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RESEARCH PAPER

Proteomic analysis of pathogenesis-related proteins (PRs) induced by compatible and incompatible interactions of pepper mild mottle virus (PMMoV) in *Capsicum chinense* L^3 plants

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Abstract

Resistance conferred by the L^3 gene is active against most of the tobamoviruses, including the Spanish strain (PMMoV-S), a $P_{1,2}$ pathotype, but not against certain strains of pepper mild mottle virus (PMMoV), termed $P_{1,2,3}$ pathotype, such as the Italian strain (PMMoV-I). Both viruses are nearly identical at their nucleotide sequence level (98%) and were used to challenge *Capsicum chinense* PI159236 plants harbouring the L^3 gene in order to carry out a comparative proteomic analysis of PR proteins induced in this host in response to infection by either PMMoV-S or PMMoV-I. PMMoV-S induces a hypersensitive reaction (HR) in *C. chinense* PI159236 plant leaves with the formation of necrotic local lesions and restriction of the virus at the primary infection sites. In this paper, *C. chinense* PR protein isoforms belonging to the PR-1, β-1, 3-glucanases (PR-2), chitinases (PR-3), osmotin-like protein (PR-5), peroxidases (PR-9), germin-like protein (PR-16), and PRp27 (PR-17) have been identified. Three of these PR protein isoforms were specifically induced during PMMoV-S-activation of *C. chinense* L^3 gene-mediated resistance: an acidic β-1,3-glucanase isoform (PR-2) (M_r 44.6; pI 5.1), an osmotin-like protein (PR-5) (M_r 26.8; pI 7.5), and a basic PR-1 protein isoform (M_r 18; pI 9.4–10.0). In addition, evidence is presented for a differential accumulation of *C. chinense* PR proteins and mRNAs in the compatible (PMMoV-I)–*C. chinense* and incompatible (PMMoV-S)–*C. chinense* interactions for proteins belonging to all PR proteins detected. Except for an acidic chitinase (PR-3) (M_r 30.2; pI 5.0), an earlier and higher accumulation of PR proteins and mRNAs was detected in plants associated with HR induction. Furthermore, the accumulation rates of PR proteins and mRNA did not correlate with maximal accumulation levels of viral RNA, thus indicating that PR protein expression may reflect the physiological status of the plant.

Key words: *Capsicum chinense*, compatible interaction, incompatible interaction, HR-induction, PMMoV, PR proteins.

Introduction

Plants have developed a range of defence mechanisms to respond to viral infection. One of the best characterized resistance responses is mediated by resistance (R) genes. Each R gene confers resistance to a specific pathogen in a gene-for-gene specific way (Flor, 1971; Hammond-Kosack and Jones, 1996). The resistance expression is governed by both R genes and matching pathogen avirulence (Avr) genes. Once direct or indirect specific recognition has taken place, a rapid, strong response is triggered at infection sites. This response is known as the hypersensitive response (HR) and it is characteristic of incompatible plant–pathogen interactions. It involves the induction of programmed cell death (PCD) and the activation of different signal transduction pathways which results in the expression of a variety of defence genes, the appearance of necrotic local lesions, and the restriction of the virus at the primary infection sites (Hull, 2002; Kang *et al*., 2005; Soosaar *et al*., 2005).

Concomitant with HR induction, there is an induction of plant defence proteins, commonly referred to as

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pathogenesis-related (PR) proteins (van Loon and van Kammen, 1970; Bol *et al*., 1990; Linthorst, 1991; Stinzi *et al*., 1993; van Loon *et al*., 1994, 2006). The accumulation of PR proteins is very often associated with a systemic acquired resistance (SAR) against a wide range of pathogens (Ward *et al*., 1991; Ryals *et al*., 1996; Durrant and Dong, 2004). However, this characteristic induction of PR proteins is not exclusive to the HR since generalized induction of defence responses also occurs in compatible interactions (van Loon, 1985; Bol *et al*., 1990; Linhorst, 1991; Jakobek and Lindgren, 1993). Recent plant microarray data have confirmed that differences between susceptibility and resistance are associated with differences in the timing and magnitude of the induced response rather than with the expression of different sets of genes (Whitham *et al*., 2003, 2006; Ishihara *et al*., 2004; Marate *et al*., 2004; Huang *et al*., 2005; Yang *et al*., 2007).

PR proteins were first discovered in tobacco leaves reacting hypersensitively to TMV (van Loon and van Kammen, 1970; Gianinazzi *et al*., 1970). Since then, PRs have been described in plant species from at least 13 families (van Loon *et al*., 2006). Most research on PR proteins has been conducted in tobacco, where five principal groups were first identified, each comprising acidic, extracellular proteins and basic, intracellular proteins (reviewed by Stinzi *et al*., 1993). More recently, new members of PR proteins have been recognized and classified into 17 structurally and functionally distinct families. Some of them have enzymatic activity, such as β-1,3-glucanase (PR-2), chitinase (PR-3, -4, -8, and -11), endoproteinase (PR-7), peroxidase (PR-9), or ribonuclease (PR-10) and possess either antifungal or antibacterial activity (reviewed in Edreva, 2005; van Loon *et al*., 2006). The biological function of PR-1 family proteins remains unclear, although some results indicated that basic tobacco and tomato PR-1 proteins had antifungal activity (Lawton *et al*., 1993; Niderman *et al*., 1995) and more recently, an enhancement of plant resistance against phytopathogenic bacteria and oomycete was observed in both *Arabidopsis thaliana* and *Nicotiana tabacum* plants that overexpressed the basic PR-1 protein from *Capsicum annuum* (Hong and Hwang, 2005; Sarowar *et al*., 2005).

In recent studies of *C. annuum* PR proteins, at least 14 defence-related genes were identified whose expression was induced by *Xanthomonas campestris* pv. *vesicatoria* infection (Jung and Hwang, 2000; Lee and Hwang, 2005) and seven defence-related genes were expressed differentially during HR in pepper leaves inoculated with TMV (Shin *et al*., 2001). However, few comparative studies about differential induction of PR proteins in compatible and incompatible virus interactions have been carried out on plants.

In *Capsicum* spp., the resistance to tobamoviruses is conferred by four seemingly allelic resistance genes (L^1–L^4) with increased effectiveness at the *L* locus (Boukema, 1980, 1982). Correspondingly, tobamoviruses have been classified in terms of increased pathogenicity as pathotypes P_0, P_1, $P_{1,2}$, $P_{1,2,3}$, and $P_{1,2,3,4}$, based on their ability to infect systemically *Capsicum* L^0, L^1, L^2, L^3, and L^4 resistant plants, respectively (reviewed in Gilardi *et al*., 1999; Genda *et al*., 2007). This *L* gene-mediated resistance is expressed as a hypersensitive response (HR) which results in the induction of necrotic local lesions (NLL) and virus confinement at the primary infection sites (reviewed in Gilardi *et al*., 1999).

In this work, a comparative analysis of PR protein accumulation in *C. chinense* PI159236 plants in response to the infection of the Spanish and Italian strains of pepper mild mottle virus (PMMoV-S and PMMoV-I, respectively) (Wetter *et al*., 1984; Alonso *et al*., 1989) is described. *C. chinense* PI159236 is a wild accession harbouring L^3 and *Tsw* genes that conferred resistance against tobamoviruses and tospoviruses, respectively (Boukema *et al*., 1980; Boiteux, 1995). This accession is used as a resistance source for these two viruses in pepper breeding programmes. Resistance conferred by the L^3 gene is active against most of the tobamoviruses including the Spanish strain (PMMoV-S), a $P_{1,2}$ pathotype, but not against certain strains of pepper mild mottle virus (PMMoV), such as the Italian strain (PMMoV-I), a $P_{1,2,3}$ pathotype (Wetter *et al*., 1984; García-Luque *et al*., 1993). Both viruses are nearly identical at their nucleotide sequence level (98%) and the PMMoV-S CP was identified as the effector of the L^3 gene-mediated HR (Berzal-Herranz *et al*., 1995; Gilardi *et al*., 1998).

In this paper, PR proteins induced in *C. chinense* plant leaves are identified and characterized by using proteomic analysis and specific antisera against tomato and tobacco PR proteins. In addition, evidence is presented for a differential accumulation of *C. chinense* PR proteins and mRNAs in the compatible (PMMoV-I)– and incompatible (PMMoV-S)–*C. chinense* interactions.

Materials and methods

Plant material, virus sources, and inoculation procedure

Capsicum chinense N. J. Jacq. PI159236 plants were maintained in growth chambers at 25 °C with a 16 h photoperiod and a light intensity of 8000 lux.

The origins of the tobamoviruses PMMoV-S and PMMoV-I, as well as the chimeric virus PVX-CPS and the parental PVX virus have already been described (García-Luque *et al*., 1990; Gilardi *et al*., 1998; Wetter *et al*., 1984). Virions were also purified as previously described (García-Luque *et al*., 1990).

Carborundum-dusted plant leaves were mechanically inoculated with one of the following: 20 mM sodium phosphate buffer pH 7.0 (inoculation buffer), PMMoV-S and PMMoV-I purified virions diluted in inoculation buffer at a concentration of 50 μg ml^{-1}. In order to express PMMoV-S CP in *C. chinense* leaves without additional PMMoV related sequences other than the elicitor ones, the PVX-CPS chimeric virus was used (Gilardi *et al*., 1998). The

chimeric virus as well as its PVX parental virus were propagated in *N. benthamiana* plants as described (Gilardi *et al*., 1998). To prevent PMMoV-S CP inserted sequence loss due to recombination events (Chapman *et al*., 1992; Gilardi *et al*., 1998), sap extracts from the systemically infected leaves of *N. benthamiana* plants at 5 days after inoculation (dpi) diluted in inoculation buffer, were used to inoculate PVX- or PVX-CPS chimeric virus onto *C. chinense* leaves. The first pair of true leaves at the 2-fully expanded leaf stage was inoculated. Samples were then taken from the inoculated leaves from 1 to 7 dpi at the same time on each day.

Protein extraction and quantification

Acidic soluble protein extracts (ASE) from inoculated leaves were obtained according to the method described by Tobìas *et al*. (1989) with several modifications. Thus, leaves were homogenized in extraction buffer (0.1 M phosphate-citrate buffer pH 2.8) using a pestle and mortar, and 1 ml buffer g^{-1} of leaf tissue. Homogenates were centrifuged for 20 min at 20 000 *g* and the supernatants were dialysed overnight at 4 °C versus 60 mM TRIS–HCl pH 6.8.

Protein extracts from apoplastic fluid (AF) were obtained by infiltrating entire inoculated leaves at 7 dpi with phosphate-citrate buffer pH 2.8 in vacuum and the AF was recovered by centrifugation at 4500 *g* for 20 min. AF extracts were dialysed overnight at 4 °C versus 60 mM TRIS–HCl pH 6.8.

Sample protein content was determined according to the method described by Bradford (1976), using bovine serum albumin (BSA) as a standard.

Electrophoretic analysis

One-dimension analytical SDS-PAGE was performed as described by Laemmli (1970), by using 12.5% and 4.5% polyacrylamide as solving and concentrating gels, respectively. 10 μg of proteins were loaded onto each lane and visualized after staining with Coomassie Blue R250.

Two-dimensional electrophoresis (2-DE) was performed by using the electrophoretic system Multiphor II (Amersham Biosciences, UK). First dimension isoelectric focusing (IEF) was carried out on commercial 11 cm Immobiline DryStrip pH 3–10 (Amersham Biosciences, UK). Proteins from AF extracts (20 μg) were precipitated in the presence of 8 vols of acetone for 1 h at 4 °C. They were then recovered by centrifugation at 10 000 *g* for 4 min. Afterwards, acetone was carefully removed and the protein samples were air-dried for 5 min at room temperature. Pellets were resuspended in 30 μl of sample IEF 3–10 loading buffer by shaking for 30 min at room temperature, and the remaining debris was removed by centrifugation at 18 000 *g* for 3 min at room temperature. Protein samples were cup-loaded near the anode of the IPG strips. Second dimension, SDS-PAGE, was carried out on commercial ExcelGel SDS, gradient 8–18 (Amersham Biosciences, UK). The first and second dimension electrophoresis were performed according to the manufacturer's recommendations. Analytical 2-D gels were stained with either Coomassie Blue R250 or silver nitrate. The analysis was performed in at least three different experiments.

Image processing and analysis

Images of the silver-stained gels were captured by a CCD camera and processed with PDQuest 7.1 software (Bio-Rad, Hercules, CA, USA). Automatic detection of protein spots, matching between control gels and those from PMMoV-infected leaves, as well as comparing the protein profiles of the different analysed gels were performed according to the manufacturer's instructions. The analysis was re-evaluated by visual inspection in order to corroborate the protein spot changes observed between mock and PMMoV-inoculated leaves.

N-terminal and internal amino acid sequencing analysis

After 2-DE, proteins were electroblotted to Immobilon-P^{SQ} Polyvinylidene Difluoride (PVDF)-membrane (Millipore, Bedford, MA, USA), stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol for 1 h and destained with 50% methanol until background coloration had disappeared. The stained protein spots were excised from five to eight membranes to yield sufficient amounts of protein for the sequencing analysis (*c*. 50 pmol of protein). N-terminal amino acid sequencing of PVDF-transferred protein spots was done on an Applied Biosystems Procise Sequencer (Perkin Elmer; Applied Biosystems). Edman degradation was performed according to the standard programme supplied by Applied Biosystems. N-terminal sequences were submitted to BLAST searching of relevant databases for protein identification (Altschul *et al*., 1997; Schäffer *et al*., 2001). Two of the N-terminally blocked proteins were analysed further by internal peptide amino acid sequencing, after trypsin digestion of the PVDF-transferred proteins, by MALDI-TOF mass spectrum and MS-MS or by peptide sequencing using nanospray ion-trap tandem mass spectrometry (nESI-IT MS/MS). The peptide identification search was performed using MASCOT (Matrix Science) (Perkins *et al*., 1999) (Proteomic Laboratory CNIC. Madrid. Spain).

Immunoblot analysis

Proteins separated in either 1-DE or 2-DE were electrotransferred onto nitrocellulose or PVDF membranes. Membranes were incubated with specific antisera raised against the tomato PR proteins: PR-1p14 (PR-1b), β-1,3-glucanases PR-2p29 and PR-2p35, and osmotin-like PR-5p23 (a gift from Dr V Conejero and P Vera) and tobacco PRs: basic β-1,3-glucanase Glu b (PR-2(I)) (a gift from Dr C Castresana) and chitinases PRQ (PR-3b(II)), and chi 32,34 (PR-3c(I); PR-3d(I)) (a gift from Dr Legrand) at the appropriate dilution. Detection of antigen–antibody complexes was carried out with peroxidase-conjugated goat anti-rabbit IgG (Nordic), and the immunoreaction was visualized with either 4-chloro-1-naphthol (Sigma) or with the ECL chemiluminescence kit (Amersham Biosciences, UK).

PR-1 cDNA cloning

The cDNA to *C. chinense* basic PR-1 was obtained by using oligonucleotide 5′-TCACTCAACACAAGCCCAAAA-3′ corresponding to *C. annuum* basic PR-1 (acc. no. AF0 53343) nt 54–74 and SMART™ RACE 3′ (Rapid amplification of cDNA Ends) (CLONTECH Laboratories Inc) method. The 762 bp-long fragment was cloned into the pGEMT easy vector (Promega), sequenced and used as probe for RNA blot analysis.

RNA isolation and northern blot hybridization

Total leaf RNA was isolated according to the method of Logemann *et al*. (1987). Twenty micrograms of total RNA were electrophoresed onto agarose–formaldehyde gels and transferred to Nytran membranes (Schleider & Schuell) in 20× SSC. Blots were prehybridized in 6× SSC, 5× Denhardt's, 30% formamide, 0.5% SDS, 100 μg ml^{-1} salmon sperm DNA at 37 °C for 2 h, and hybridized at either 37 °C or 42 °C overnight in a buffer identical to the prehybridization one but without Denhardt's solution, and supplemented with 10% (w/v) dextran sulphate. The probes used were obtained from cDNA clones of tomato: an extracellular PR-1 (P4) protein, an intracellular basic β-1,3-glucanase GLUB (PR-2(I)), an acidic CHI3 (PR-3(II)) and a basic CHI9 (PR-3(I)) chitinase

(a gift from Dr Van Kan) (Van Kan *et al*., 1992; Danhash *et al*. 1993), an osmotin-like PR-5p23 protein (Rodrigo *et al*., 1993) (a gift from Dr P Vera), and of *C. chinense* basic PR-1 protein. ^{32}P-labelled DNA probes were prepared using the rediprime DNA labelling system (Amersham Biosciences, UK). After hybridization, blots were washed twice in 1× SSC, 0.1% SDS at room temperature and at 42 °C, respectively, followed by a third wash in 0.2× SSC, 0.1% SDS at 58 °C. Northern blots were stripped of radioactive probes for rehybridization according to Sambrook *et al*. (1989). Loading of the lanes was confirmed by ethidiun bromide staining of the RNA.

For viral RNA detection, the clone pT-CPS containing the 593 bp from PMMoV-S CP (Gilardi *et al*., 1998) was used. ^{32}P-labelled riboprobes-specific for plus and minus polarity were obtained after transcription with the T3 and T7 polymerases, respectively. Prehybridization, hybridization, and washes were carried out as in Sambrook *et al*. (1989).

Results

Differential induction of PR proteins in C. chinense *plants infected with PMMoV-S and PMMoV-I*

C. chinense (L^3L^3) plants were inoculated with PMMoV-S and PMMoV-I viruses. The PMMoV-S virus induced necrotic local lesions (NLL) on the inoculated leaves which were visible at 3–4 dpi, being the virus localized at the primary infection site, a typical hypersensitive reaction (HR). By contrast, PMMoV-I evaded L^3 gene-mediated resistance and spread systemically in these plants. PMMoV-I infection did not result in the development of any visible symptoms in the inoculated leaves and symptoms of mottling appeared in the upper non-inoculated leaves from 7 dpi onwards.

In order to characterize PR proteins specifically induced during PMMoV-S-HR activation in *C. chinense* leaves, an approach using 2-DE analysis of proteins obtained from mock-, PMMoV-S-, and PMMoV-I-inoculated leaves was used.

Initially, the optimal time point and the protein extraction method allowing reproducible differences in protein expression among mock- and virus-inoculated leaves were determined by SDS-PAGE analysis. From this assay, it was established that major differences in the protein accumulation pattern from inoculated leaves were observed at 7 dpi in intercellular (AF) protein extracts (Fig. 1; data not shown). At this time point, SDS-PAGE of both total (ASE) and intercellular (AF) (Fig. 1) protein leaf extracts showed that a 15 kDa protein band was detected in both virus-infected leaves. Furthermore, a 14 kDa protein band was only visualized in the HR-inducing virus. Three other protein bands of *c*. 45, 29, and 26 kDa also accumulated differentially in the AF extracts from PMMoV-S inoculated leaves. Therefore, for subsequent 2-DE analysis, AF protein extracts from 7 dpi leaves were used.

Analysis of 2-DE gels (Fig. 2) also showed differential protein expression among virus- and mock-inoculated leaves, as expected from preliminary SDS-PAGE analysis.

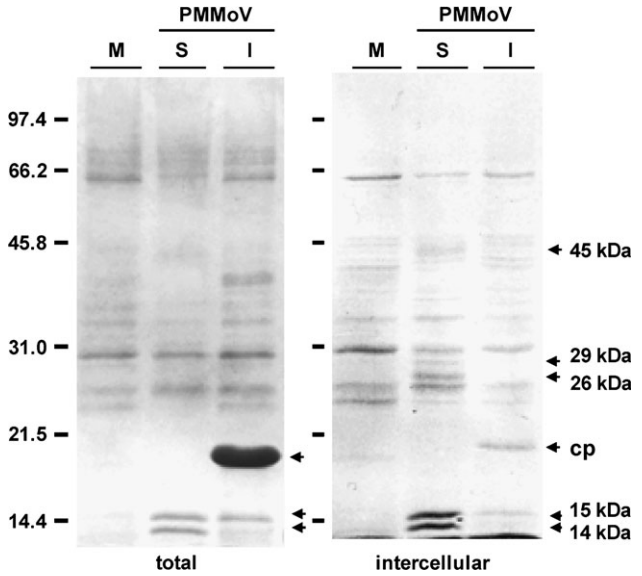

Fig. 1. Differential induction of PR protein in *C. chinense*-infected plants. Coomassie blue staining of SDS-PAGE-separated proteins from total acidic extract (left panel) and intercellular fluid (right panel) from the inoculated leaves from mock-inoculated (M) and infected plants with the S and I strains of PMMoV at 7 dpi. PMMoV capsid protein (cp). Numbers on the left indicate the Mrs from markers. Arrowheads point to the protein accumulated only in infected extracts. Numbers on the right indicate the calculated molecular mass.

The image analysis was performed visually on three different experiments, and with the PDQuest 7.1 application from Bio-Rad in the best resolved gels. Reproducible 2-DE patterns of soluble *C. chinense* AF proteins were obtained by using IEF in pH 3–10 IPG strips and a horizontal 8–18% gradient SDS-PAGE for the second dimension (Amersham Biosciences). This analysis detected a large number of protein spots, 59, in PMMoV-S inoculated leaf AF protein extracts. A lower number of spots, 41 and 27, were observed in PMMoV-I inoculated and control mock-inoculated leaf AF protein extracts, respectively.

The spots that were visually identifiable in the virus-infected extracts, but either absent or present at very low levels in control extracts, were selected for further analysis by N-terminal, as well as internal amino acid sequencing, whenever needed. Some spots common to the three AF extracts from inoculated leaves were also analysed. Selected spots were numbered from 1 to 17 (Fig. 2; Table 1) and it was found that at least six of these spots increased dramatically in the AF extract from PMMoV-S inoculated leaves, where a HR was induced (Fig. 2; Table 1). They corresponded to proteins of 43.9 and 44.6 kDa with pl 5 and 5.1 (spots 1 and 2, respectively), 28.5 kDa with pI 5.9 (spot 6), 26.8 kDa with pI 7.5. (spot 11), 16.2 kDa with pI 7.9 (spot 15) and a 14.7 kDa protein with pI 7.7 (spot 14). From these spots, those corresponding to proteins of 26.8 kDa (spot 11) and 14.7 kDa (spot 14) were only detected in these HR developing leaves (Fig. 2B).

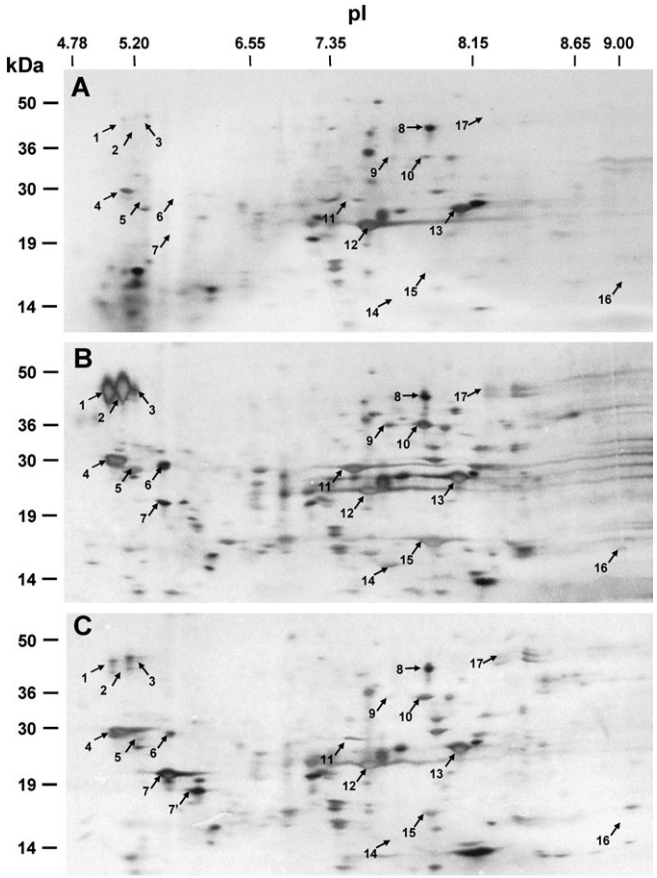

Fig. 2. Representative 2-D gel electrophoresis of apoplastic proteins extracted from mock and PMMoV-infected *C. chinense*-inoculated leaves at 7 dpi. Proteins (20 μg) from mock (A), PMMoV-S (B) or PMMoV-I (C) *C. chinense*-inoculated leaves were separated by 2-DE and stained with silver. The positions of analysed protein spots are denoted by numbered arrows. Arrows in gels A and C are in the corresponding position of gel B. The pI marker positions are indicated by numbers on top of the gel. Numbers on the left indicate the molecular mass markers.

Five of the analysed proteins were identified on the basis of amino acid sequence data obtained by their N-terminal amino acid sequence (spots 2, 10, 11, 12, and 13; Table 1). For the other six spots (spots 1, 4, 6, 8, 14, and 15) N-terminal sequence analysis was unsuccessful, suggesting that mature proteins were blocked at the N-terminus. From these proteins, two of them (spots 4 and 6), were identified from internal peptide sequences obtained from tryptic spot digestion and MALDI-TOF MS and MS/MS or nESI-IT MS/MS.

Database searching of the aa sequences obtained, permitted the identification of the proteins (Table 1). Most of them corresponded to proteins involved in defence and stress responses, such as peroxidases (spot 2), chitinases (spots 4, 6, and 10), and an osmotin-like protein (spot 11).

In order to identify the remaining selected protein spots, 2DE blots were incubated with antisera raised against different tomato and tobacco PR proteins and against PMMoV CP. From these analysis (Figs 2, 3), spots 7 and 7′ were identified as the PMMoV CP, spots 3, 5, and 17 as β-1,3-glucanase (PR-2) proteins; spot 9 as chitinase (PR-3) protein, and spots 14 and 16 as PR-1 proteins. The assay also revealed that PMMoV-S infection induced the accumulation of two PR-1 basic isoforms, of 15 kDa and pI 9 and 18 kDa and pIs between 9.4 and 10, respectively (Fig. 3). These isoforms were not detected in AF extracts from either PMMoV-I or mock-inoculated leaves (Figs 2, 3).

Differential accumulation kinetics of PR proteins in C. chinense *plants infected with the HR-inducer PMMoV-S and the non HR-inducer PMMoV-I*

In order to gain insight into the accumulation pattern of PR proteins in both compatible and incompatible virus interactions the accumulation kinetics of selected proteins were analysed from 1–7 dpi.

For this purpose, total acidic soluble protein extracts (ASE) were obtained at consecutive dpi from either PMMoV-I-, PMMoV-S-, or mock-inoculated *C. chinense* leaves. Protein extracts were electrophoresed, blotted onto nitrocellulose, and incubated with different tomato and tobacco PR protein specific antisera. Protein extracts from buffer mock-inoculated leaves sampled at 7 dpi were used as controls.

Kinetics of pathogenesis-related (PR) protein accumulation in the inoculated leaves of *C. chinense* plants showed a distinct accumulation pattern in incompatible interaction (PMMoV-S) compared to that of the compatible one (PMMoV-I) depending on the PR protein. Thus, the PR-1 protein accumulated at higher levels as early as 2 dpi. This protein was also induced in PMMoV-I inoculated leaves, but it accumulated to a lower extent throughout the infection (Fig. 4). A similar accumulation pattern was observed for the PR-2 (β-1,3-glucanase) immunoreacting with the anti-tomato PR-2p29 protein, as well as for the band immunoreacting to the tobacco basic PR-2 Glu b antisera. By contrast, the accumulation of the PR-2 β-1,3-glucanase band immunoreacting to the tomato PR-2p35 protein immunoserum was only detected during the outcome of the incompatible interaction, though a noticeable increase was observed throughout the time of the infection.

On the other hand, both acidic and basic chitinase PR-3 bands and PR-5 accumulated to a similar extent in both compatible and incompatible interactions and were detected from day one on. A steady increase in these proteins for at least 5–7 dpi was observed and the PR-5 accumulation pattern was rather complex, showing a steady increase at earlier stages of infection, the time of the increase depending on the viral strain, followed by a decrease and then a further increase.

Table 1. *Protein spots identified in* C. chinense *plants infected with either the S (incompatible) or I (compatible) strains of PMMoV, and accumulation ratio in PMMoV-S-infected versus mock-inoculated control plants, PMMoV-I-infected versus mock-inoculated control plants, and PMMoV-S-infected versus PMMoV-I-infected plants*

Spot no.	Protein/accession no.	Calculated M_r/pI	Identified peptides[a]	Cross-reacting antisera	Ratio PMMoV-S-infected versus control	Ratio PMMoV-I-infected versus control	Ratio PMMoV-S-infected versus PMMoV-I infected
1	NI	43.9/5.0	Blocked^{N}		35.75	8.91	4.01
2	Peroxidase PR-9/ CAA50597.1	44.6/5.1	ATFYASTC^{N} L		–[b]	–	3.69
3	Acidic β-1,3 glucanase PR-2	44.2/5.2	ND	Anti-tomato PR-2p35	(219.61)[c]	-	–
4	Acidic chitinase PR-3(II)/gil19187	30.2/5.0	EGNQMGSGFGR^{I}		2.94	4.71	0.63
5	Acidic β-1,3 glucanase PR-2	26.6/5.2	ND	Anti-tomato PR-2p29	–	–	2.94
6	Endochitinase PR-3(II)/P27054	28.5/5.9	LECDGANPQTVAR^{I}		–	–	1.54
7	PMMoV CP	19.3/5.9	ND	Anti-PMMoV CP	–	–	0.30
7′	PMMoV CP	16.1/6.1	ND	Anti-PMMoV CP	–	–	0.34
8	NI	38.7/7.9	Blocked^{N}		2.56	1.26	2.06
9	Basic chitinase PR-3(I)	33.3/7.7	ND	Anti-tobacco PR-3(I)(P32-34)	–	–	13.25
10	Endochitinase PR-3(I)/P52405	33.5/7.9	EQCGSQAGGALCAPG^{N}	Anti-tobacco PR-3(I)(P32-34)	6.05	2.23	2.72
11	Osmotin-like protein PR-5/CAC34055.21/ AF297646.1	26.8/7.5	AKFDVRNNCDYTVwAASTPV^{N}		(359.36)[c]	–	–
12	Germin-like protein PR-16/ AB112080.1	22.2/7.6	AVLDFCVG^{N}		0.5	0.37	1.35
13	NtPRp27-like protein PR-17/ AY185207.1	23.6/8.1	VDYSVTNTASNTPGG^{N}		3.98	3.68	1.08
14	PR-1 protein	14.7/7.7	Blocked^{N}	Anti-tomato PR-1p14	(52.15)[c]	–	–
15	NI	16.2/7.9	Blocked^{N}		–	–	2.98
16	PR-1 protein	15.0/9.0	ND	Anti-tomato PR-1p14	(9.78)[c]	–	–
17	Basic β-1,3 glucanase PR-2	40.0/8.3	ND	Anti-tomato PR-2p29	–	–	0.87

[a] N, Amino terminal peptide; I, Internal peptide; NI, Non-identified; ND, non-determined.
[b] Spots not detected in control mock-inoculated plants.
[c] Ratio of spots only detected in PMMoV-S-infected AP extracts versus background.

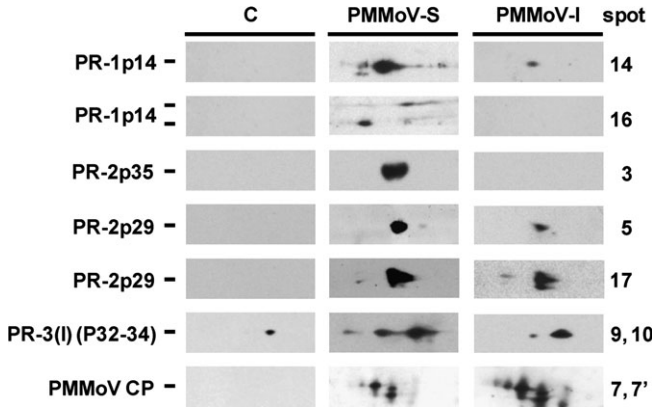

Fig. 3. Western blot detection of PR proteins on 2-DE gels of intercellular fluid extracted at 7 dpi from mock and PMMoV-S or PMMoV-I *C. chinense*-inoculated leaves. 2-D blots were reacted with the specific antisera indicated on the left of the figure: tomato basic PR-1p14 protein, β-1,3-glucanases PR-2p35 and PR-2p29. Basic (b) chitinases were detected with tobacco PR-3(I) (P32–34) antiserum. PMMoV CP was detected by using PMMoV specific antiserum. The number of the corresponding protein spot is indicated on the right. The antisera used are indicated on the left of the figure.

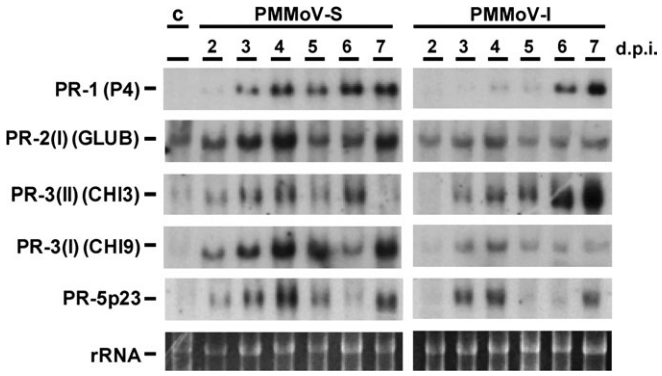

Fig. 5. Accumulation kinetics of PR gene expression in PMMoV-S- and PMMoV-I- inoculated leaves from *C. chinense* plants. Total RNA (20 μg) from PMMoV-S- and PMMoV-I-inoculated *C. chinense* leaves and mock-inoculated ones were analysed by northern blot at the times indicated at the top of the figure. Blots were hybridized to tomato specific probes against a basic PR-1 (P4); a basic β-1,3-glucanase PR-2(I) (GLUB), an acidic PR-3(II) (CHI3) and a basic PR-3(I) (CHI9) chitinase (Van Kan *et al*., 1992; Danhash *et al*., 1993), and a PR-5p23 gene (Rodrigo *et al*., 1993). rRNA corresponded to ribosomic RNA stained with ethidium bromide and used as a loading control.

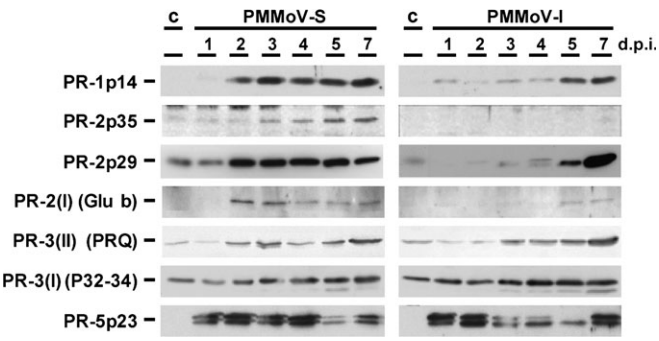

Fig. 4. Accumulation kinetics of PR proteins in PMMoV-S or PMMoV-I *C. chinense*-inoculated leaves. Total protein extracts (10 μg) from *C. chinense* leaves inoculated with buffer (c), PMMoV-S and PMMoV-I were extracted at the times indicated at the top of the figure. Mock-inoculated plants were assayed at 7 dpi. Proteins were separated in SDS-PAGE, transferred to PVDF membranes and reacted with the specific antisera indicated on the left of the figure, as in legend to Fig. 3. PR-2(I) (Glu b): tobacco basic β-1,3-glucanase antiserum. PR-3(II) (PRQ): tobacco acidic chitinase PRQ antiserum. PR-5p23: tomato osmotin-like PR-5p23 antiserum.

Differential accumulation kinetics of PR protein mRNAs in C. chinense *plants infected with the HR-inducer PMMoV-S and the non-HR-inducer PMMoV-I*

To compare the accumulation kinetics of pepper PR proteins with the time-dependent expression of their corresponding mRNAs, the total RNA from PMMoV-S and PMMoV-I inoculated leaves were isolated daily from 2–7 dpi and the RNAs were analysed by northern hybridization using specific probes of PR mRNAs (Fig. 5).

The accumulation pattern of the mRNAs corresponding to the PR-1, PR-2(I) (GLUB), PR-3(I) (CHI9), and PR-5 was different for PMMoV-S and PMMoV-I-infected leaves. Thus, the mRNA detected by the tomato PR-1 probe accumulated faster and at higher levels in PMMoV-S inoculated leaves than in the PMMoV-I ones. In the PMMoV-S inoculated leaves, the PR-1 mRNA increased rapidly from 2 dpi up to 7 dpi, the last time point assayed. However, in the PMMoV-I inoculated leaves PR-1 mRNA could only be detected from 3 dpi onwards, and to a lower extent. No expression was detected in mock inoculated leaves at 7 dpi.

A similar accumulation pattern was observed among the PR-2(I) (GLUB), PR-3(I) (CHI9), and PR-5 mRNAs. In the three cases, an increased accumulation from 2–3 dpi up to 4 dpi, was observed at the time at which the HR was visualized, then decreased, and peaked at 7 dpi. Furthermore, the accumulation levels were higher for the incompatible interaction, and in the case of PR-3(I) (CHI9) and PR-5 mRNAs an earlier detection (2 dpi) was observed (Fig. 5) in the PMMoV-S-infected leaves. The accumulation pattern for these mRNAs is different from that observed in the accumulation of their corresponding proteins (Fig. 4), indicating that, besides their transcriptional regulation, other factors such as PR protein stability and antisera specificity could account for the accumulation pattern observed.

By contrast, and in accordance with the data obtained in the western blot analysis (Fig. 4), the accumulation of the PR-3(II) (CHI3) mRNA was similar in both compatible and incompatible interactions, albeit being detected earlier (2 dpi) in the incompatible one.

It is noteworthy to point out that, in control plants, the PR-2(I) (GLUB), and PR-3(I) (CHI9) mRNAs were also detected, although to a lesser extent than in infected plants.

Cloning and analysis of the C. chinense *basic PR-1 mRNA*

The presence of specific proteins immunoreacting to the basic tomato PR-1p14 antisera only during the induction of the HR (Fig. 3), prompted the isolation of the cDNA to the *C. chinense* basic PR-1 protein, to corroborate that this isoform of the protein accumulates specifically during the elicitation of the HR in this host.

Based upon the nucleotide sequence of a previously published sequence of a basic PR-1 from *C. annuum* (Kim and Hwang, 2000), an oligonucleotide was used to clone the basic PR-1 from *C. chinense*, by using the RACE 3′ method. The 762-bp-long cDNA fragment was sequenced and it was found to encode a 162 long-polypeptide, highly homologous to that from *C. annuum* and to a lesser extent to the basic and acidic isoforms of tobacco and tomato plants, respectively. Thus, its sequence was 99% identical to the *C. annuum* PR-1 protein (accession no. O65157; Kim and Hwang, 2000); 91% and 86% to *N. tabacum* PR-1 proteins PRb-1b (accession no. Q04106; Eyal *et al.*, 1992) and PRP1 (accession no. P11670; Payne *et al.*, 1989), respectively, and 76% to *S. lycopersicum* PR-1a1 (accession no. Q08697; Tornero *et al.*, 1994). By contrast, though the identity of the *C. chinense* PR-1 sequence at the nucleotide level with that from *C. annuum* was 98%, it was lower for those from tobacco and tomato, sharing sequence identities of 76% and 77%, respectively.

Basic C. chinense *PR-1 (PR-1b) is specifically accumulated during viral HR induction*

To corroborate that the accumulation of the *C. chinense* basic PR-1 protein was specific to the HR induction, a similar experiment was carried out with extracts from *C. chinense* leaves inoculated with either the systemically infecting PVX or the chimeric HR-inducer PVX-CPS viruses, that permitted the expression of the elicitor of the L^3-mediated resistance in *C. chinense* leaves (Gilardi *et al.*, 1998). As shown in Fig. 6A, the more basic PR-1 immunoreacting spots were only detected in the incompatible interaction, but not in the compatible one.

To assess whether this induction was observed at the transcriptional level, northern blot analysis was carried out on the total RNA extracted from PMMoV-infected leaves on several post-inoculation days by using the *C. chinense* basic PR-1 clone described above.

The assay (Fig. 6B) revealed that in the PMMoV-S infection the PR-1b mRNA accumulated from 2 dpi on with a noticeable increase at 4 dpi, and then decreased up to 6 dpi. By contrast, a faint band was only detected in the PMMoV-I-infected leaves at 4 dpi.

To determine whether this time-point did reflect major changes in viral accumulation, viral RNAs were analysed from 1 dpi up to 7 dpi by using specific probes for viral RNA of both polarities (Fig. 6C). The data revealed that, in the PMMoV-S-infected leaves, a major accumulation of RNA was detected at 4 dpi and 7 dpi. However, in the PMMoV-I infected leaves, the accumulation levels of viral RNA at 4 dpi were much lower than at later post-inoculation days (Fig. 6C). Thus, the data indicated that the accumulation of *C. chinense* basic PR-1 mRNA was not correlated with the viral content of the leaves, but might be associated with the physiological status of the plant instead.

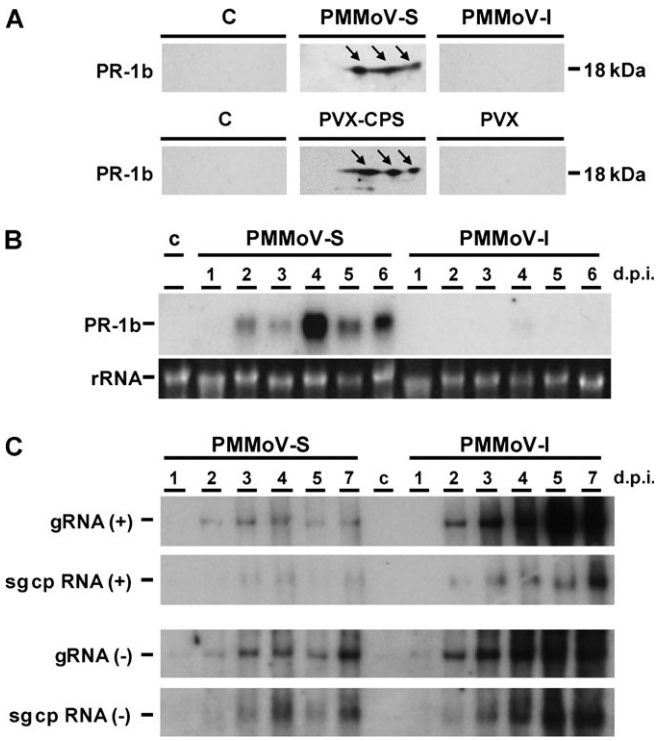

Fig. 6. (A) Western blot detection of basic PR-1b protein on 2-DE gels of intercellular fluid extracted at 7 dpi from *C. chinense* leaves inoculated with the HR-elicitor viruses PMMoV-S and chimeric PVX-CPS and the non HR-elicitor viruses PMMoV-I and PVX. 2-DE blots were incubated with tomato basic PR-1p14 protein specific antisera. Only the area of the gel corresponding to the IP of *c*. 10 is shown. (B) Accumulation kinetics of basic PR-1b mRNA in *C. chinense* leaves inoculated with buffer (c), PMMoV-I and PMMoV-S. Total RNA was extracted at the times indicated at the top of the figure. Control RNA was extracted at 7 dpi. Blots were hybridized to the basic PR-1 *C. chinense* probe. rRNA corresponded to ribosomic RNA stained with ethidium bromide and used as loading control. (C) Accumulation kinetics of viral RNAs in the inoculated leaves of *C. chinense* plants. 20 μg of total RNA extracted at the times indicated at the top of the figure were assayed by northern blot analysis and hybridized to specific probes for viral RNA of plus and minus polarity. C, control plant. gRNA, genomic RNA; sg cp RNA, subgenomic coat protein mRNA.

Discussion

The infection of *C. chinense* plants by either the incompatible PMMoV-S or the compatible PMMoV-I viruses is associated with dramatic changes in the protein pattern of the host. These changes are more prominent in

the apoplastic fluid (AF) at 7 dpi, as revealed by the SDS-PAGE analysis carried out. In these analysis, the presence of several protein bands (45, 29, 26, and 14 kDa) was observed associated with the incompatible interaction, and an additional band of 15 kDa was present in both the compatible and incompatible interactions, albeit at lower levels. As assayed here, the proteomic analysis of the AF protein extracts of *C. chinense* leaves was complementary to the SDS-PAGE analysis and also to both protein and mRNA expression analysis as shown in previous studies (Canovas *et al*., 2004; Sappl *et al*., 2004; Ingle *et al*., 2007). The protein changes observed in the SDS-PAGE analysis were corroborated by the 2-DE analysis and the differences in the protein profiles of the three treatments were enhanced in this assay, so that the number of protein spots differentially detected in PMMoV-S AF protein extracts was higher than the number observed in SDS-PAGE analysis. It was observed that out of the 59 protein spots detected in PMMoV-S AF protein extracts, 13 and 24 were not detected in either PMMoV-I or control AF extracts, respectively.

Of the 17 protein spots selected for further characterization, 15 were successfully identified, either by inmunospecific detection analysis or protein sequence analysis. Two of them, spots 7 and 7′, were identified as PMMoV CP, thus suggesting that proteins other than extracellular proteins were extracted in AF protein extracts. A cytoplasmic contamination of AF protein extracts could not be ruled out. Nevertheless, because tobamoviruses accumulate in leaf veins and petioles (Hull, 2002), it is also possible that vascular exudates containing PMMoV CP protein could be released during the extraction procedure. Nine of the protein spots, besides the viral coat protein and an unidentified protein (spot 15) were induced *de novo* after viral infection with either the HR-elicitor virus or both viruses, being absent in the extracts from control mock-inoculated plants. Most of these spots (5) corresponding to peroxidase PR-9 (spot 2), acidic β-1, 3-glucanase PR-2 (spot 5), acidic endochitinase PR-3 (spot 6), basic chitinase PR-3 (spot 9), and basic β-1, 3-glucanase PR-2 (spot 17) were detected in both virus-infected plants. However, four other spots corresponding to an acidic β-1,3-glucanase PR-2 (spot 3), an osmotin-like PR-5 protein (spot 11), and two PR-1 proteins (spots 14 and 16) were only detected in the AF extracts from the HR-eliciting virus. Further analysis using specific antisera corroborated these data, although it did reveal that the 14.7 kDa PR-1 protein (spot 14) was also present in the PMMoV-I-infected plants. Thus, the data revealed that there are specific and common changes in both types of interactions, although the intensity of the response is higher during HR-elicitation.

27 out of 59 protein spots detected in PMMoV-S-inoculated leaf AF protein extracts were common to the three treatments. Four of them were identified as an acidic chitinase PR-3 (spot 4), a basic endochitinase PR-3 (spot 10), a germin-like protein PR-16 (spot 12), and a PRp27-like PR-17 protein (spot 13), although to a much lower extent in the control plants than in the virus-infected extracts, except for the germin-like protein (GLP) (spot 12) (22.2 kDa; pI 7.6) whose accumulation decreased in the PMMoV-infected leaves.

The GLP family is a large and heterogeneous group of proteins with amino acid identities ranging from 25% to 100% (Bernier and Berna, 2001) and with different patterns of expression (Bernier and Berna, 2001; Godfrey *et al*., 2007). The N-terminal sequence from the GLP protein detected in *C. chinense* leaves has a 100% identity with that of a GLP protein (AB112080.1) from *N. tabacum*, but it has no homology with the N terminus from the *Ca*GLP1 protein (AY391748) described in *C. annuum* (Park *et al*., 2004*a*). In addition, the accumulation data observed suggested a different expression pattern for the *C. chinense* GLP protein and the *C. annuum* *Ca*GLP1 protein, whose mRNA was specifically induced during the HR in *C. annuum* (Park *et al*., 2004*a*). Taken together, these data suggested that a different GLP protein had been detected in *C. chinense* leaves.

Moreover, our data showed that the PRp27 protein expression was induced after viral infection in *C. chinense*, showing that this protein is well conserved among plant species, further expanding previous analysis in tobacco cultured BY2 cells (Okushima *et al*., 2000).

In all cases, except for the acidic chitinase PR-3 (spot 4) and the basic β-1,3-glucanase PR-2 (spot 17), the extent of protein accumulation in the incompatible interaction was higher than in the compatible one, indicating that the host response is, in general, higher in the incompatible interaction, although it does depend on the protein, thus pointing out the complexity of the host–virus interaction and the differential regulation of their expression.

Three different β-1,3-glucanase PR-2 isoforms have been identified in this study with characteristic expression patterns during the plant–virus interaction. Thus the acidic β-1,3-glucanase (spot 3) of M_r 44.2 and pI 5.2, detected with the tomato β-1,3-glucanase PR-2-p35 inmunoserum, was only expressed during the elicitation of the HR, whereas the acidic β-1,3-glucanase (spot 5) of M_r 26.6; pI 5.2 and the basic one (spot 17), M_r 40.0; pI 8.3, both detected with the tomato β-1,3-glucanase PR-2p29 inmunoserum, were expressed in both types of interactions although at an opposite ratio depending upon the protein. Their expression is regulated at the transcriptional level, since the accumulation kinetics of both protein and mRNA is similar (Figs 4, 5). Therefore, our data indicate that, as in *C. annuum*, the *C. chinense* β-1,3-glucanases are a family of proteins of both acidic and basic pIs that are up-regulated in the PMMoV-S virus incompatible interaction (Kim and Hwang, 1994). At variance with our data, in the bacteria- and fungi-infected *C. annuum* plants,

these proteins are strongly induced in both the compatible and incompatible interactions (Kim and Hwang, 1994; Jung and Hwang, 2000), that could be indicative of either the different defence signalling pathways triggered by viruses and other pathogens (Murphy *et al*., 1999) or that the strength of the elicitation is higher in compatible fungi or bacteria than in compatible viruses.

Four different chitinases of both acidic and basic pIs were identified in *C. chinense*-infected plants. The analysis of their amino acid sequences indicated that all of them belong to the PR-3 family of PR proteins. They are highly homologous to other Solanaceae PR-3 proteins, such as potato (Beerhues and Kombrink, 1994), tomato (Danhash *et al*., 1993), and pepper (Kim and Hwang, 1996), except for spot 6 which showed homology to a class IV chitinase Pvchi4 (P27054) from *Phaseolus vulgaris* (Margis-Pinheiro *et al*., 1991). The N-terminal amino acid sequence of the basic chitinase spot 10 has a 93% identity with the 32 kDa b1 chitinase from *C. annuum*, although the empirical calculated pI for each of them was quite different, pI 7.9 and 9.0, respectively (Kim and Hwang, 1996). Two of them (spots 4 and 10) are expressed in virus-infected and control mock-inoculated leaves, whereas the acidic endochitinase (spot 6) of M_r 28.5 and pI 5.9 was only detected in *C. chinense*-infected leaves. In all cases, their expression was up-regulated after viral infection. At variance with most of the proteins analysed in the present study, the acidic chitinase spot 4 (M_r 30.2) accumulated to a higher extent in the compatible interaction than in the incompatible one, the molecular basis of this differential expression being unknown at present. The accumulation kinetics of its mRNA indicates that its accumulation is regulated at the transcriptional level, although the differences encountered among the kinetics of mRNA and proteins are indicative that these proteins are very stable, thus contributing stability to the accumulation pattern of the protein.

Spot 11 was one of the proteins accumulating to a higher extent in the incompatible PMMoV-S–*C. chinense* interaction and was identified as an osmotin-like protein belonging to the PR-5 group of PR proteins. In *C. annuum* two different genes encoding PR-5 proteins have been described. One of them, the *CAOSM1* gene, encodes an acidic protein with a predicted pI of 5.91 (Hong *et al*., 2004). The other one, designated *PepTLP*, encodes a polypeptide of 225 amino acids with a predicted molecular mass of 24 kDa and a pI of 7.5 (Kim *et al*. 2002). The N-terminal sequence of *C. chinense* spot 11 shared a high degree of identity, 85%, with that of the *C. annuum* PepTLP protein, whereas the homology to CAOSM1 protein was only of 77%. The empirically calculated pI of spot 11 was 7.5, well in accordance with the predicted pI of PepTLP mature peptide (Kim *et al*., 2002). In the western blot analysis, by using the tomato PR-5p23 immunoserum, two protein bands of 23 kDa and 26 kDa were detected in both the compatible and the incompatible interaction, thus suggesting that, in *C. chinense* as well as in *C. annuum*, there were at least two different PR-5 isoforms. The accumulation kinetics of the PR-5 proteins and mRNA was similar, indicating that its expression is regulated at the transcriptional level. In contrast to the accumulation kinetics of PR-1 mRNA, in which a steady increase in accumulation is observed throughout the post-infection period analysed, PR-5 mRNA accumulation was complex, with two peaks at 4 dpi and 7 dpi, in both compatible and incompatible interactions. A similar expression pattern, although presenting the maximal accumulation rate at 12 hours post-inoculation (hpi) and 72 hpi, was described for the *PepTLP* gene in unripe pepper fruit after being inoculated with the fungi *Colletotrichum gloeosporioides* (Kim *et al*., 2002), indicating that this is a general phenomenon for PR-5 expression. This pattern was also encountered in the basic PR-3 and PR-2 mRNAs. The pattern is not related to the accumulation level of viral RNA, since the maximum accumulation rates of virus take place at 7 dpi in the compatible interaction, whereas higher accumulation rates of viral RNA are detected in the incompatible interaction at 4 dpi and 7 dpi. Therefore, the complex transcriptional regulation of PR-5, basic PR-3, and PR-2 mRNAs may reflect some type of physiological cycling after viral infection, paralleling the viral replication rounds in the incompatible interaction, but not in the compatible one.

As in *Arabidopsis*, tomato, and tobacco plants (Joosten *et al*., 1989; Niderman *et al*., 1995; Laird *et al*., 2004), the pattern of the *C. chinense* PR protein in the 15 kDa range is complex, with at least three different proteins as detected by immunoblot analysis of the 2-D electrophoresis assay, thus indicating that in *C. chinense* the PR-1 protein comprises a family of proteins. One of the proteins (pI 7.7) is induced in both the compatible and incompatible interaction, at both the mRNA and protein levels albeit to a lower extent in the compatible interaction. The other two proteins of pI 9 and >9.4 are only detected in the incompatible interaction, as corroborated by the data produced when plants were infected with the PMMoV-S HR elicitor in the context of a heterologous virus. These data suggest that the basic PR-1 isoforms are specifically induced in *C. chinense* leaves and are associated with the HR defence response.

The deduced amino acid sequence of the *C. chinense* basic PR-1 protein, as determined in this study, was 99% identical to the basic CABPR1 protein from *C. annuum*, whereas its homology with the PR-1 protein of *C. chinense* described by Hamada *et al.* (2005) was lower, only 59%, thus supporting the assumption that PR-1 proteins of *C. chinense* are a protein family.

It is the differential expression pattern for the 15 kDa range of proteins which brings about the complexity of the induction of their expression. At this point it is

noteworthy to point out that the kinetics of the basic PR-1 mRNA in either the compatible or the incompatible PMMoV–*C. chinense* interactions (Fig. 6B) were similar to those found in *C. annuum* infected with TMV and PMMoV (Shin *et al*., 2001), but at variance with previous data in *C. annuum* infected with bacteria in which the expression of this gene was also associated with the bacterial compatible infection (Kim and Hwang, 2000). Maximal induction of gene expression of the basic PR-1 mRNA (Fig. 6B) takes place at 4 dpi, the time point at which maximal gRNA of the incompatible virus takes place. However, this time point does not coincide with the higher viral accumulation for the compatible interaction, thus ruling out the possibility that its induction is associated with viral RNA levels.

The subcellular location of these protein isoforms is at present unknown, although they are detected in the extracellular apoplastic extract, thus indicating an extracellular location. It is also possible that breakage of the cell as a consequence of the HR might release the proteins located in another subcellular compartment such as the vacuole. Future work addressing this question will elucidate whether, in *C. chinense*, the basic PR-1 protein is located in the extracellular space or the vacuoles as has been described for the closely related tobacco PRB-1b or for the basic isoform of the PR-1 protein in tobacco plants (Bol *et al*., 1990; Sessa *et al*., 1995).

The increased expression of basic PR-1, PR-2, PR-3, and PR-5 protein genes correlates well with the timing of the outcome of the HR induced by PMMoV-S in *C. chinense* leaves. This suggests a co-ordinated induction of these genes as part of the defence response triggered by the incompatible virus. However, with the only exception of the putative antiviral function of the PR-10 RNase (Park *et al*., 2004*b*), a direct involvement of PR proteins on plant virus resistance has not been shown in other plant hosts (reviewed in van Loon *et al*., 2006). For this reason, their role might be related to the enhanced host resistance against further superinfection by other plant pathogens, such as fungi and bacteria, as has been described for certain PR proteins (Durrant and Dong, 2004; reviewed in van Loon *et al*., 2006). On the other hand, studies carried out on tobacco senescent plants were indicative that the accumulation of PR transcripts might be related to the mechanism of senescence and cellular damage (Obregon *et al*., 2001; reviewed in van Loon *et al*., 2006), so that expressed PR-proteins might have a role in the mobilization of nutrients from the virus-damaged tissues, or in the protection against viral cellular injury (Espinoza *et al*., 2007).

In conclusion, it has been determined that in *C. chinense* plants, as in other hosts, the infection by both compatible and incompatible viral strains of PMMoV induces the accumulation of a set of proteins. With few exceptions, the data revealed that both reactions are qualitatively similar, but differing in the extent and timing of the response. In addition, the 2-D approach has allowed the identification of different PR protein isoforms in *C. chinense* leaves and their specific accumulation pattern in both the compatible and incompatible PMMoV–*C. chinense* interactions, thus improving the results obtained by SDS-PAGE analysis and allowing an easy identification of differentially expressed proteins in both type of interactions. Furthermore, it has been shown that each protein has a characteristic expression pattern thus highlighting the complexity of their regulation.

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