performed in order to study the effect of PPV inoculation on POX and PPO isozyme expression in the symplastic and apoplastic compartments of peach and apricot leaves.

Native PAGE revealed the presence of four POX activity bands in the symplastic fraction from peach (Fig. 3). This POX isozyme pattern was quite similar to that shown by inoculated peach plants (Fig. 3). In the apoplastic space, five POX activity bands were observed. However, bands were more intense in the apoplastic samples. Moreover, two additional POX bands, with higher mobility ($R_{F,S}$ 0.475 and 0.598), appeared more specifically only in the apoplastic fraction. In the apoplastic space from inoculated plants, an increase in the lower mobility band (R_F 0.230) was observed (Fig. 3).

In the symplast from apricot cv. Real Fino, three POX activity bands were observed, the band with R_F 0.370 being the main one whereas the minor bands had $R_{F,S}$ of ~ 0.132 and 0.220, respectively (Fig. 3). In the apoplastic space, a new POX activity band with low mobility (R_F 0.170) appeared. In the symplast from apricot cv. SEO, five POX activity bands appeared. In the apoplastic space, the lower mobility band (R_F 0.132) was not detected, whereas the bands with $R_{F,S}$ of 0.220 and 0.540 were stronger (Fig. 3). In the apoplast from inoculated SEO plants, the band with R_F 0.170 does not appear. Two apoplastic bands (R_F 0.540 and 0.370) were stronger in the resistant cultivar than in

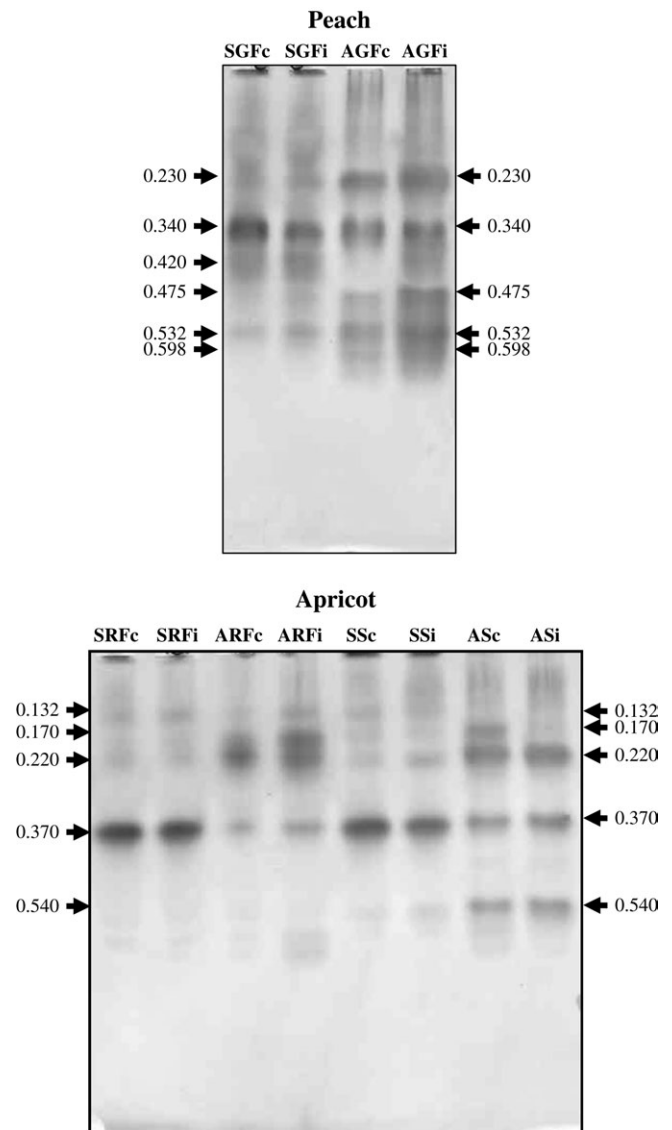


Fig. 3. Effect of PPV infection on the pattern of native peroxidase isozymes from symplastic and apoplastic fractions of peach and apricot. Gels were stained for peroxidase activity with 4-methoxy- α -naphthol (Ros Barceló, 1998). A 10 μ g or 3 μ g aliquot of proteins was loaded for peach and apricot, respectively. SGFc, symplast from control cv. GF305; SGFi, symplast from infected cv. GF305; AGFc, apoplast from control cv. GF305; AGFi, apoplast from infected cv. GF305; SRFc, symplast from control cv. Real Fino; SRFi, symplast from infected cv. Real Fino; ARFc, apoplast from control cv. Real Fino; ARFi, apoplast from inoculated cv. Real Fino; SSc, symplast from control cv. SEO; SSi, symplast from infected cv. SEO; ASc, apoplast from control cv. SEO; ASi, apoplast from infected cv. SEO.

the susceptible cultivar, under both control and inoculated conditions (Fig. 3). On the other hand, results on this gel system do not agree with the results in Fig. 1 regarding POX activity. Probably, gel staining with 4MN is more a qualitative than a quantitative probe.

IEF revealed that most of the POX activity detected in the apoplastic space from both peach and apricot was cationic in nature (data not shown).

Analysis of the isoenzyme pattern of PPO by native PAGE revealed five PPO activity bands in the symplastic fraction from peach. In the upper part of the gels, a low mobility band appeared (R_F 0.082). Below this band, two bands with intermediate mobility were present as well as two additional bands with higher mobility (Fig. 4). PPV inoculation resulted in the disappearance of one of the intermediate mobility bands (R_F 0.140) in the leaf symplast from peach (Fig. 4). In the apoplastic space from peach, six PPO activity bands appeared. In this case, the two bands with the highest mobility (R_F 0.410 and 0.480) were more specific for this compartment, showing an increase in inoculated plants. The gel results emphasize that the increase detected in apoplastic PPO activity in PPV-inoculated plants was also evident from the results obtained by native PAGE (Fig. 4).

PPO activity showed fewer bands in apricot than in peach. In the symplast from apricot cv. Real Fino, native PAGE revealed the presence of three PPO activity bands ($R_{F,s}$ of \sim 0.098, 0.160, and 0.290, respectively) (Fig. 4). The apoplastic space showed basically the same bands, but

the band with intermediate mobility (R_F 0.160) showed a stronger intensity, whereas the band with the highest mobility (R_F 0.290) was barely detected.

In the symplast from apricot cv. SEO, only two PPO activity bands were observed (Fig. 4). However, in the apoplastic space, four PPO activity bands were detected, and three of them ($R_{F,s}$ 0.130, 0.160, and 0.440) seemed to be more specific for this compartment. A band with PPO activity (R_F 0.160) disappears in the apoplastic space from inoculated SEO plants (Fig. 4). However, in apricot, and similar to POX activity, the PPO activity observed in gels does not mirror the total apoplastic PPO activity observed by kinetic analyses (Fig. 1).

SDS-PAGE analysis showed that certain proteins were increased mostly in the apoplastic fraction from PPV-inoculated peach leaves. Specifically, bands of \sim 39, 34, 30, and 18 kDa showed an increase in intensity due to PPV inoculation. In the symplastic fraction, an increase in some polypeptides was also observed in inoculated plants (37, 29, 23, 21, and 18 kDa) (Fig. 5). In apricot, the changes detected were lower in comparison with those observed in peach. In the apoplast from cv. Real Fino, a band of 29 kDa was increased. Similarly, in the apoplastic fraction from cv. SEO, the bands of 43 and 46 kDa were also intensified. In the symplastic fraction from PPV-inoculated 'Real Fino' plants, a decrease in the proteins of 25.8, 26, 23, 18.7, and 18.1 kDa took place, whereas no important changes were detected in the symplast from 'SEO' plants (Fig. 5).

Since the major changes caused by PPV inoculation in the protein pattern determined by SDS-PAGE were observed in peach, 2D protein expression was analysed only in peach. Proteins extracted from the peach leaf apoplast were analysed by 2D gel electrophoresis. To improve the resolution of the 2D maps of the apoplastic proteins, extracts were separated in the first dimension using a linear pH gradient of 4–7. Some of the protein spots resolved by 2D electrophoresis, and after silver staining, were excised and analysed by MALDI-TOF MS. The distribution of protein was substantially different between the apoplastic and symplastic extracts (data not shown). The 2D separation of apoplastic fluid revealed that the majority of the polypeptides in the apoplastic fluid had low isoelectric points, in the range of pI 4–6 (Fig. 6). From the 22 selected spots, and after database searching, some sequence similarity with other proteins was found for only four of them. Spot number 9 showed similarity to thaumatin-like protein from *P. persica* and was induced by PPV inoculation. Spots 16 and 17 showed similarity to the isoforms MDL5 and MDL4, precursors of R-(+)-mandelonitrile lyase (MDL) from *Prunus serotina*, respectively. Finally, spot 21 also showed similarity with MDL from *P. serotina* (Table 2), and a decrease in the intensity of these three last polypeptides was produced in PPV-infected peach leaves. However, most of the

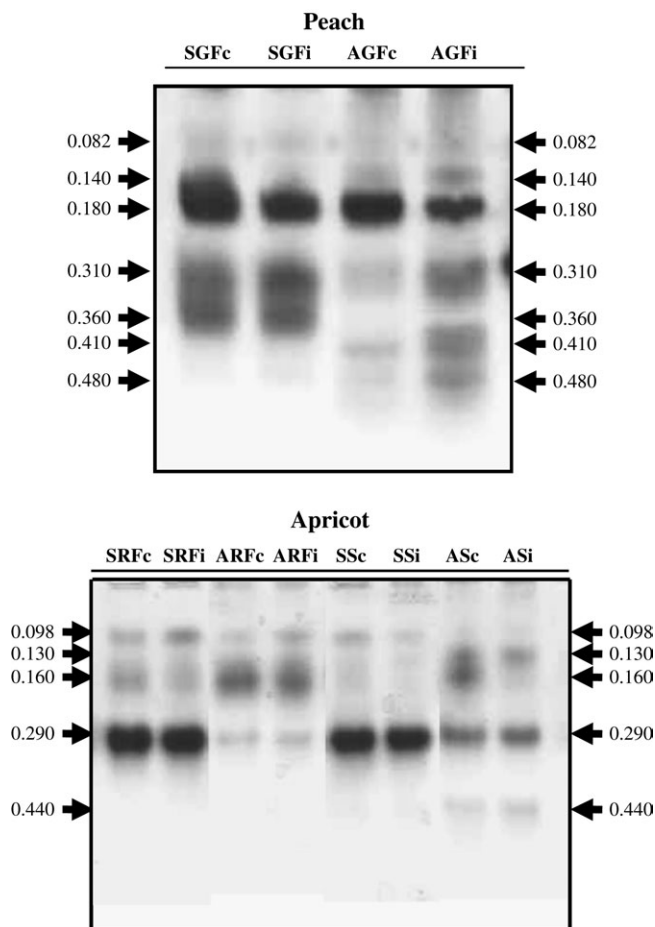


Fig. 4. Effect of PPV infection on the pattern of native PPO isozymes from the symplastic and apoplastic fractions of peach and apricot leaves. Staining of PPO isoenzymes was carried out with 10 mM catechol and 5 mM MBTH (Cantos *et al.*, 2002). For other details, see Fig. 3.

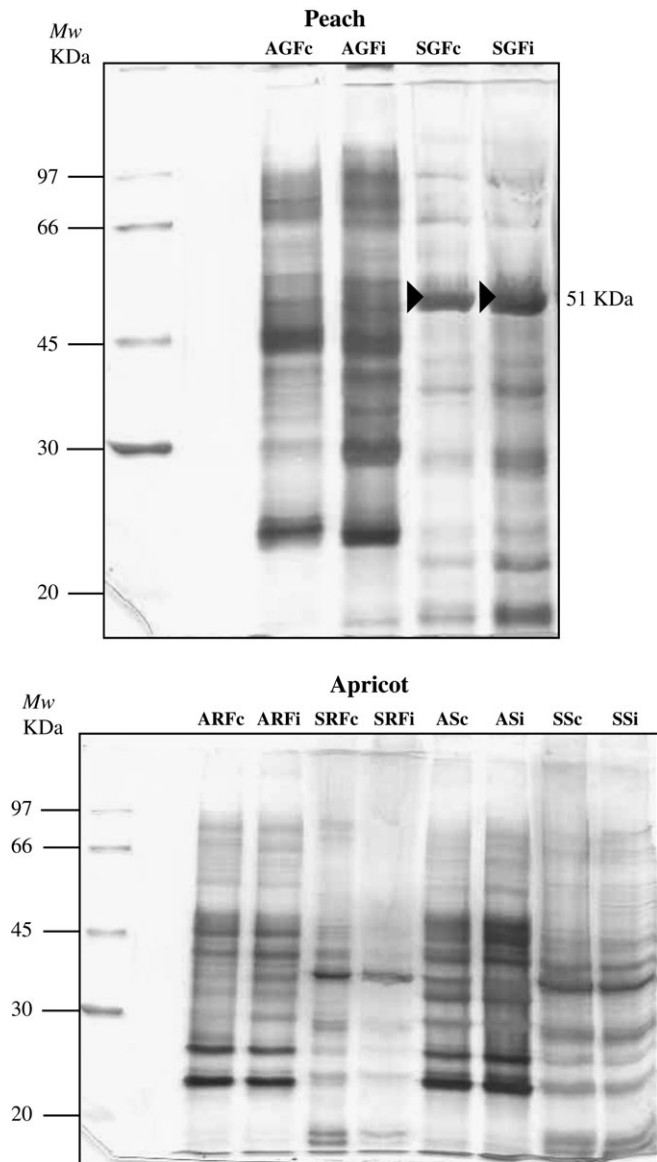


Fig. 5. Fingerprinting of total proteins, resolved by SDS-PAGE, in the symplast and apoplast of peach and apricot leaves. A 7 or 4 μ g aliquot of protein was loaded in gels for peach or apricot, respectively. Gels were silver stained according to Blum *et al.* (1987). For other details, see Fig. 3.

selected spots, from a total of 22, showed no homology with known proteins.

Discussion

The apoplastic space is a cellular compartment little studied, especially in woody plants. Moreover, there is scant information about the apoplastic antioxidant system response to biotic stresses, and this information is non-existent in the PPV-*Prunus* interaction. The level of symplastic contamination in the apoplastic fractions was evaluated by two different approaches: the G6PDH activity and the use of protein fingerprints analyses. Both

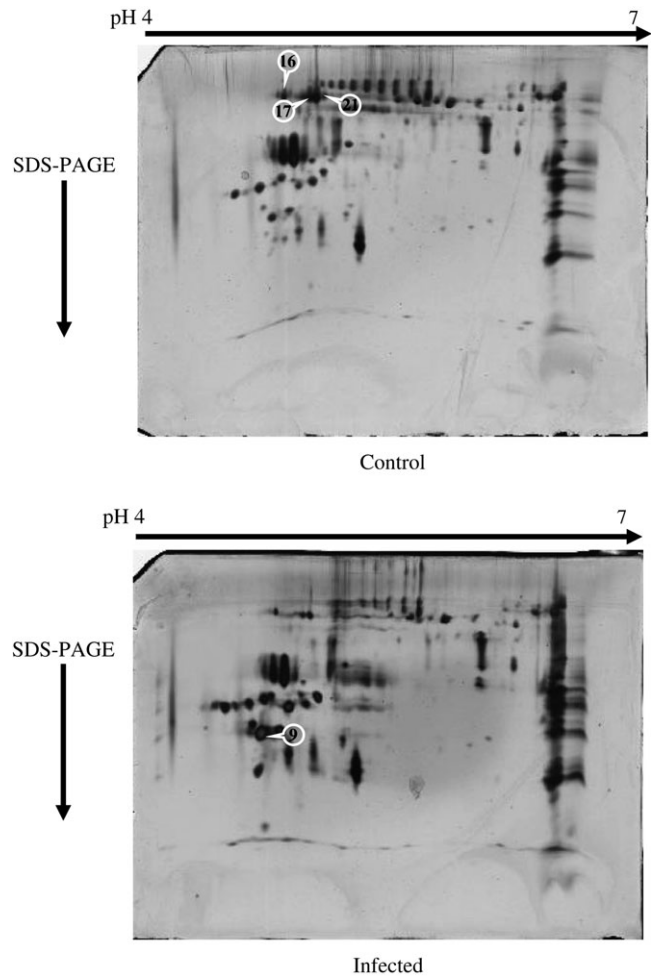


Fig. 6. Reference 2D electrophoretic patterns of apoplast extracts obtained from control and PPV-infected peach plants. Proteins were resolved using a linear pH gradient of 4–7 in the first dimension (25 μ g of proteins) and 12% SDS-PAGE in the second dimension. Protein spots marked with white arrows in the figure were identified by mass spectrometry and database search.

showed that the contamination by symplastic constituents was very low in the apoplastic fractions from both peach and apricot.

In the apoplastic space from peach and apricot, both *p*HMB-sensitive and *p*HMB-insensitive APX were identified. To avoid confusion, *p*HMB-sensitive APX and *p*HMB-insensitive APX should be renamed as class I APX and class III APX, respectively (Ros Barceló *et al.*, 2006). These enzymes have also been identified in the soluble and chloroplastic fractions from apricot and peach (Hernández *et al.*, 2004a, 2006b). In a recent paper, Ros Barceló *et al.* (2006) described class I APX in the apoplastic fraction of *Populus alba* and class III APX in the apoplast of *Citrus aurantium*. However, these authors did not find any APX activities in the apoplastic space from *Zinnia elegans* or *Eucalyptus camaldulensis* (Ros Barceló *et al.*, 2006), and similar results were observed in the apoplastic fraction from mature pea leaves (Hernández *et al.*, 2001).

Table 2. Proteins detected by 2D electrophoresis in the leaf apoplast of peach plants infected with PPV after MALDI-TOF MS

Spot no.	Mascot score	Theoretical M _r	Variation in expression	Accession code	Sequence similarity
9	61	27	Increase	gi/19879966	Thaumatococcus-like protein
16	110	61	Decrease	gi/3676473	R-(+)-mandelonitrile lyase isoform MDL5 precursor
17	144	62	Decrease	gi/4105129	R-(+)-mandelonitrile lyase isoform MDL4 precursor
21	39	61	Decrease	gi/288116	Mandelonitrile lyase

Class I APXs are involved in the removal of H₂O₂ in a reaction strictly dependent on ascorbate (de Pinto and de Gara, 2004). However, class III APXs are the classical secretory plant peroxidases and are responsible for cell wall lignification and other cell wall-stiffening process, which conclude in the maturation of the cell wall (Ros Barceló *et al.*, 2006).

However, the other enzymes of the ASC–GSH cycle, i.e. MDHAR, DHAR, and GR, were absent in the apoplastic space from apricot and peach leaves. These results are in accordance with data described previously for mature pea leaves, where the ASC–GSH cycle enzymes and class III APX were not located in the apoplastic space (Hernández *et al.*, 2001). Nonetheless, in monocotyledonous plants, such as barley and oat, Vanacker *et al.* (1998*a, b*) described the presence of all the ASC–GSH cycle enzymes in the apoplastic space.

In the apoplastic space from peach and apricot, values found for SOD activity are similar to data described for Scots pine needles, and pea, oat, and barley leaves (0.1–2.5% of total activity) (Streller and Winsgle, 1994; Vanacker *et al.*, 1998*a, b*; Hernández *et al.*, 2001). In response to PPV infection, SOD activity only increased in the apoplastic space of the PPV-resistant apricot cultivar (cv. SEO), not in the susceptible cultivars (apricot cv. Real Fino and peach cv. GF305). This suggests a decreased capacity to eliminate O₂⁻ in the apoplastic space of PPV-susceptible cultivars, compared with the PPV-resistant cultivar (cv. SEO), under long-term PPV-infection conditions.

In peach, the increase in apoplastic H₂O₂ correlated with the rise in NADH-POX and PPO activities. Some authors have reported that the presence of H₂O₂ in the apoplastic space is correlated with NAD(P)H oxidation (Bestwick *et al.*, 1998; Ros Barceló, 1998). In sunflower plants exposed to ozone, both the plasma membrane-bound NAD(P)H oxidase complex and cell wall NAD(P)H peroxidases contributed to apoplastic H₂O₂ generation (Ranieri *et al.*, 2003). However, in apricot leaves, no increases in apoplastic NADH-POX were observed, so the increases in apoplastic H₂O₂ were not correlated with NADH oxidation. Furthermore, the quinones produced by oxidation of phenolics can undergo one-electron reduction, forming semiquinone radicals that can produce O₂⁻ and others reactive oxygen species (ROS) such as H₂O₂ (Appel, 1993). PPV infection produced an increase in PPO activity in both cv. Real Fino (100%) and cv. SEO (40%), where

a rise in SOD was also detected. SOD is a H₂O₂-producing enzyme that also may have contributed to the observed increases in apoplastic H₂O₂. Recently, a role for apoplastic SOD in the regulation of H₂O₂, required for the development of the secondary cell wall, has been described (Karlsson *et al.*, 2005).

Class I APX and POX showed a significant increase only in the resistant apricot cultivar, not in the PPV-susceptible apricot cultivar, that showed no changes in class I APX and a reduction in POX in its apoplastic space. However, in peach (also PPV susceptible), an increase in class I APX and POX was also noticed in infected plants. These increases were in parallel with the increases in the apoplastic H₂O₂ levels. In the present work, a possible function for H₂O₂ in the increases in apoplastic class I APX and POX is suggested in peach and apricot plants, although other intracellular signals cannot be ruled out. H₂O₂ is a membrane-permeable molecule that has been demonstrated to function as a diffusible intercellular signal (Karpinski *et al.*, 1999; Neill *et al.*, 2002) and a possible role for H₂O₂ in APX and POX induction has been described previously (Wu *et al.*, 1997; Karpinski *et al.*, 1999; Yoshimura *et al.*, 2000; Hernández *et al.*, 2004*b*).

Although H₂O₂ is scarcely toxic by itself unless at very high concentrations, it can damage membranes, particularly following its reduction to ·OH radicals by transition metals (Fenton reaction). To avoid oxidative damage, apoplastic H₂O₂ must be under control by the antioxidant mechanisms present in the apoplast, including APX and POX activities (Ros Barceló *et al.*, 2006). In this sense, the antioxidant capacity of apricot cv. SEO (PPV resistant) seems to control apoplastic H₂O₂ levels more properly than cv. Real Fino (PPV susceptible), for which a higher increase in H₂O₂ took place. However, in peach cv. GF305 (also PPV susceptible) and as mentioned above, the increase in apoplastic H₂O₂ was also accompanied by a rise in class I APX and POX, but, in this case, the observed increase in these H₂O₂-scavenging enzymes seems to have failed to regulate the apoplastic H₂O₂ levels and did not avoid damage to the membranes. In this respect, the levels of H₂O₂ detected in the apoplastic space of inoculated peach leaves were much higher (nearly 50%) than those observed in both apricot cultivars. Therefore, the H₂O₂ contents are controlled more in the apoplastic space of cv. SEO than in the PPV-susceptible cultivars; this confirms the conclusion that long-term PPV infection produces an oxidative

stress only in susceptible plants (Hernández *et al.*, 2004a, 2006b). This observation was also supported by the fact that only in the susceptible plants did PPV infection produce an increase in electrolyte leakage in leaves. Electrolyte leakage is caused by lipid hydroperoxidation of the cell membranes and is usually associated with disruption of cellular functions (Yoshimura *et al.*, 2004). Hydrogen peroxide and lipid peroxidation, as well as electrolyte leakage measurements, are routinely analysed parameters used to evaluate the extent of oxidative stress in tissues. The observed increases in leaf electrolyte leakage correlated with the increase in apoplastic H₂O₂ and in leaf lipid peroxidation and protein oxidation observed previously in leaves of peach cv. GF305 and apricot cv. Real Fino, both susceptible to PPV (Hernández *et al.*, 2004a, 2006b). Similar observations have been described in leaf tissues from lentil plants under salinity conditions (Bandeoglu *et al.*, 2004) and in tobacco leaves infected with *Pseudomonas syringae* (Mittler *et al.*, 1999). All these data demonstrate that an increase in ROS generation is correlated with membrane damage and is involved in the injuries induced by different stress situations in plants.

In the present work, most of the NADH oxidation in the apoplastic space of *Prunus* species is due to NADH-POX and not NAD(P)H oxidase. Similar results were described by Patkowski and Urbanek (2003) for the apoplastic space of tomato leaves: DPI, a specific inhibitor of NAD(P)H oxidase, inhibited NADH peroxidase activity by only 15%, whereas 1 mM KCN completely inhibited NADH oxidation. It has been reported that the enzyme system which is the major source of ROS in French bean cells appears to be dependent on an exocellular peroxidase according to its relative insensitivity to DPI (Bolwell *et al.*, 2002). Since the NADPH oxidase complex is associated with the plasma membrane (Rao and David, 1999), the present results confirming that most of the NADH oxidation in the apoplast is due to NADH-POX and not NADPH oxidases also support the fact that the symplastic contamination of the apoplastic fractions was very low.

PPV infection induced changes in the protein present in the apoplast, according to the 2D analysis. Only homology with thaumatin-like protein and MDL was obtained. MDL is a flavoprotein involved in the catabolism of (R)-amygdaline. In *P. serotina*, MDL is localized in cell walls and vacuoles (Swain and Poulton, 1994). However, no studies of the subcellular localization of MDL have been conducted in peach leaves. The present results, including the low contamination levels obtained, point out that MDL could be localized in the apoplastic space, although a vacuolar localization should not be excluded. Thaumatin-like protein is a pathogenesis-related (PR) protein that has been shown to have inhibitory effects on the hyphal growth and spore germination of many fungi *in vitro* (Kuwabara *et al.*, 2002). However, information about the changes in thaumatin-like protein in virus-infected plants

is scarce. In the present case, the 2D analysis showed that PPV infection produced an induction of thaumatin-like protein in the apoplastic space from peach leaves. This induction could also be mediated by H₂O₂, the levels of which increased in the apoplast from infected peach leaves. The exact meaning of this response in peach plants is not known, but it can be speculated that thaumatin-like protein, like other PR proteins, forms part of a general response mechanism to unfavourable conditions, by providing protection from an opportunist pathogen attack of the plant in a state of weakness (Dani *et al.*, 2005).

In the present work, most of the selected polypeptides were not identified in database searches. This suggests that most of the functions of the apoplastic space remain unknown, at least in *Prunus*. In the apoplastic space from *Oryza sativa* and *Arabidopsis thaliana*, from the selected spots many of them produced no identification in databases searches. As in the present work, no homologies with antioxidant enzymes were found (Haslam *et al.*, 2003). Also, this in part reflects the paucity of information on the protein present in the apoplastic space, particularly in woody plants such as peach.

As a general conclusion, and regarding the results for antioxidant enzymes as well as for H₂O₂ accumulation and electrolyte leakage, it can be stated that long term-PPV infection produced an imbalance in the apoplastic antioxidant system of susceptible peach and apricot, giving rise to an oxidative stress in their leaf apoplastic space, that may contribute to the deleterious effects produced by PPV infection in leaves from inoculated susceptible plants.

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