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RESEARCH PAPER

Analysis of the antioxidant response of *Nicotiana benthamiana* to infection with two strains of *Pepper mild mottle virus*

A. Hakmaoui[1,*], M. L. Pérez-Bueno[1,*], B. García-Fontana[1], D. Camejo[2], A. Jiménez[2], F. Sevilla[2] and M. Barón[1,†]

[1] Department of Biochemistry, Molecular and Cellular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Profesor Albareda 1, 18008 Granada, Spain

[2] Department of Biology of Stress and Plant Pathology, CEBAS, Consejo Superior de Investigaciones Científicas (CSIC), PO Box 164, 30100 Murcia, Spain

* These authors contributed equally to this work.

† To whom correspondence should be addressed. E-mail: *mbaron@eez.csic.es*

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Abstract

The present study was carried out to investigate the role of reactive oxygen species (ROS) metabolism in symptom development and pathogenesis in *Nicotiana benthamiana* plants upon infection with two strains of *Pepper mild mottle virus*, the Italian (PMMoV-I) and the Spanish (PMMoV-S) strains. In this host, it has been shown that PMMoV-I is less virulent and plants show the capability to recover 21 d after inoculation. Analyses of oxidative stress biomarkers, ROS-scavenging enzyme activities, and antioxidant compounds were conducted in plants at different post-infection times. Only PMMoV-I stimulated a defence response through: (i) up-regulation of different superoxide dismutase isozymes; (ii) maintenance of adequate levels of three peroxiredoxins (2-Cys Prx, Prx IIC, and Prx IIF); and (iii) adjustments in the glutathione pool to maintain the total glutathione content. Moreover, there was an increase in the level of oxidized glutathione and ascorbate in the recovery phase of PMMoV-I-infected plants. The antioxidant response and the extent of oxidative stress in *N. benthamiana* plants correlates to: (i) the severity of the symptoms elicited by either strain of PMMoV; and (ii) the high capacity of PMMoV-I-infected plants for symptom recovery and delayed senescence, compared with PMMoV-S-infected plants.

Key words: Antioxidant defence, biotic stress, *Pepper mild mottle tobamovirus*, ROS, tobamovirus.

Introduction

In some compatible virus–host plant interactions, oxidative stress is manifested as an increase in specific physiological parameters such as lipid peroxidation, protein oxidation, and electrolyte leakage, accumulation of hydrogen peroxide (H_2O_2), and an imbalance in the antioxidative systems at the subcellular level (Clarke *et al*., 2002; Hernández *et al*., 2004, 2006; Díaz-Vivancos *et al*., 2006). Furthermore, some authors have proposed that disturbances in the photosynthetic electron transport chain during viral pathogenesis enhance the generation of reactive oxygen species (ROS) in the chloroplast (Torres *et al*., 2006; Díaz-Vivancos *et al*., 2008).

Abbreviations: ASC, ascorbate; ASC–GSH cycle, ascorbate–glutathione cycle; APX, ascorbate peroxidase; CAT, catalase; CP, viral coat protein; CO-protein, carbonylated protein; 2-Cys Prx, 2-cysteine plastid peroxiredoxin; DAB, 3,3'-diaminobenzidine; D1, PsbA protein PSII reaction centre; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; dpi, days post-inoculation; GR, glutathione reductase; GSH and GSSG, reduced and oxidized glutathione; H_2O_2, hydrogen peroxide; LHCII, major light-harvesting complex of PSII; LOX, lipoxygenase; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; O_2^-, superoxide radical; OEC, oxygen-evolving complex; PAR, photosynthetically active radiation; PMMoV-S and PMMoV-I, Spanish and Italian strains of the *Pepper mild mottle virus*; Prx, peroxiredoxin; PSII, photosystem II; PsbO, PsbP, and PsbQ, 33, 24, and 16 kDa extrinsic proteins of the photosystem II oxygen-evolving complex; ROS, reactive oxygen species; SOD, superoxide dismutase.

The production of ROS is a common feature of incompatible and compatible plant–pathogen interactions (Bolwell *et al*., 1998, 2002; Torres *et al*., 2006). However, little attention has been devoted to resolving the actual role of ROS in symptom development and pathogenesis, in particular in compatible plant–virus interactions (Riedle-Bauer, 2000; Stone *et al*., 2000; Venisse *et al*., 2001). Contradictory results were reported in compatible plant–virus interactions regarding the levels of antioxidant enzymes (Riedle-Bauer, 2000; Hernández *et al*., 2001, 2004; Clarke *et al*., 2002). Clarke *et al*. (2002) proposed that a decline in antioxidant enzymes with a consequent increase in ROS may be necessary for the establishment of infection, replication, and spread of the virus. After a detailed study of resistant and susceptible cultivars of *Prunus armeniaca* inoculated with *Plum pox virus* (PPV), Hernández *et al*. (2006) suggested that ROS could activate defence genes and regulation of antioxidant enzymes could be of importance in determining susceptibility or resistance to the plant viruses. Riedle-Bauer (2000) studied oxidative stress in *Cucumber mosaic virus*-infected *Cucumis sativus* and *Zucchini yellow mosaic virus*-infected *Cucurbita pepo* plants, and concluded that virus-enhanced peroxidation via formation of ROS is involved in the development of both mosaic and yellowing symptoms in virus-infected tissues. In a review, De Gara *et al*. (2003) concluded that alterations in the activities of ROS-scavenging enzymes could be a key step in the activation of the phytopathogenic response.

In previous studies, it was shown that the oxygen-evolving complex (OEC) of photosystem II (PSII) was the target of the *Pepper mild mottle tobamovirus* (PMMoV) in the chloroplast. *Nicotiana benthamiana* plants infected with two strains of PMMoV—Italian (PMMoV-I) and Spanish (PMMoV-S) strains—exhibited an inhibition of PSII from 7 to 21 days post-inoculation (dpi) (Rahoutei *et al*., 2000). The accumulation levels of several chloroplastidic proteins involved in both the photosynthetic electron transport chain and the Benson–Calvin cycle decreased in *N. benthamiana* plants upon infection with PMMoV-S (Pérez-Bueno *et al*., 2004; Pineda *et al*., 2010).

The aim of this work is to gain further knowledge on the role of ROS metabolism in viral pathogenesis. *Nicotiana benthamiana* plants were infected with PMMoV-S and PMMoV-I. PMMoV-S is the most virulent strain, inducing more dramatic symptoms; in contrast, plants infected with PMMoV-I are able to recover from their symptoms. Analysis of oxidative stress biomarkers and quantification of ROS-scavenging capacity by different enzymes and antioxidant compounds were carried out in plants at different times post-infection. The impact of the oxidative stress in the chloroplast during the infection was also investigated by analysis of PSII protein composition. The results suggest that the antioxidant response, and the extent of oxidative stress, correlate with the differences in virulence between the two PMMoV strains analysed. Moreover, the antioxidant response elicited by PMMoV-I could be associated with the capacity for symptom recovery.

Materials and methods

Plants and treatments

Nicotiana benthamiana Gray plants were cultivated in a growth chamber at 100 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation), generated by cool white fluorescent lamps, with a 16/8 h light/dark photoperiod, a temperature of 23 ºC, and a relative humidity of 60–70%. Plants with 6–7 fully expanded leaves were inoculated in the three lower leaves (inoculated leaves), using 25 μl of inoculum per leaf (50 μg ml^{-1} PMMoV protein in 20 mM sodium phosphate/biphosphate buffer, pH 7.0). Mock-inoculated plants treated with virus-free buffer were used as controls. The Italian and Spanish strains of PMMoV were isolated in Sicily (Italy) and Almería (Spain), respectively (Wetter *et al*., 1984; Alonso *et al*., 1989). Leaves were harvested at 7, 14, 21, and 28 dpi.

Visual symptoms are clearly established at 7 dpi with PMMoV-S or PMMoV-I. New leaves which developed after inoculation (S leaves) showed severe wrinkling and curling. No symptoms were observed in those leaves already developed at the time of the inoculation (AS leaves). Inoculated and AS leaves were not analysed in this study. At 14 dpi, stunting of the plants was evident, and symptoms were more severe in PMMoV-S-infected plants. Moreover, PMMoV-I-infected plants 21 dpi developed new non-curly leaves similar to corresponding leaves in control plants, and grew in height, during the so-called recovery phase of the infection (Pineda *et al*., 2008).

Enzymatic assays

All enzyme extractions were performed at 4 °C. Leaves (1 g fresh weight) were homogenized in 2 ml of ice-cold medium containing 50 mM potassium phosphate/biphosphate pH 7.8, 0.1 mM EDTA, 5 mM cysteine, 1% (w/v) polyvinylpolypyrrolidone (PVPP), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.2% (v/v) Triton X-100. For the ascorbate peroxidase (APX) activity assay, 20 mM sodium ascorbate was added to the extraction buffer. The extracts were filtered through two layers of nylon cloth and centrifuged at 8000 *g* for 20 min at 4 °C. The supernatant was then filtered through Sephadex G-50 M PD10 columns (Amersham Pharmacia Biotech., Wien, Austria) equilibrated with the extraction buffer.

Catalase (CAT; EC 1.11.1.6) activity was determined spectrometrically by following the dismutation of H_2O_2 at 240 nm for 1 min, as described by Aebi (1984). Activities of enzymes in the ascorbate–glutathione (ASC–GSH) cycle were determined as described by Jiménez *et al*. (1998).

Isoenzymes of superoxide dismutase (SOD; EC 1.15.1.1; Cu/Zn-SOD, Mn-SOD, and Fe-SOD) were separated by PAGE in 10% (w/v) polyacrylamide gels. SOD activity bands were detected in gels by photochemical nitroblue tetrazolium (NBT) staining according to Beauchamp and Fridovich (1971), loading 100 μg of protein in each well. To identify Cu/Zn-SOD, Mn-SOD, and Fe-SOD isoenzymes, their activities were specifically inhibited by using 2 mM KCN and 5 mM H_2O_2 prior to staining of gels. KCN inhibits Cu/Zn-SODs, while H_2O_2 inactivates both Cu/Zn-SODs and Fe-SODs. The isozyme activities were quantified on an image analyser (GeneTools; Syngene, Cambridge, UK).

Determination of H_2O_2 and oxidative stress parameters

The H_2O_2 concentration in the leaf was determined immediately after isolation using 4-aminoantipyrine and phenol as donor substrates (Frew *et al*., 1983).

The extent of lipid peroxidation in leaves was estimated by quantification of thiobarbituric acid-reactive substances (TBARS) according to Cakmak and Horst (1991). Protein oxidation, quantified as carbonylated protein (CO-protein) content, was measured by reaction with 2,4-dinitrophenylhydrazine, as described by Levine *et al*. (1994) and modified by Prasad (1996).

Lipoxygenase (LOX; 1.13.11.12) activity was assayed by linoleic acid reaction, following the formation of a diene by LOX-catalysed hydroxyperoxidation according to Suurmejier *et al*. (1998).

Ascorbate and glutathione determination

For the extraction of total ascorbate and total glutathione, leaves were homogenized in 5% (w/v) metaphosphoric acid and 10% (w/v) perchloric acid in a 1 mM β-phenanthroline disulphonic acid solution. The resulting acid extract was incubated for 30 min at 4 °C in the dark

and centrifuged at 12 000 *g* for 10 min (Jiménez *et al*., 1997). ASC and DHA (dehydroascorbate) levels in the supernatant were determined by high-performance liquid chromatography (HPLC), as described by Castillo and Greppin (1988). GSH and GSSG (reduced and oxidized glutathione, respectively) derivatization of the supernatant was carried out as previously described (Fariss and Reed, 1987), and HPLC analysis was conducted as described in Asensi *et al*. (1994).

Protein extraction, SDS–PAGE, and western blotting

Frozen leaves from different treatments were ground in liquid nitrogen. Proteins were extracted from 50 mg of leaf tissue ground in liquid N_2 by adding 200 µl of extraction buffer [100 mM Tris–HCl pH 6.8, 5 mM PMSF, 4% SDS, 30% glycerol, 200 mM dithiothreitol (DTT)].

Gel electrophoresis was performed on a 15% (w/v) polyacrylamide gel with a 6% (w/v) stacking gel, loading 30 µg of protein in each well. Western blotting was carried out according to Berzal-Herranz *et al*. (1995). For protein immunodetection, rabbit antisera against the OEC proteins, light-harvesting complex of PSII (LHCII; a kind gift from Dr P. Böger. University of Konstanz, Germany), D1 (Agrisera, Sweden), and peroxiredoxins (Prxs) (anti 2-Cys Prx, Prx IIC, and Prx IIF were gifts from Dr Lázaro Paniagua) were used. The antigen–antibody complex was detected with goat anti-rabbit alkaline phosphatase–IgG (Sigma Aldrich) using NBT/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche) as substrate. Protein was determined with Bio-Rad Protein Assay (Bio-Rad, USA). The western blot filters were photographed in a Chemi Doc XRS (Bio-Rad, USA), and quantification of the bands was carried out by Photoshop CS5 (Adobe).

Statistical analysis

Values presented are means ±SD of at least three replicates. When analysis of variance (ANOVA) showed significant treatment effects, Duncan's multiple range test was applied to compare the means at $P < 0.05$ using SPSS software.

Results

Changes in PSII protein pattern due to viral infection

Figure 1 shows representative results of the immunodetection of the most significant PSII subunits during pathogenesis. Levels of the three OEC proteins (PsbO, PsbP, and PsbQ) were reduced in PMMoV-S-infected plants to different extents relative to the control. At 21 dpi the levels of PsbO and PsbP were reduced to 40% of control values and those of PsbQ to 20%. The accumulation of these three proteins decreased dramatically at 28 dpi. In PMMoV-I-infected plants, however, the OEC protein levels showed a smaller decrease at 21 dpi, down to 70–60% of the control values for PsbO and PsbP, and to 80% in the case of PsbQ. Interestingly, in the recovery phase, the levels of PsbO and PsbP were comparable with those in control plants.

The accumulation levels of the D1 protein decreased to 50% of control levels in plants infected by both viral strains at the end of the infection period. This was accompanied by diminished levels of LHCII, to 25% and 75% of the controls in PMMoV-S- and PMMoV-I-infected plants, respectively. In the recovery phase (28 dpi) of PMMoV-I-infected plants, the content on most of the PSII subunits analysed was similar or even higher than that found at 21 dpi. This contrasts with the further decrease found at 28 dpi in PMMoV-S-infected plants.

Oxidative stress biomarkers

The levels of lipid peroxidation, protein oxidation, and ROS accumulation are oxidative stress parameters commonly used as biomarkers to assess the extent of cell damage produced under different stress conditions.

Malondialdehyde (MDA) is a breakdown product of peroxidation of membrane lipids. Already at 7 dpi the level of MDA increased 3-fold in PMMoV-S-infected plants compared with control values and continued increasing throughout the infection. In plants infected with PMMoV-I, the least virulent strain, the increase in the level of MDA accumulation was lower, ~1.5-fold of the control, and was apparent only at 7 and 14 dpi (Fig. 2A); between 21 and 28 dpi, no significant differences were found with respect to the control.

The LOX activity increased greatly during the infection with PMMoV-S, especially towards the end of the period analysed, reaching a value ~30 times larger than that of the control. In contrast, this activity only showed a small increase (2- to 3-fold) in PMMoV-I-infected plants at 21 and 28 dpi (Fig. 2B).

A remarkably high content of H_2O_2 was observed at 7 and 14 dpi in leaves of PMMoV-S- and PMMoV-I-infected plants relative to the control levels (Fig. 2C). During the recovery phase of plants inoculated with the PMMoV-I strain, the values were comparable with those of the control, whereas leaves of PMMoV-S-inoculated plants showed lower levels at the same number of dpi. These results correlated with the data obtained by 3,3'-diaminobenzidine (DAB) staining of leaf tissue (data not shown).

ROS may cause irreversible oxidation of proteins by carbonylation, resulting in structural and/or functional alterations. In PMMoV-S-infected plants, protein oxidation increases by 60% at 21 dpi, compared with the control, whereas in PMMoV-I-infected plants the increase is only 40%. At 28 dpi, the accumulation of CO-proteins was very high (3- to 4-fold) in plants infected with the PMMoV-S strain; however, in PMMoV-I-infected plants

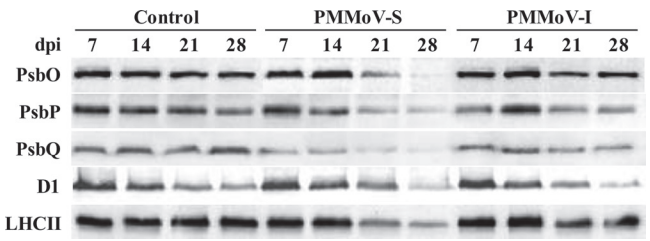

Fig. 1. Western blots of PsbO, PsbP, PsbQ, D1, and LHCII proteins in extracts from control and symptomatic leaves from PMMoV-infected *N. benthamiana* plants carried out at different dpi.

protein oxidation decreased to control levels during the recovery phase (Fig. 2D).

ROS-scavenging capacity

To investigate the role of ROS-scavenging metabolism during pathogenesis in *N. benthamiana* infected with PMMoV, the activities of several antioxidant enzymes were determined. SOD activities were analysed on non-denaturing electrophoresis gels (Fig. 3). Five isozymes were detected: two Mn-SODs (named Mn-SOD I and Mn-SOD II, in order of increasing mobility), one Fe-SOD, and two Cu/Zn-SODs (Cu/Zn-SOD I and II, in order of increasing mobility). The most remarkable change in Mn-SODs was found in PMMoV-I-infected plants, with the enhancement of isoform II activity throughout the infection, reaching 2-fold control values at 28 dpi; the activity of isoform I increased by 1.2-fold only during the last infection steps (21–28 dpi). Fe-SOD and Cu/Zn-SOD I activities decreased throughout the PMMoV-S infection, reaching around 70% and 50%, respectively, of control levels in the last infection steps. In contrast, PMMoV-I induced an increase in Fe-SOD and Cu/Zn-SOD I activities by 2- and 1.5-fold, respectively. Cu/Zn-SOD II activity increased drastically (4- to 6-fold) throughout the infection with PMMoV-I.

The activity of CAT was found to be lower in the infected plants compared with controls (Fig. 4A). In contrast, the APX activities increased from 14 dpi onwards in plants inoculated with both virus strains (Fig. 4B), the increase being highest (~4-fold) in the case of PMMoV-I-infected plants at 28 dpi. Glutathione reductase (GR) activities were lower in plants infected with both virus strains (Fig. 4C) but increased back to control levels at 28 dpi. Monodehydroascorbate reductase (MDHAR) activity (Fig. 4D) showed an increase in PMMoV-S-infected plants from 7 dpi until 21 dpi, and then decreased to reach values similar to control plants at 28 dpi. In PMMoV-I-infected plants, a significant increase in this enzyme activity was only observed at 21 dpi.

Prxs, the most recently identified group of H_2O_2-decomposing antioxidant enzymes, were also investigated during pathogenesis. Representative data obtained by immunodetection of Prxs (2-Cys Prx, Prx IIC, and Prx IIF) are shown in Fig. 5. The infected plants contained less plastid 2-Cys Prx towards the last stages of the infection: 70% of control values at 21 dpi with both viral strains, and 60% and 40% of control values with strains PMMoV-S and PMMoV-I, respectively, at 28 dpi (Fig. 5). The levels of the cytosolic Prx IIC were negligible in the last infection steps with PMMoV-S. In PMMoV-I-infected plants, however, Prx IIC decreased until 21 dpi but increased up to 1.5-fold of the control values in the recovery phase (28 dpi). The accumulation levels of the mitochondrial Prx IIF decreased to 75% of the control at 21 dpi and 40% at 28 dpi during the infection with the PMMoV-S strain, whereas it increased by 1.5-fold at 28 dpi with the PMMoV-I strain.

Ascorbate and glutathione content

A decrease in ASC content was observed only in plants infected with PMMoV-S from 14 dpi (Fig. 6A). However, DHA

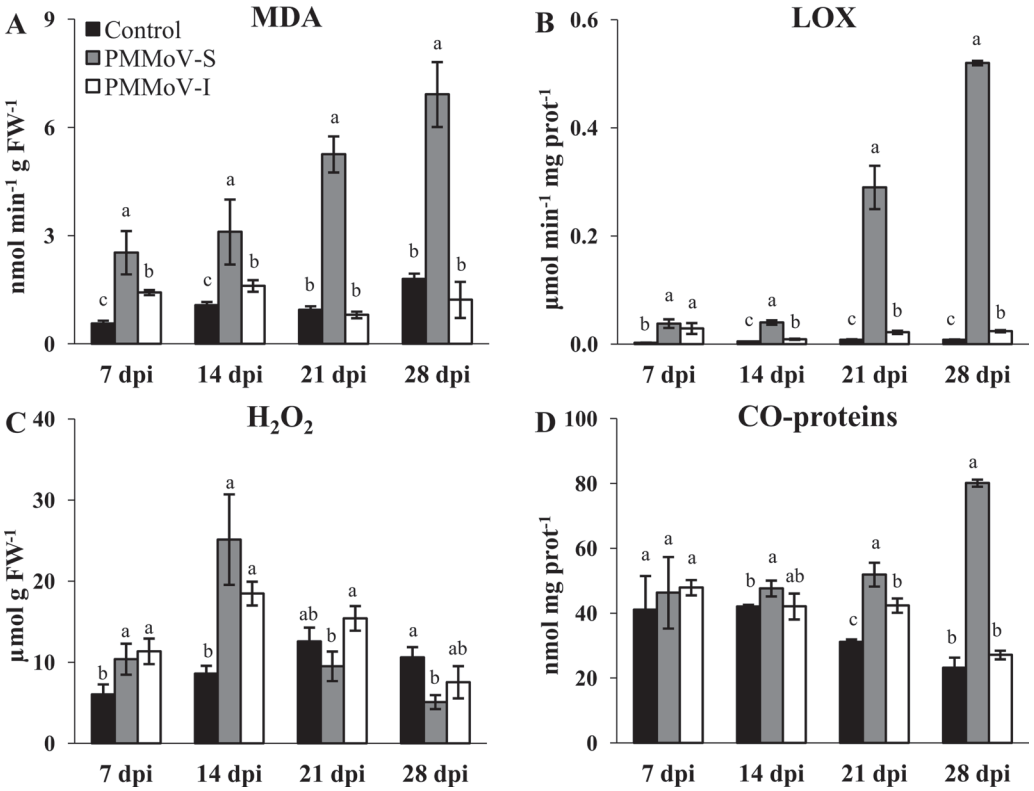

Fig. 2. Changes in MDA content (A), lipoxygenase activity (B), H_2O_2 (C), and carbonylated proteins (D) in control and symptomatic leaves from PMMoV-infected plants as a function of dpi. Different letters in the same block indicate significant differences between means ($P < 0.05$) according to Duncan's test.

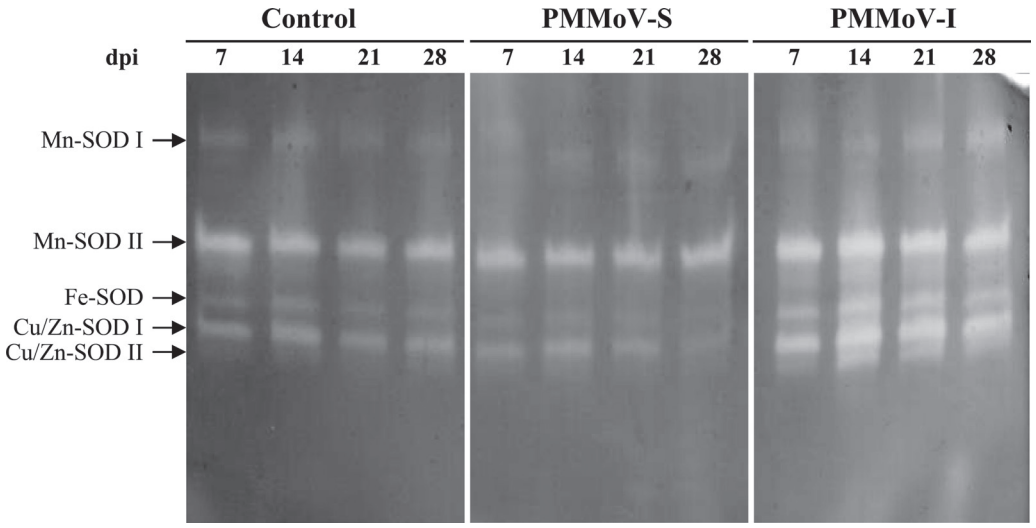

Fig. 3. Separation of SOD isoforms by non-denaturating gel electrophoresis in samples from control and symptomatic leaves from PMMoV-infected *N. benthamiana* plants at different dpi.

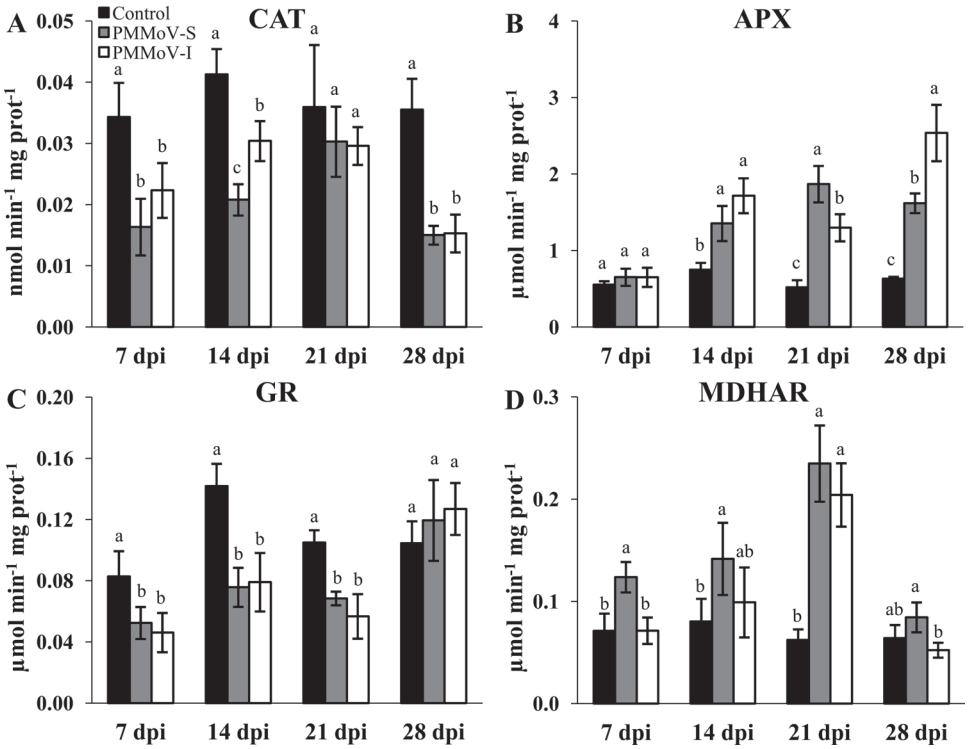

Fig. 4. Changes in enzyme activities of CAT (A), APX (B), GR (C), and MDHAR (D) in control and symptomatic leaves from PMMoV-infected *N. benthamiana* plants as a function of dpi. Different letters in the same block indicate significant differences between means ($P < 0.05$) according to Duncan's test.

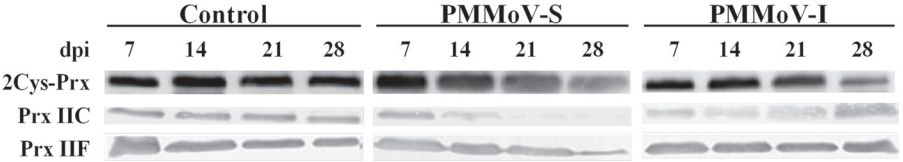

Fig. 5. Western blots of Prx proteins in extracts from control and symptomatic leaves from PMMoV-infected *N. benthamiana* plants carried out at different dpi.

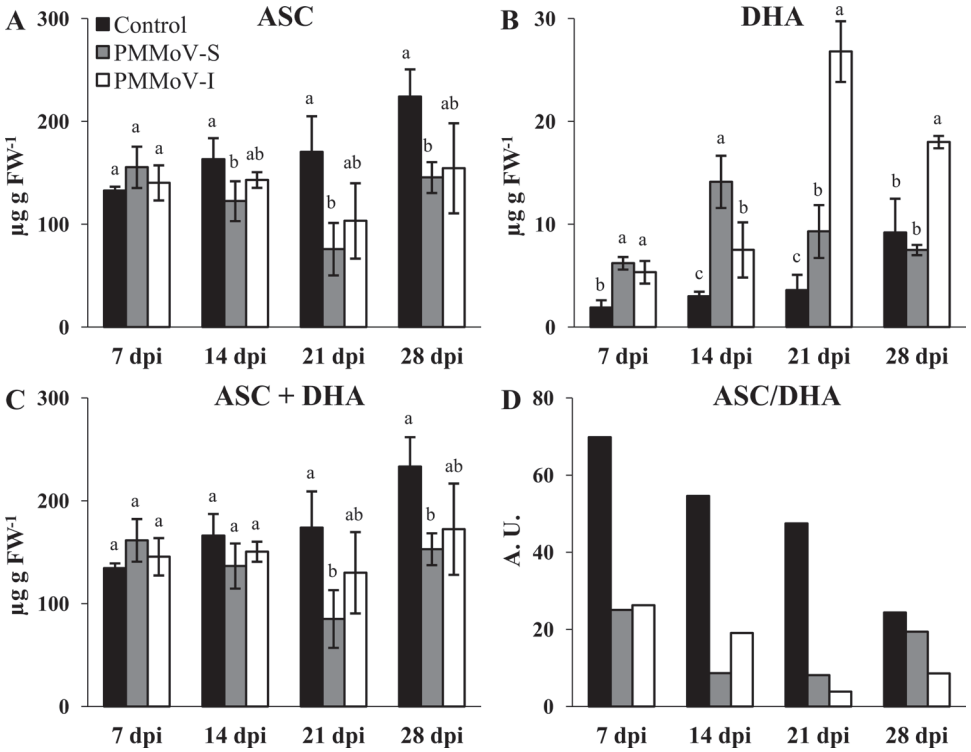

Fig. 6. Quantification of reduced (ASC) and oxidized (DHA) ascorbate (A, B) in control and symptomatic leaves from PMMoV-infected *N. benthamiana* plants at different dpi. (C, D) Ascorbate pool size and redox state. Different letters in the same block indicate significant differences between means ($P < 0.05$) according to Duncan's test.

increased from 7 dpi onwards (Fig. 6B): PMMoV-S-infected plants showed a maximum increase in DHA content, ~4-fold of the control values, at 14 dpi. In PMMoV-I-infected plants, the maximum DHA accumulation, by 7-fold of control values, was reached at 21 dpi. At 28 dpi it remained high in PMMoV-I-infected plants, whereas PMMoV-S-infected plants showed similar values to those of control plants. In consequence, the size of the ascorbate pool (ASC+DHA) was only decreased in PMMoV-S-inoculated plants towards the end of the infection (21 and 28 dpi) and no significant effect was found at any time point in those plants inoculated with the PMMoV-I strain (Fig. 6C). Moreover, the ascorbate pool was more oxidized in plants infected by both viral strains although at 28 dpi PMMoV-S inoculated plants showed values similar to those in the control.

Significant changes in GSH content were observed only in PMMoV-S-infected plants (Fig. 7A). On the other hand, the accumulation level of GSSG was only affected at 28 dpi, increasing slightly in the case of PMMoV-S and drastically in the case of PMMoV-I (Fig. 7B). However, the glutathione pool size was only decreased in plants inoculated with the PMMoV-S strain throughout the infection, and no significant effect was observed in the case of the PMMoV-I strain (Fig. 7C). Interestingly, in PMMoV-S-inoculated plants the glutathione pool is more oxidized at any analysed time point relative to the control. In contrast, the redox state of the glutathione pool was unaffected in plants inoculated with PMMoV-I until 21 dpi and was more oxidized in the recovery phase (Fig. 7D) due to an increase in the levels of GSSG (Fig. 7B).

Discussion

The decrease in PSII electron transport efficiency during PMMoV infection reported by Rahoutei *et al*. (2000) could enhance the production of ROS, such as singlet oxygen ($^1O_2$; Foyer and Harbison, 1994). ROS can oxidize chloroplastidic proteins, especially PSII subunits such as D1 (particularly sensitive to oxidants), and inhibit their *de novo* synthesis, thus inhibiting partial reactions of photosynthesis and eventually leading to further photoinhibition (Takahashi and Murata, 2008). A decrease in the contents of PSII proteins was observed in infected plants (Fig. 1). The extent of the decrease in D1 and OEC proteins during the infection with PMMoV-I, relative to the effects of PMMoV-S, was consistent with the lower virulence of the PMMoV-I strain.

In *N. benthamiana* plants inoculated with PMMoV-S and PMMoV-I, there is an increase in the levels of H_2O_2 (Fig. 2C). Similar effects were reported during infection of *Pisum sativum* with *Plum pox virus* (Díaz-Vivancos *et al*., 2008). Stomatal closure and limitation of CO_2 uptake during PMMoV pathogenesis (Chaerle *et al*., 2006) might drive a photorespiratory burst of O_2^- and H_2O_2 that could be considered as the first line of defence against the pathogen, and could also be involved in oxidative modifications (Malolepsza and Rózalska 2005; Torres *et al*., 2006; Shetty *et al*., 2007). Histochemical H_2O_2 staining (data not shown) confirmed that the accumulation of H_2O_2 is an early event during pathogenesis, being evident even before the symptoms appeared (7 dpi).

Membrane degradation related to lipid peroxidation and protein oxidation is a marker of senescence (Elstner, 1990; Jiménez

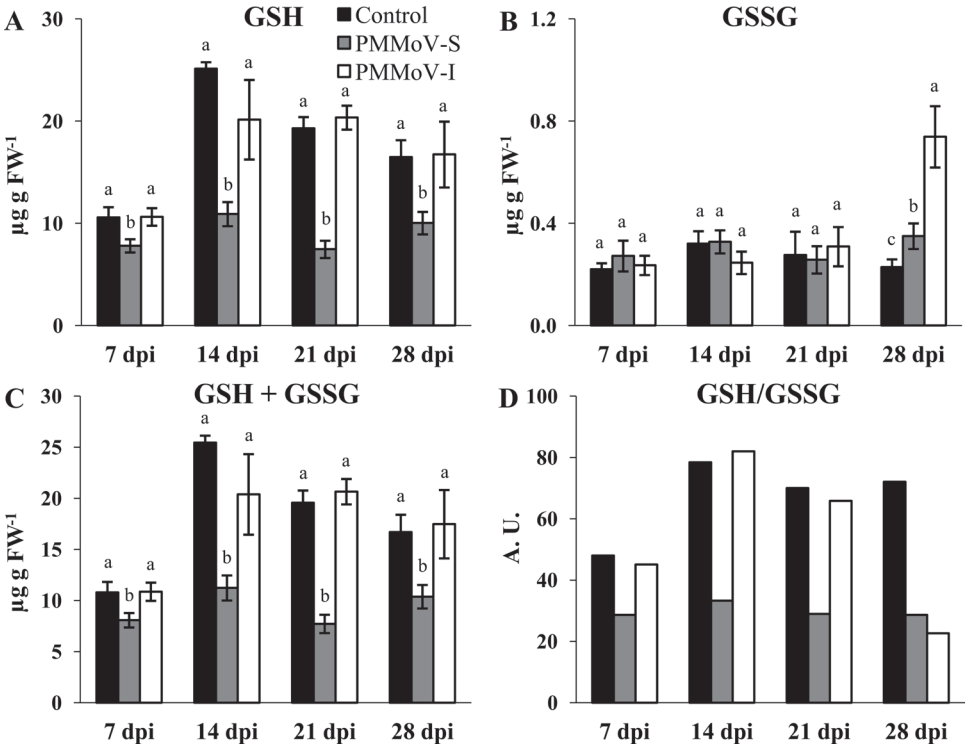

Fig. 7. Quantification of reduced (GSH) and oxidized (GSSG) glutathione (A, B) in control and symptomatic leaves from PMMoV-infected *N. benthamiana* plants at different dpi. (C, D) Glutathione pool size and redox state. Different letters in the same block indicate significant differences between means ($P < 0.05$) according to Duncan's test.

et al., 1998; Vanacker *et al*., 2006). The accumulation levels of LOX mRNA as well as an increase in LOX activity have been reported for several plant–pathogen systems (Koch *et al*., 1992; Melan *et al*., 1993; Gullner *et al*., 2010). In the present work, the dramatic increase in LOX activity during the last stages of PMMoV-S infection may account for the enhanced lipid oxidation. The remarkable rise in oxidation of lipids and proteins in PMMoV-S-infected plants and the substantial increase in LOX activity, especially at the last time points analysed (Fig. 2), together with previous results on lipid peroxidation (Rahoutei *et al*., 1999; Sajnani *et al*., 2007), inhibition of PSII (Rahoutei *et al*., 2000), and the ultrastructure of chloroplasts from PMMoV-infected leaves (Pérez-Bueno *et al*., 2006), are consistent with an accelerated senescence in PMMoV-S-infected plants. In contrast, PMMoV-I-infected plants exhibited slight changes in LOX activity as well as in lipid (7 and 14 dpi) and protein (21 dpi) oxidation. During the recovery phase in PMMoV-I-inoculated plants, the biomarkers of oxidative stress analysed showed values comparable with those of the control (Fig. 2).

Although the primary oxidative burst following pathogen recognition occurs in the apoplast (De Gara *et al*., 2003; Díaz-Vivancos *et al*., 2006; Torres *et al*., 2006), ROS produced in other cell compartments may also play a role in defence. Uncoupling or inhibition of the photosynthetic machinery in the chloroplasts and photorespiration associated with chloroplasts, peroxisomes, and mitochondria can lead to high ROS levels (Torres *et al*., 2006). Plants infected with PMMoV-I showed a higher capacity to eliminate O_2^- than those infected with PMMoV-S, due to higher activities of all SOD isoenzymes, particularly Cu/Zn-SOD II; in contrast, in PMMoV-S-infected plants, all isoenzymes (except Cu/Zn-SOD II) show decreased activities between 7 and 21 dpi. The higher MDA and CO-protein levels measured in PMMoV-S-infected plants could be due to a higher accumulation of O_2^- associated with the decreased SOD activities in different cell compartments. These data are consistent with previous reports suggesting that SODs played an important role in regulating ROS levels in different cell compartments during senescence and viral pathogenesis (del Río *et al*., 1998; Riedle-Bauer, 2000; Hernández *et al*., 2001).

The APX activity is thought to play the most essential role in ROS scavenging. At 14 dpi, the H_2O_2-scavenging capacities of APX and CAT seemed to be overwhelmed, with a consequent increase in foliar H_2O_2 (Fig. 2C). At 21 and 28 dpi, infected plants showed a decrease in H_2O_2 content, with respect to the values at 14 dpi, that could be related to an increase in APX activity (Fig. 4B). The total APX activity analysed here was the sum of contributions by APX isozymes located in the chloroplast (stroma and bound to thylakoid membranes), mitochondrion, microbodies, and cytosol, whereas CAT is found in the mitochondrion, peroxisomes, and cytosol. The expression of APXs increases under different abiotic stress conditions and is related to tolerance (reviewed by Gill and Tutej, 2010). Moreover, the decrease in CAT activity reported here was also found in other host–pathogen systems (Clarke *et al*., 2002; Yi *et al*., 2003; Hernández *et al*., 2006).

The combined rate of ASC production by reduction of DHA plus *de novo* synthesis could not match the rate of ASC oxidation in PMMoV-infected leaves; this resulted in a significant rise in

DHA content and a decrease in the ASC/DHA ratio during the infection with both viral strains. The increase in MDHAR activity was described in other host–virus compatible interactions (Hernández *et al*., 2006). ASC has been linked to leaf senescence (Jiménez *et al*., 1998; Foyer, 2004; Vanacker *et al*., 2006).

Differences between the infections with the two viral strains were most striking in the glutathione pool and the GSH/GSSG ratio. In contrast to PMMoV-S-infected plants, PMMoV-I-infected plants maintained their total glutathione content throughout the infection and the GSH/GSSG ratio at values similar to the controls until 21 dpi; at 28 dpi, GSSG increased dramatically. Modulation of cellular glutathione content has been described to transmit information through diverse signalling mechanisms (Gómez *et al*., 2004); glutathione has been implicated in a mechanism to protect proteins from oxidative damage and regulation of defence-related genes (Mou *et al*., 2003; Pavet *et al*., 2005). Artificial elevation of cellular GSH and activation of GSH-related enzymes can markedly suppress necrotic disease symptoms and in some cases also virus multiplication (Gullner *et al*., 1999).

In addition to CAT and APX, Prxs can also act as H_2O_2-decomposing enzymes in different cell organelles. Prxs reduce alkyl hydroperoxides and peroxynitrite and work as redox sensor proteins (Barranco-Medina *et al*., 2008). The 2-Cys Prx is thought to play a key role in the antioxidant defence of the chloroplast (Dietz *et al*., 2006; Kim *et al*., 2009). Thus, the lower levels of the chloroplastidic 2-Cys Prx during the infection with PMMoV-S could lead to subsequent photoinhibition in the chloroplast. On the other hand, the decrease in the mitochondrial Prx IIF content, found only after 21 and 28 dpi of inoculation with PMMoV-S, may influence the levels of H_2O_2 in this organelle.

In contrast to PMMoV-S, the stable levels of Prx IIF during the infection with PMMoV-I and the observed increase in the cytosolic Prx IIC during the recovery phase could contribute to the delay in the senescence process in the PMMoV-I-infected plants. An up-regulation of Prx IIC protein has been reported under abiotic stress such as high salinity (Horling *et al*., 2003), whereas Prx IIF expression could be rather stable upon abiotic stress conditions (Gama *et al*., 2007). Three Prx proteins in poplar (Prx IIC, Prx IIF, and Prx Q) were affected during compatible and incompatible interactions with two different races of the fungus *Melampsora laricii populina* (Rouhier *et al*., 2004; Gama *et al*., 2007): in the compatible interaction, the content of Prx IIF increased and the content of Prx IIC and Prx Q decreased, in the first hours of infection. The opposite effect was found in the incompatible interaction. Moreover, the expression of 2-Cys Prx did not change upon infection in these experimental systems. The physiological meaning of these variations is still uncertain, but it appears clear that Prxs are important players in plant stress defence and plant–pathogen interactions.

Conclusions

The extent of the oxidative stress in *N. benthamiana* caused by infection with PMMoV correlates to the virulence of the strain. Furthermore, the capacity of PMMoV-I-infected plants to recover from their symptoms contrasts with the early senescence of PMMoV-S-infected plants. Plants inoculated with PMMoV-I, but not with PMMoV-S, exhibited: up-regulation of all SOD isozymes; maintenance of adequate levels of chloroplastidic 2-Cys Prx, cytosolic Prx IIC, and mitochondrial Prx IIF; and adjustments in the glutathione pool to maintain the levels of total glutathione content throughout the infection. A remarkable increase in the GSSG and DHA content was noteworthy in the recovery phase. It is suggested that these combined defence responses allow PMMoV-I-infected plants to recover from the symptoms and delay senescence compared with PMMoV-S-infected plants.

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