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RESEARCH PAPER

Characterization of a pathogenesis-related protein 4 (PR-4) induced in *Capsicum chinense* L^3 plants with dual RNase and DNase activities

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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 as $P_{1,2,3}$ pathotype, such as the Italian strain (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, a *C. chinense* PR-4 protein induced during both the compatible and the incompatible interactions has been identified. It was strongly associated with HR induction and to a lesser extent with the compatible interaction, but only in the later stages of infection. Moreover, it was found to accumulate during the necrogenic reaction induced by Potato virus X. The *C. chinense* PR-4 protein belongs to the PR-4 protein subgroup II, based on the absence of a hevein domain. Furthermore, it is shown that the purified protein does not have chitinase activity, as previously proposed for PR-4 proteins. Instead, it has both RNase and DNase activity, although its contribution to the bulk activity of nucleases in infected plants is very low.

Key words: *Capsicum chinense*, compatible interaction, DNase activity, HR-induction, incompatible interaction, necrogenic interaction, PMMoV, PR-4 protein, RNase activity.

Introduction

In *Capsicum* spp., the resistance against tobamoviruses is conferred by four seemingly allelic series of 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 restriction at the primary infection sites (reviewed in Gilardi *et al.*, 1999).

Concomitant with HR elicitation, there is an induction of certain plant proteins, commonly referred to as pathogenesis-related (PR) proteins (Bol *et al.*, 1990; Linthorst, 1991; Stinzi *et al.*, 1993; van Loon *et al.*, 1994, 2006; Edreva, 2005; Elvira *et al.*, 2008). Pathogenesis-related proteins (PRs) have been defined as plant proteins that are induced in pathological or related situations. Although some of them are implicated in plant defence, they have not been identified because of their antimicrobial activity, but only because of their accumulation in infected plants (van Loon, 1997). The two criteria used for a protein to be considered as PR are that the protein must be induced by a pathogen in tissues that do not express it and that the induction has to be detected in two independent plant–pathogen interactions or confirmed by two independent laboratories (van Loon and van Strien, 1999).

Besides being induced by pathogens, PRs synthesis can also be developmentally controlled in an organ-specific manner in healthy plants (reviewed in van Loon *et al.*, 2006).

Of the 17 groups of PR proteins, the PR-4 protein is one of the less extensively studied. The first PR-4 proteins to be described were from potato (Stanford *et al.*, 1989), and were termed win-1 and win-2, from wound-inducible proteins. Since then, several PR-4 proteins from different hosts such as tomato, tobacco, *Arabidopsis*, wheat, pepper, *C. chinense*, aerial yam, cabbage, barley, and maize have been described (Broekaert *et al.*, 1990; Friedrich *et al.*, 1991; Linthorst *et al.*, 1991; Caruso *et al.*, 1993; Potter *et al.*, 1993; Chevalier *et al.*, 1995; Gregersen *et al.*, 1997; Rompf and Kahl, 1999; Lee *et al.*, 2001; Park *et al.*, 2001; Shin *et al.*, 2001; Ryang *et al.*, 2002; Bravo *et al.*, 2003; Hamada *et al.*, 2005; Park *et al.*, 2005).

All PR-4 proteins have a common C-terminal Barwin domain. This term comes from a basic barley seed protein which is able to bind a chitin analogue weakly, namely the tetrameric β-(1,4) oligosaccharide of *N*-acetylglusosamine (Svensson and Svendsen, 1992). Both the secondary and tertiary structure of this protein has been determined (Ludvigsen and Poulsen, 1992*a*, *b*). PR-4 proteins have been classified as endochitinases (Neuhaus *et al.*, 1996) due to the fact that one of them (tobacco CBP20) (Ponstein *et al.*, 1994) was found to have weak chitinase activity (Brunner *et al.*, 1998). The PR-4 family classification proposal (Neuhaus *et al.*, 1996) is similar to that of the plant chitinase family, in that both contain structural subclasses characterized by the presence (class I) or absence (class II) of a conserved N-terminal cysteine-rich domain corresponding to the mature hevein, a small antifungal protein isolated from rubber plant (*Hevea brasiliensis*) latex (Van Parijs *et al.*, 1991). This hevein domain shares a partial identity with several chitin-binding domains in other proteins (Broekaert *et al.*, 1990).

PR-4 protein is known to be induced not only by pathogen attack, but by ethylene in *Arabidopsis* and peach plants (Gu *et al.*, 2002; Ruperti *et al.*, 2002) as well as by O_3 in *Arabidopsis* plants (Rao *et al.*, 2002).

In spite of efforts to establish a function for the PR-4 II subgroup proteins, no activity has previously been reported except for the PR-4 subgroup I in barley and tobacco (Hejgaard *et al.*, 1992; Ponstein *et al.*, 1994), albeit later studies with subgroup II PR-4 proteins from wheat showed that both wheatwin1 and wheatwin2 inhibited spore germination and hyphal growth in *in vitro* assays (Caruso *et al.*, 1996, 2001*b*). In addition, Caporale *et al.* (2004) showed that wheatwin1 protein has ribonuclease activity and recent findings have shown that the antifungal activity of the native and mutated forms of wheatwin1 protein was related to their ribonuclease activity (Bertini *et al.*, 2009). In this context, it should be noted that PR-4 proteins were recently found in the extrafloral nectar of *Acacia*, where their presence was described as protecting the extrafloral nectar from fungal infestation, although unfortunately the data did not discriminate between the PR-4 I and II subgroups (González-Teuber *et al.*, 2009).

In previous work (Elvira *et al.*, 2008), PR-induction was analysed in *C. chinense* (L^3L^3) leaves infected with two distinct isolates of Pepper Mild Mottle Virus (PMMoV) that either induced the HR (S strain) or systemically infected (I strain) the plants. From the different induced PR proteins characterized, no PR-4 was identified. In addition, two proteins of low M_r were found to be the most abundant induced by PMMoV-S. One of them was identified as a PR-1 protein (Elvira *et al.*, 2008) while the other one could not be identified. This unknown protein accumulated as much as the PR-1 protein in PMMoV-S-*C. chinense* (L^3L^3) inoculated leaves, prompting us to purify and characterize it.

In this work, the isolation and characterization of the PR-4 protein induced in *C. chinense* (L^3L^3) plants after infection with either the compatible or incompatible strain I or S of PMMoV are presented, as well as its association with the necrogenic response against the Potato Virus X (PVX) and the S strain of PMMoV. It has been determined that the purified PR-4 is a bifunctional enzyme with both RNase and DNase activity, thus confirming previous data on PR-4 protein RNase activity (Caporale *et al.*, 2004) and extending our findings to include DNase activity being, to our knowledge, the first time a PR protein has been described as having this activity.

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 (unless otherwise stated) with a 16 h photoperiod and light intensity of 8000 lux.

In other experiments, plants were maintained at 25 °C and then switched to 32 °C after being inoculated, and subsequently maintained either at 32 °C or returned to 25 °C at 72 h post inoculation (h.p.i) and maintained at this temperature for the rest of the experiment.

The origins of the tobamoviruses PMMoV-S and PMMoV-I, as well as that of the chimeric virus PVX-GFP, have already been described (Wetter *et al.*, 1984; García-Luque *et al.*, 1990; Baulcombe *et al.*, 1995). 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, PMMoV-I purified virions diluted in inoculation buffer at a concentration of 50 μg ml^{-1} and PVX-GFP-infected *N. benthamiana* sap extracts. The first pair of true leaves at the 2-fully expanded leaf stage was inoculated and samples from the inoculated leaves were taken from 1 to 7 d after inoculation (d.p.i).

Purification of C. chinense *PR-4 protein*

C. chinense PR-4 protein was purified from intercellular fluid (AF) extracts obtained from 250 g of *C. chinense* PMMoV-S-inoculated leaves at 7 d.p.i as described by Elvira *et al.* (2008). AF extracts were dialysed overnight at 4 °C against 20 mM MES buffer pH 6.0, and then concentrated 30-fold by using Macrosep centrifugal concentrators with a 3000 Da molecular mass cut-off membrane (Pall Filtron Corp. Northborough, MA. USA). The AF concentrated protein extracts were filtered through a 0.20 μm filter, diluted to a concentration of 5 mg ml^{-1} in 20 mM MES buffer pH 6.0 and applied to a cation exchange HiTrap SP 5 ml column (GE Healthcare) equilibrated with the same buffer, according to the manufacturer's instructions. Bound fractions were eluted with

a 0–200 mM NaCl continuous ionic strength gradient in the same buffer.

The eluted fractions were dialysed against 20 mM MES buffer pH 6.0 and concentrated by using Microsep microconcentrators with a 3000 Da molecular mass cut-off membrane. Proteins in the different eluted fractions were resolved according to their molecular mass by Fast protein liquid chromatography (FPLC) on a Superdex 75 column (GE Healthcare).

Further PR-4 protein purification was carried out by high-pressure liquid chromatography (HPLC) on a C18 reverse-phase column Spherisorb S5ODS2 (Hichron). Protein samples were loaded on the column and protein fractions were eluted by an acetonitrile gradient. Fractions were dialysed against 50 mM TRIS-HCl pH 6.8 buffer, concentrated as above, and analysed by SDS-PAGE.

Purified protein was analysed by MALDI-TOF mass spectrometry. The MALDI experiments were performed on a BIFLEX III (Bruker-Franzen Analytik, Bremen, Germany) with a nitrogen laser.

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

Electrophoretic analysis

One-dimension analytical SDS-PAGE was performed as described by Laemmli (1970), using 12.5% and 4.5% polyacrylamide as solving and concentrating gels, respectively. Either 2 μg or 10 μg of proteins were loaded onto each lane and visualized after staining with Coomassie Blue R250.

Isoelectric focusing

To determine the pI of the protein, 2 μg of purified PR-4 protein were loaded onto an ampholine PAGplate pH 3.5–9.5 along with the standard Broad pI Kit, pH 3–10, electrophoresed, and stained with Coomassie blue R250 as indicated by the manufacturer (GE Healthcare).

β-1,3-glucanase and chitinase activity determination

To determine β-1,3-glucanase activity, the in-gel detection method described by Pan *et al.* (1989) was followed using laminarin as substrate and 2,3,5-triphenyltetrazolium chloride as reagent.

For chitinase detection activity, the in-gel method described by Pan *et al.* (1991) was followed using glycol-chitin as substrate, except that no electrophoresis was performed after loading the samples. Polyacrylamide gels containing glycol-chitin were stained with Calcofluor White and photographed under UV.

RNase and DNase activity determination.

To determine purified PR-4 protein RNase activity, the method described by Caporale *et al.* (2004) was used, except that total RNA from *N. benthamiana* plants was used in the assays and the reaction mixture was incubated for 30 min at room temperature. RNA was purified from the reaction mixture by passing it through a column from the RNeasy Mini Kit for RNA purification (Qiagen) following the manufacturer's instructions. RNA was loaded onto a 1% agarose gel containing 0.75 μg ml^{-1} of ethidium bromide and analysed by electrophoresis. RNAs were visualized under UV light. To analyse the stability of the PR-4 RNase activity, the purified protein was either boiled for 15 min, autoclaved or heated at 95 °C for 5 min in the presence of 100 mM DTT before the assay. RNase activity in the presence of either 5 or 50 mM EDTA, 10 mM DTT or 20 U of RNasin in the reaction mixture was also assayed.

The in-gel detection of RNase activity in plant extracts was performed according to Yen and Green (1991), except that 5 μg of total protein from AF extracts were loaded onto the gels. Samples were prepared in SDS-sample buffer lacking reducing agents, and incubated at 37 °C for 15 min before loading. After electrophoresis and washing, gels were incubated for 1 h at room temperature. TRIS-HCl buffer at pH 7.5 was used for washing and incubation. Pictures were taken under UV after staining the gels for 1 h with 2 μg ml^{-1} of ethidium bromide in 10 mM TRIS-HCl, pH 7.5.

To determine the DNase activity of the purified PR-4 protein, 4 μg of purified pUC18 DNA, either undigested or linearized with *Eco*RI were incubated with 4 μg of the purified protein in a total volume of 25 μl in the presence of 10 mM TRIS-HCl pH 7.5 containing 10 mM imidazole and 5 mM NaCl, in either the presence or absence of 2.5 mM $MgCl_2$ for 1.5 h at room temperature. 10 μl of the reaction mixtures were directly loaded onto 1% agarose gels containing 0.75 μg ml^{-1} of ethidium bromide. After electrophoresis, DNA was visualized under UV light.

The in-gel detection of DNase activity in plant extracts was carried out according to Thelen and Northcote (1989), except that gels contained 0.4 mg ml^{-1} of either double- or single-stranded calf thymus DNA as described in Yen and Green (1991). Samples were prepared as indicated above. Gels were incubated overnight at room temperature, in either the presence or absence of 10 mM $MgCl_2$, and visualized in the same way as for the in-gel detection of RNA.

N-terminal and internal amino acid sequencing analysis

Internal peptides were obtained by trypsin hydrolysis of 30 μg of purified protein with 2 μg of trypsin in 10 mM Na phosphate buffer pH 8.0, 1.125 mM DTT (dithiothreitol), and 2.5 mM iodoacetamide. Peptides were purified by HPLC on a C18 reverse-phase column Spherisorb S5ODS2 (Hichron) as above. Purified protein or internal peptides were sequenced on an Applied Biosystems Procise Sequencer (Perkin Elmer; Applied Biosystems). Edman degradation was carried out according to the standard programme supplied by Applied Biosystems. N-terminal sequences were submitted to BLAST searching of relevant databases for protein identification (Altschul, 2001; Schäffer *et al.*, 2001).

PR-4 cDNA cloning

The cDNA to *C. chinense* PR-4 was obtained by RT-PCR on total RNA (0.5 μg) extracted from the PMMoV-S-inoculated leaves of *C. chinense* at 2–7 d.p.i, as described in Tenllado *et al.* (1994) by using oligonucleotides: 5′-CCAAGCT$_{18}$ and 5′-CGATTCAACA-CATA(T/C)ACAGGAACTCAAGC as 3′ and 5′ primers, respectively. The nucleotide sequence of the 5′ primer was determined from the peptide sequence obtained. The amplified 390 bp-long fragment was cloned into pGEM®-T easy vector (Promega) and sequenced. From the obtained sequence, an oligonucleotide 5′-TGGTAGCCCCGTCGATCAGTGTCC was used in a SMART™ RACE 5′ (Rapid Amplification of cDNA Ends) (Clontech Laboratories Inc) assay, to get the 5′-end of the sequence. The 477 bp-long fragment was cloned into the pGEM®-T easy vector (Promega) and sequenced.

Analysis of the sequence obtained was carried out by BLAST (Altschul, 2001; Schäffer *et al.*, 2001).

RNA isolation and Northern blot hybridization

Capsicum chinense total leaf RNA extraction, from either inoculated or systemically infected leaves, and Northern blot hybridization was carried out at the same time of the day as described in Elvira *et al.* (2008). Blots were hybridized to the ^{32}P-labelled amplified 390 bp-long fragment corresponding to the 3′ region of the PR-4 cDNA by using the Rediprime II random prime labelling system (GE Healthcare).

Total RNA from *Nicotiana benthamiana* plants was extracted by the Trizol method, and further purified by using the RNeasy Mini Kit for RNA purification (Qiagen) following the manufacturer's instructions.

Southern blot analysis

Capsicum chinense genomic DNA was purified according to Doyle and Doyle (1990). Southern blot analysis was performed according to Sambrook *et al.* (1989). For this purpose, 15 μg lots of genomic DNA were digested each with the restriction enzymes *Eco*RI, *Hin*dIII, and *Xba*I, respectively, loaded onto an 1.2% agarose gel and blotted. Blots were hybridized to the probe as described above for Northern blot analysis.

Molecular-building procedure

The three-dimensional model for the *C. chinense* PR-4 sequence was built using a knowledge-based protein modelling method based on the given pairwise target-template sequence alignment to the experimentally NMR-resolved structure of the barwin protein from barley seed as template (PDB code 1bw4). An average structure calculated from the internal coordinates of the 20 NMR-conformations of 1bw4 was taken as the final template. Computational resources from the SWISS-MODEL homology modelling server (Arnold *et al.*, 2006) were used to achieve the final structural model for *C. chinense* PR-4. The stereochemical quality of that model and its structural self-consistency were assessed with PROCHECK (Laskowski *et al.*, 1993) and Verify-3D (Eisenberg *et al.*, 1997) programs, respectively. The assignment of the secondary structure elements to the PR-4 model was predicted by DSSP (Kabsch and Sander, 1983). For superposition of the model-template conformations, a combinatorial extension algorithm for computing the pairwise structural alignment (Shindyalov and Bourne, 1998) was used. The final figure was created with PyMOL Release 0.99 (http://www.pymol.org/).

Results

Protein purification and characterization

Among the different proteins induced by PMMoV-S in *C. chinense* inoculated leaves, a protein of M_r 14 kDa (P14) was one of the most abundant proteins detected in *C. chinense* AF leaf extracts after PMMoV-S HR elicitation in this host. It was readily visible in SDS-PAGE analysis of total and extracellular *C. chinense* leaf protein extracts (Elvira *et al.*, 2008), but its relative amount with respect to total proteins in AF extracts was higher than in total extracts. In order to characterize the P14 protein, this protein was purified from the crude extracellular fluid (AF) using a cationic exchange column as described in the Materials and methods. SDS-PAGE analysis confirmed that the first peak after the void volume contained the P14 protein (data not shown). It then underwent further purification by being passed through a Superdex 75 column, and the major protein peak corresponded to a single band as revealed by SDS-PAGE analysis (Fig. 1A) that co-migrated with the unidentified P14 protein. IEF analysis also showed the presence of a unique band with a pI of 7.5 (Fig. 1B). This protein was further characterized as a PR-4 protein.

To determine whether the protein had either β-1,3-glucanase or chitinase activity, the methods described by Pan *et al.* (1989, 1991) were used. No β-1.3 glucanase or chitinase activity was detected (Fig. 2A; data not shown).

To establish whether the protein had RNase or DNase activity or both, the methods described by Caporale *et al.* (2004) and those in the Material and methods were used.

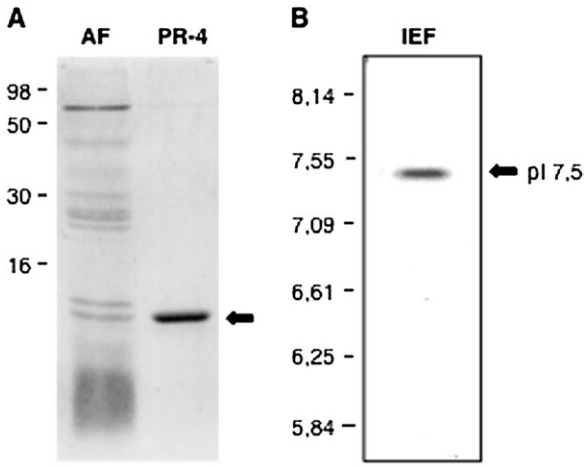

Fig. 1. Electrophoretic analysis of purified PR-4 protein from *C. chinense* leaves. (A) SDS-PAGE analysis of intercellular fluid proteins (AF) (10 μg) from PMMoV-S-inoculated leaves at 7 d.p.i, and purified PR-4 protein (2 μg). Numbers on the left indicate the M_rs from marker proteins. (B) Isoelectric focusing electrophoresis (IEF) of purified PR-4 protein (2 μg). Numbers on the left indicate isoelectric points of marker proteins.

The data (Fig. 2B, C) indicated that the purified protein had both activities. Moreover, the data revealed that, in the case of RNase activity, the protein was very stable, being active after heat treatment and autoclaving, and its RNase activity proved to be insensitive to EDTA (5 mM and 50 mM), DTT (10 mM), and to RNasin treatment. It lost its activity only after heating at 95 °C in the presence of 100 mM DTT (Fig. 2B). The protein also had DNase activity, being active against both circular and linear plasmid DNA. The addition of Mg^{2+} cations enhanced DNase activity on plasmid circular DNA, but DNase activity was lost by heating in the presence of DTT (Fig. 2C).

These data prompted the analysis of the contribution of both P14 protein RNase and DNase activity in the total activity of the extract. In-gel analysis revealed (Fig. 3) that *C. chinense* AF extracts contained both RNase and DNase activity protein bands. Two major bands of RNase activity with an apparent M_r of 28 kDa and 18 kDa, respectively, were observed in infected and mock-inoculated leaf extracts. A minor band with an apparent M_r of 13.5 kDa was detected in these assays and a DNase band with an apparent M_r of 37 kDa was also observed in either double or single stranded DNA-containing gels. No DNase activity bands at the expected mobility of P14 protein were detected, thus indicating that the contribution of the P14 activity (further characterized as a PR-4 protein) is scarce.

To characterize the purified protein further, amino acid sequence analysis of the *N*-terminus was carried out on an Applied Biosystems Procise Sequencer (Perkin Elmer; Applied Biosystems). Edman degradation was performed according to the standard program supplied by Applied Biosystems. Analysis of the obtained sequence by BLAST searching on relevant databases for protein identification (Altschul 2001; Schäffer *et al.*, 2001) showed that the

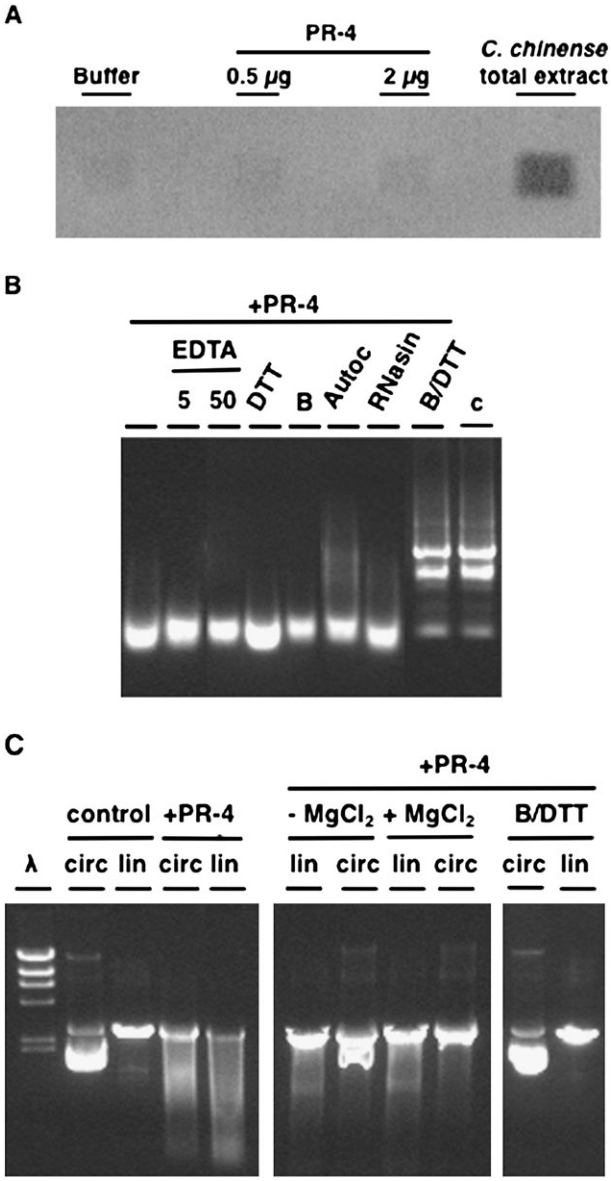

Fig. 2. Analysis of enzymatic activities of purified *C. chinense* PR-4 protein. (A) In-gel analysis of chitinase activity of 0.5 μg and 2 μg of purified PR-4 protein. Buffer: negative control using protein buffer (TRIS-HCl 50 mM, pH 6.8). *C. chinese* total extract corresponds to 10 μg of the intercellular fluid extract from PMMoV-S-inoculated leaves at 7 d.p.i. The gel was stained with Calcofluor White and observed under UV light. (B) RNase activity of 4 μg of purified PR-4 protein assayed on 12 μg of *N. benthamiana* total RNA. Agarose gel electrophoresis analysis of the purified RNAs after the assays in the presence of either EDTA (5 mM and 50 mM) or DTT (10 mM) or Rnasin (20 U) or after the purified PR-4 protein have been subjected to different treatments: B, boiled for 15 min; Autoc, Autoclaved; B/DTT, boiled for 5 min in the presence of 100 mM DTT before the assay; c: negative control using protein buffer as in (A). Gels were stained with ethidium bromide and observed under UV light. (C) DNase activity of 4 μg of purified PR-4 protein assayed on 4 μg of pUC18 plasmid DNA. Agarose gel electrophoresis analysis of 10 μl of the reaction mixture. Circ, circular pUC18 DNA; lin, pUC18 DNA linearized with *Eco*RI restriction enzyme; control: negative control using protein buffer as in (A). λ, λ phage digested with *Hin*dIII. The DNase activity was assayed in either the presence (left, middle, and right panels) or absence of 2.5 mM $MgCl_2$ (middle panel) or after boiling the PR-4 protein in the presence of 100 mM DTT (B/DTT). Gels were stained as in (B).

sequence (DEGTATYYTAPYVPS) shared a high degree of homology with a putative protein of *Vitis vinifera* (acc. no. CAN65920) of unknown function that has a transferase family domain.

However, the low amount of recovered picomoles for each amino acid in the analysis, in spite of the amount of purified protein assayed, seemed to indicate that the protein of interest was blocked, and the sequence obtained could correspond to a contaminant protein. For this reason, the purified protein was subjected to further HPLC purification by loading the protein sample onto a C18 reverse-phase column and a major peak was observed at a retention time of 32 min. Using mass spectrometry the purified protein was calculated to have an M_r of 13.342 kDa.

Amino acid sequence analysis of the N-terminus of the major peak was unsuccessful, thus indicating that the protein was blocked at its N-terminus. After trypsin hydrolysis, an internal fragment was liberated and further characterized by amino acid sequencing. The amino acid sequence obtained (NTYTGTQATV) shared a high sequence identity to the amino acid sequence (NTYTGTQETV) of a tomato PR-4 protein (Linthorst *et al.*, 1991), thus indicating that the purified protein did correspond to a *C. chinense* PR-4 type protein.

To characterize the protein further, its cDNA was obtained by RT-PCR on total RNA as described in the Materials and methods. Analysis of the overlapping clones revealed that the sequence contained a total of 692 bp, coding for a unique polypeptide of 143 aa.

Analysis of the sequence obtained by BLAST searching on relevant databases for nucleic acid and protein identification (Altschul, 2001; Schäffer *et al.*, 2001) showed that it shared a high degree of sequence identity (99%) with a partial clone encoding for a putative PR-4 from *C. chinense* (acc. no. CB185055) and 97% with a *C. annuum* sequence-encoding a PR-4 protein (acc. no. CB185055). At the amino acid level, the protein is closely related to the PR-4 protein from *C. annuum* (AAF63520; Shin *et al.*, 2001) sharing a 92% of sequence identity. It also shared a high degree of sequence identity with other proteins from *Solanum lycopersicon* (86%) (PR-protein P2, X58548; Linthorst *et al.*, 1991); *Nicotiana tabacum* (86%) (PR-4B, CAA41437; Friedrich *et al.*, 1991), *N. tabacum* (85%) (PR-4A, CAA41437; Linthorst *et al.*, 1991); *Vitis vinifera* (79%) (CAN80017); *Solanum tuberosum* (73%) (CAA 31855.1); *Capsicum chinense* (73%) (BAD11073) (Hamada *et al.*, 2005), and to a lesser extent with those from aerial yam (67%) (AAB94514.1) (Rompf and Kahl, 1999); cabbage PR-4 (62%) (AAN23106; AAN23106.2; Ryang *et al.*, 2002; Park *et al.*, 2005); wheat (PR-4b) (62%) (CAA06857.1; Caruso *et al.*, 1999); *Lycoris radiata* LrPR4 (61%)

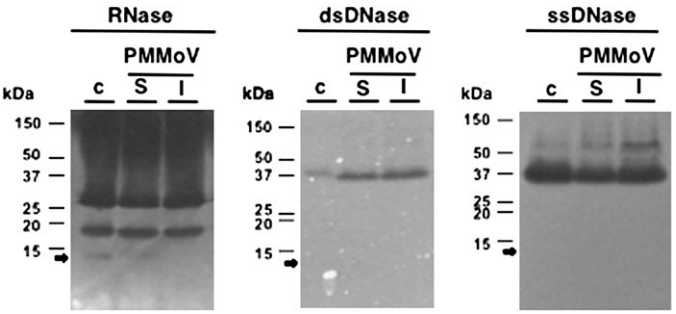

Fig. 3. In-gel analysis of RNase and DNase activity of the intercellular fluid of *C. chinense* infected leaves with either the compatible (I) or the incompatible (S) strains of PMMoV at 9 d.p.i. C, control mock-inoculated plants. 5 μg of AF extracts were loaded onto each lane. ss, single-stranded DNA; ds, double-stranded DNA. DNase activity in the presence of 10 mM $MgCl_2$ is displayed. Numbers on the left of the figures represent the M_r markers. Gels were stained with 2 μg ml^{-1} of ethidium bromide and observed under UV light. Arrows indicate the predicted position of the P14 protein.

(ACI31201.1; Li *et al.*, 2009); wheat PR-4a and barley (60%) (CAA06856.1, CAA71774; Caruso *et al.*, 1999; Gregersen *et al.*, 1997); and maize (55%) (CAA57674; Chevalier *et al.*, 1995).

Therefore, both the nucleotide and amino acid sequence analysis revealed that the purified protein corresponded to a PR-4 protein and, incidentally, showed the high degree of sequence conservation achieved by these proteins in the plant kingdom.

Computer analysis of the encoded protein revealed that it might contain a 23 aa long putative signal peptide that would be expected to function for translocation through the endoplasmic reticulum yielding an extracellular protein. The predicted cleavage site is located at position 23–24 between the alanine–glutamine bond, as has been described for tobacco PR-4a and PR-4b, tomato PR-P2, as well as for other proteins such as acidic chitinases, glucanases, and hevein (Linthorst *et al.*, 1991), indicating again the high conservation of this protein. The result is a mature protein with a molecular mass of 13480.92 Da and a pI of 7.77, well in accordance with the empirically observed data.

Three-dimensional structure of the protein

The sequence alignment of *C. chinense* PR-4 and barwin protein shows a ratio of aligned/gap positions of 120/3 with 68% sequence identity as yielded by the BLASTP algorithm.

The structural alignment of barwin with the PR4 model shows a possible identical fold (a six-stranded double-psi beta barrel), with an average all-atom root-mean-square deviation of 0.6 Ångströms (Fig. 4A), in spite of slight differences of one residue length for the prediction of some secondary elements, i.e. helix 3, and strands 4 and 5 (Fig. 4B). One significant feature is the absence of helix 1 in the PR4 model. On the other hand, the model presents a deletion of two residues in the first gamma turn (residues 20–22) and a deletion of one residue in the eighth beta turn (residues 94–97) of barwin.

PR-4 protein is encoded by a small multigene family in C. chinense

To determine whether the PR-4 protein is encoded in *C. chinense* by either a single gene or by a multigene gene family, Southern blot analysis of genomic DNA was performed as described in the Material and methods. The analysis revealed the existence of several bands (Fig. 5), indicating that the PR-4 protein is encoded by a small multigene family in this host, thus expanding previous data on PR-4s from *C. chinense* (Hamada *et al.*, 2005).

The PR-4 mRNA is expressed in both compatible and incompatible interactions

To verify whether the PR-4 mRNA was associated with the HR induction as revealed by protein analysis, the mRNA expression pattern was assayed on total RNA extracted from the inoculated and systemic leaves on several post-inoculation days in both compatible (PMMoV-I infection) and incompatible (PMMoV-S infection) interactions.

As shown in Fig. 6A and data not shown, increasing amounts of PR-4 mRNA were detected in the PMMoV-S inoculated leaves from 2 d.p.i up to 5 d.p.i, decreasing at 6 d.p.i. A very faint band was also detected at both 5 d.p.i and 6 d.p.i in the PMMoV-I inoculated leaves, albeit to the same extent. No mRNA was detected in either control or mock-inoculated plants, thus indicating that its expression was not induced by carborundum rubbing. Instead, it was strongly associated with the induction of the HR reaction, and to a lesser extent with the compatible interaction, but only during the later stages of infection. At this point it should be noted that leaf abscission of the inoculated leaves took place at 7–9 d.p.i, but only in the PMMoV-S inoculated leaves. On the other hand no signal was detected in the upper non-inoculated leaves at 3, 7, and 14 d.p.i (data not shown), thus reinforcing data indicating that it is mainly associated with the HR reaction.

It has already been established that L^3 gene-mediated resistance is sensitive to temperature, being inactive at temperatures above 30–32 °C. At these temperatures, the incompatible virus invades the plant systemically, and a further incubation of the plant at 25 °C for 2 d results in the formation of NLLs in the upper non-inoculated leaves. To determine how temperature affects PR-4 expression, plants were maintained at either 25 °C or 32 °C for 6 d.p.i with either the compatible or the incompatible viral strain. Another group of plants was subjected to a temperature shift from 32 °C to 25 °C after 3 d, and subsequently incubated at 25 °C for three or five additional days. RNA blot analysis of both the inoculated and upper non-inoculated leaves (Fig. 6B; data not shown) revealed that PR-4 mRNA is mainly expressed in association with the HR, and late in the infection in the compatible interaction, but only when plants were maintained at 25 °C for the

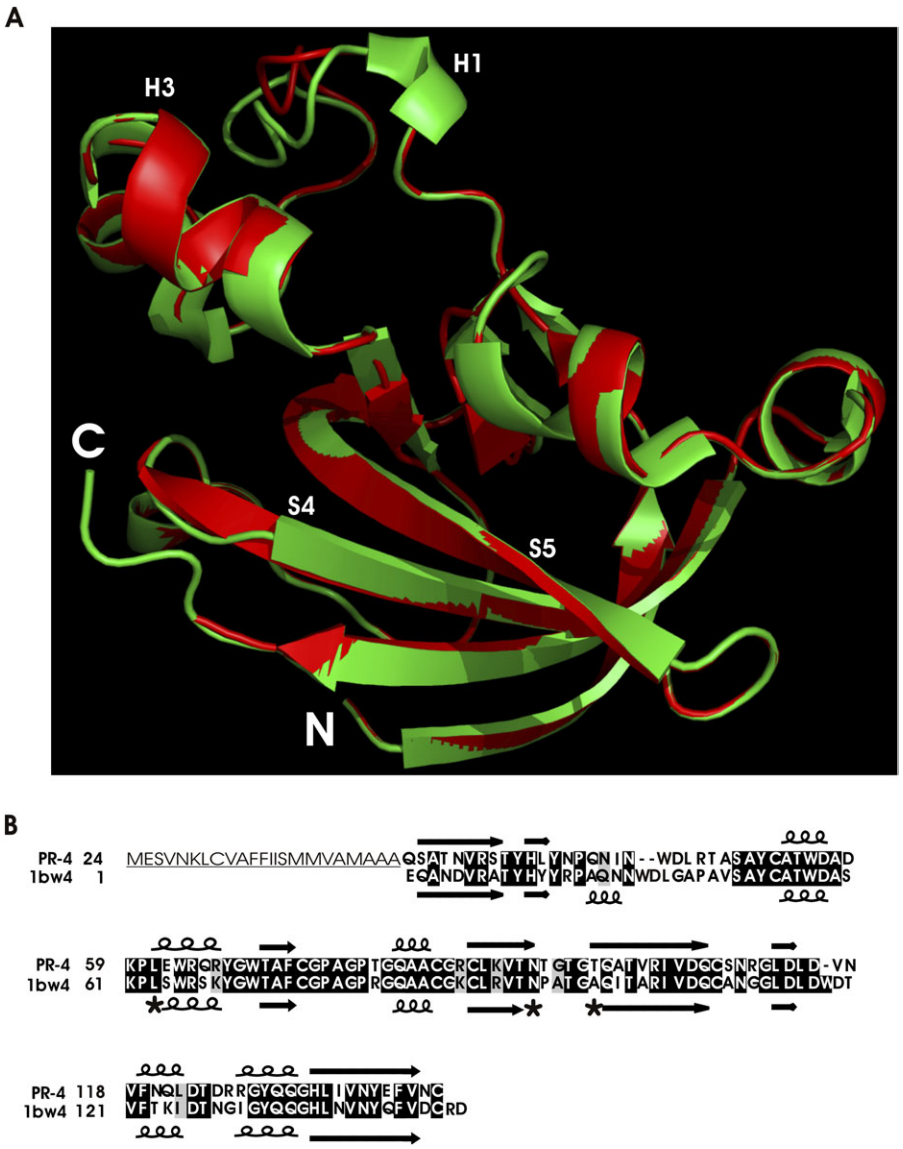

Fig. 4. (A) Ribbon diagram for the backbone structure of PR-4 model (in red) superimposed on Barwin protein (1bw4, in green), N- and C-termini are labelled. H1, H3, S4, and S5 labels point to helix 1, helix 3, and strands 4 and 5, respectively. (B) Structure-based sequence alignment of PR4 and 1bw4. Identical and similar residues are shaded in black and grey, respectively. Residue positions are shown on the left, starting at the the first model's residue for PR-4. The secondary structure elements corresponding to helices and beta strands are depicted on the alignment. Asterisks mark one residue-length differences for some secondary structure predictions by DSSP. The alignment was drawn with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). The sequence corresponding to the putative signal peptide is underlined.

entire period assayed. Furthermore, when plants inoculated with either virus were maintained at 32 °C for 6 d no PR-4 mRNA was detected in either the inoculated leaves or in the upper systemic leaves.

To determine whether PR-4 expression is associated with either necrosis or with the cell death HR, which is determined by a programmed cell death (PCD), *C. chinense* plants were infected with the necrosis-inducing potato virus X-GFP, which gives rise to necrotic spots only on the systemically-infected leaves. Northern blot analysis of PR-4 expression revealed that the PR-4 mRNA did accumulate in the upper leaves, but not in the inoculated leaves that did not develop necrosis (Fig. 6C).

All the data taken together indicate that the PR-4 mRNA expression of the isoform described here is associated with the expression of necrosis either due to an incompatible interaction (PMMoV-S) or to a compatible interaction (PVX-GFP), although, in this case, during the later stages of infection, thus reflecting the damage inflicted on the cell.

Discussion

In this work, a PR-4 protein has been identified and characterized in *C. chinense* plants that had not been determined previously in the proteomic analysis of PR

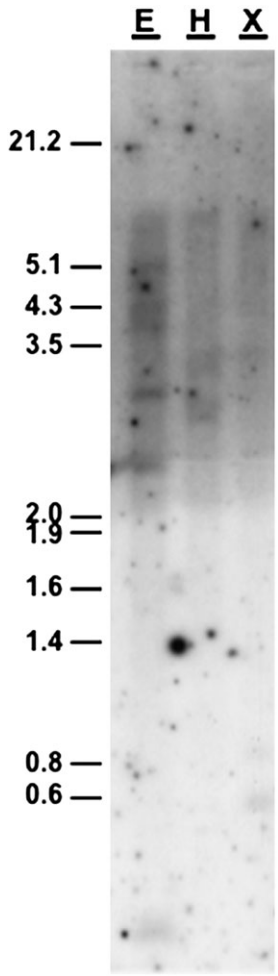

Fig. 5. Analysis by Southern blotting of the *PR-4* gene from *C. chinense*. The probe against the sequence was hybridized with genomic DNA previously digested with *Eco*RI (E), *Hin*dIII (H), and *Xba*I (X). On the left, the molecular weight markers correspond to those from λ phage digested with *Eco*RI and *Hin*dIII and are given in kb.

proteins induced in the intercellular space during incompatible viral interactions (Elvira *et al.*, 2008). A protein with a pI of 7.5 and a M_r of 13.342 was purified by cation-exchange chromatography and gel filtration analysis. Subsequent amino acid sequence analysis of an internal peptide, and further sequence analysis of the cDNA clones obtained revealed that the protein corresponded to a PR-4 protein.

The fact that this protein could not be sequenced by Edman degradation, indicated that its first aa had been modified. This phenomenon is widespread in nature, since a homologue protein from tobacco, such as the so-called CBP20, belonging to the I subgroup (Ponstein *et al.*, 1994) along with a PR-4a from tobacco and a P2 from tomato could not be subjected to Edman degradation (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991; Ponstein *et al.*, 1994).

Based on the absence of a chitin-binding domain in the N region of the protein, the PR-4 protein described here belongs to subgroup II, whereas the protein previously described for *C. chinense* has this chitin binding domain and therefore belongs to subgroup I (Hamada *et al.*, 2005). It has been proposed that class II PR-4 derived from class I after losing the hevein domain (chitin-binding domain) (Friedrich *et al.*, 1991, and references herein).

The homology modelling procedure, previously described, enables us to confirm that the plant defence protein barwin from barley is highly homologous to *C. chinense* PR-4 protein, similar to that described for wheat PR-4 proteins (Bertini *et al.*, 2009). The barwin, like the PR-4 model, shares a six-stranded double-psi beta barrel structural domain. Among the commonly observed beta barrels, the double-psi beta barrel fold consists of two interlocked motifs and was first recognized as the common core borne by the plant defence protein barwin (Ludvigsen and Poulsen, 1992*a*) and the fungal endoglucanase V (Davies *et al.*, 1993). Structural and functional studies performed with diverse protein super-families bearing this beta barrel fold suggest a common functional role in either catalysis or cofactor binding for the psi loop motifs because of the clustering of protein active sites around these psi loops (Castillo *et al.*, 1999). Furthermore, in barwin the residues immediately before the first psi loop (spanned by residues 52–65) seem to be involved in the ligand-binding site (Ludvigsen and Poulsen, 1992*b*). Therefore the substitution R79T in the middle of the psi loop displayed by the PR-4 model could account for some kind of specificity in the functional properties of *C. chinense* PR-4.

Although the precise subcellular localization of this protein, along with other PR-4 proteins, has not yet been determined, computer analysis of its aa sequence predicts the existence of a signal peptide, and that a mature protein is presumably synthesized as a preprotein that undergoes removal of the signal peptide to give rise to the 13 kDa mature protein. As the protein lacks a C-terminal extension, it must be secreted to the apoplast, well in accordance with the fact that *C. chinense* PR-4 protein was readily purified from the AF extracts of *C. chinense* PMMoV-S inoculated leaves. Thus suggesting that the most likely localization of the protein is the apoplastic space, as suggested for other PR-4 proteins such as tomato PR-P2 and tobacco PR-4 proteins (Linhorst *et al.*, 1991). However, further work is required to corroborate this assumption.

As described in tobacco, tomato, wheat, hot pepper (*C. annuum*), and yam, the PR-4 protein in *C. chinense* comprises a small gene family (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991; Rompf and Kahl, 1999; Caruso *et al*, 2001*a*; Park *et al.*, 2001; Bertini *et al.*, 2006).

In spite of PR-4 proteins being described as chitinases, no detectable levels of chitinase activity were observed for the purified *C. chinense* PR-4 protein. Although, from our analysis, a low specific chitinase activity could not be ruled out as described for the tobacco CBP20 protein (Ponstein *et al.*, 1994). Instead, it was found that this protein had both RNase and DNase activity.

PR-4a from wheat and LrPR4 from *Lycoris radiata* have previously been described as having ribonuclease activity (Caporale *et al.*, 2004; Li *et al.*, 2009). The data presented here not only corroborate these findings but expand them further by showing that *C. chinense* PR-4 protein also has

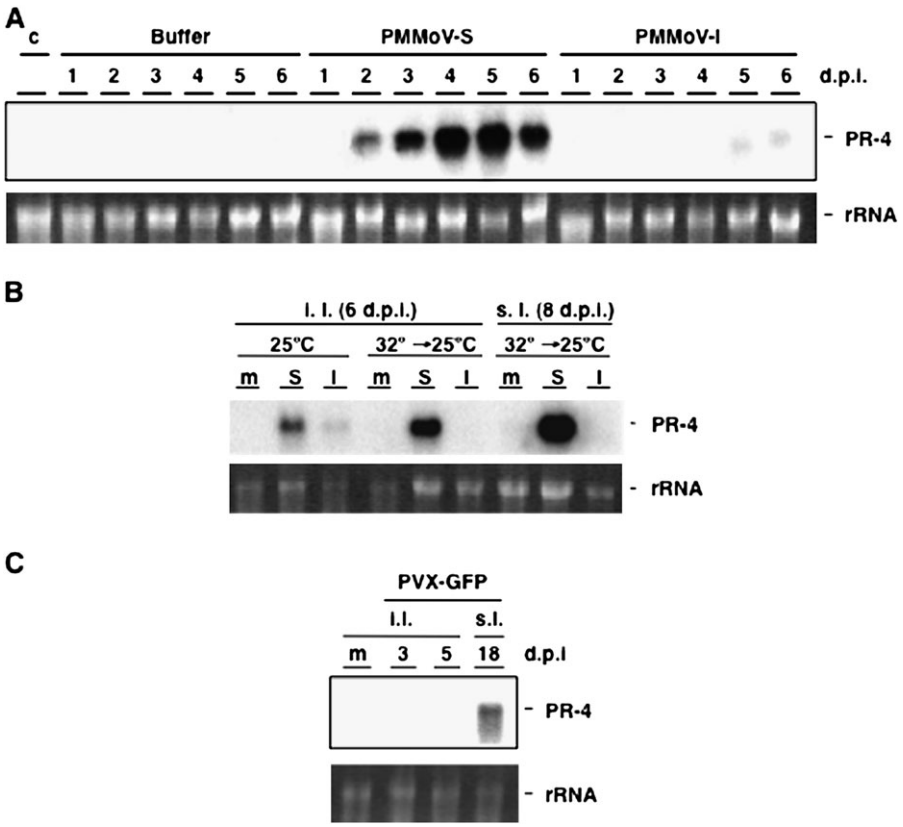

Fig. 6. Northern blot analysis of PR-4 mRNA expression in *C. chinense* leaves. Total RNA (20 μg) was analysed by Northern blot at the times indicated at the top of the figure. Blots were hybridized to the PR-4 mRNA probe. rRNA corresponds to ribosomic RNA stained with ethidium bromide and used as a loading control. (A) Time-course of the accumulation of PR-4 mRNA in PMMoV-S- and PMMoV-I-inoculated *C. chinense* leaves, mock-inoculated ones (buffer), and uninoculated ones (c). (B) Accumulation of PR-4 mRNA in the inoculated (i.l.) and systemic (s.l.) leaves of *C. chinense* plants infected with either the incompatible (S) and compatible (I) strain of PMMoV and subjected to the temperature regimes indicated at the top of the figure. m, mock-inoculated plants. (C) Accumulation of PR-4 mRNA in the inoculated (i.l.) and systemic (s.l.) leaves of PVX-GFP-inoculated *C. chinense* plants. m, mock-inoculated plants.

DNase activity. To our knowledge this is the first time that a PR protein has been described as possessing such activity.

Caporale *et al.* (2004) pointed out the putative amino acids involved in RNase activity namely H11, H113, D92, and R7 of wheatwin1 PR-4 based on the RNase A activity model, in spite of the low similarity found between these two proteins (60%). The results presented here indicated that the PR-4 from *C. chinense* is not of the RNase A, neither RNase B, RNase C, or human placenta RNase type, since it was not inhibited by RNasin, an RNase inhibitor capable of inhibiting these RNases (Balwit, 1996). Nevertheless, these two conserved His residues must be important for PR-4 protein ribonuclease activity, as mutations of either each one or both His residues partially impaired the ribonuclease activity of the wheatwin1 protein (Bertini *et al.* 2009). On the other hand, the presence of the six conserved cysteine residues presumably involved in disulphide bonds, whose positions are strictly conserved in the PR-4 described here, is consistent with the high stability of the protein. Thus only heating of the protein in the presence of the reducing agent DTT is able to disrupt its RNase activity and DNAase activity and is probably associated to its resistance to proteolytic attack.

The contribution made by PR-4 to the total RNase and DNase activity, that increases during pathogen infection in the intercellular fluid (Barna *et al.*, 1989; Mittler and Lam, 1997) and in total extracts (Lusso and Kuc, 1995) remains an open question. However, from in-gel analysis of the RNase and DNase activity in *C. chinense* AF extracts it seems that its contribution is insignificant. In any case, the multifunctional activity found indicates that it is a novel type of nuclease, at variance with LrPR4 from *Lycoris radiata* (Li *et al.*, 2009) and CaPR-10 from *C. annnum* (Park *et al.*, 2004) as it has DNase activity.

PR-4 mRNA has been detected in inoculated leaves from both the compatible and incompatible interactions, although the timing is earlier and accumulation is higher for the incompatible viral strain. The increased PR-4 mRNA expression correlates well with the timing of the outcome of the HR induced by PMMoV-S in *C. chinense* leaves, following an expression pattern different from that of other *C. chinense* PR mRNAs (Elvira *et al.*, 2008), thus suggesting differences in their induction signalling pathways as also described for CaPR-4 and CaPR-1 proteins from hot pepper (Park *et al.*, 2001).

The fact that PR-4 mRNA is only detected in the inoculated leaves of PMMoV-I-infected plants grown at 25 °C but not in those plants that were grown at 32 °C might indicate that its expression is temperature dependent, as it has been described for the tobacco PR-1 protein (Van Loon, 1975; White *et al.*, 1983; Yalpani *et al.*, 1991).

No PR-4 mRNA was detected in the systemic leaves of either PMMoV-S- or PMMoV-I infected plants (data not shown). The finding that PR-4 mRNA isolated from hot pepper is induced in the systemic leaves (Park *et al.*, 2001) is at variance with what it is found in *C. chinense* plants, and might be ascribed to a different pattern of induction of distinct PR-4 genes from the small multigene family.

PR-4 mRNA could only be detected in the systemic leaves of plants that had undergone necrosis, either because of HR elicitation or because of infection with the necrogenic virus PVX-GFP. This suggests that some molecular events associated with programmed cell death (PCD) during the HR are common to those of necrotic symptom developments in susceptible plant tissues (Kiba *et al.*, 2006; Komatsu *et al.*, 2010).

Whether the role of PR-4 as a ribonuclease and DNase might contribute to the depletion of RNA and DNA during stress conditions, such as necrosis or viral infection, it remains an open question since the bulk of RNA and DNA accumulated within the cell, and PR-4 protein seems to accumulate in the extracellular space. However, it is possible that once the plasma membrane integrity is compromised, PR-4, like other nucleases, might have access to the endocellular DNA and RNA as it has been pointed out for the fragmentation of DNA at the onset of the HR (Mittler and Lam, 1997). One of the most plausible roles for the induced extracellular *C. chinense* PR-4 protein would be the breakdown and scavenging of RNA and DNA fragments liberated from damaged plant cells that undergo either PCD or necrosis, as suggested for other nucleases induced during HR and senescence (reviewed by Sugiyama *et al.*, 2000).

Finally, the finding of the nuclease activity in *C. chinense* PR-4 protein might reflect its putative role not only in mobilizing compounds during the senescence programme, but also in a protective role by degrading DNA or RNA of either foreign, invading pathogens, or of the host. In fact, it has been demonstrated that applying an extracellular RNase to tobacco leaves inhibits the growth of both TMV, inducing a delay in the appearance of the symptoms, as well as a reduction in viral accumulation (Trifonova *et al.*, 2007), and a fungal pathogen, although it does not have a direct effect upon pathogen germination or hyphal growth (Galiana *et al.*, 1997). Thus, the antimicrobial effect in plants of the PR-4 described here is a seemingly complex process that requires the concourse of other hydrolytic enzymes such as proteases, chitinases, and glucanases to act in the defence and remobilization of compounds in the plant.

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