# INDUCTION OF CINNAMATE 4-HYDROXYLASE AND PHENYLPROPANOIDS IN VIRUS-INFECTED CUCUMBER AND MELON PLANTS

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**Abstract**

In the present work, we have studied the induction of *p*-coumaric, caffeic and ferulic acids in the plant-pathogen systems, *Cucumis sativus* and *Cucumis melo* infected with either prunus necrotic ringspot virus (PNRSV) or melon necrotic spot virus (MNSV), respectively, after hydrolysis with β-glucosidase or esterase. These hydroxycinnamic acids constitute the major UV-light absorbing compounds at 320 nm and were analysed by HPLC/ESI-MS. They were found in undigested samples mainly forming esters with glucose: 1-*O*-coumaroyl-β-glucose, 1-*O*-caffeoyl-β-glucose, and 1-*O*-feruloyl-β-glucose. Cinnamic acid 4-hydroxylase (C4H, EC 1.14.13.11), the second enzyme of the plant phenylpropanoid pathway, plays a pivotal role in the synthesis of

these hydroxycinnamic acids. Thus, we have isolated and characterised a cDNA clone encoding this enzyme from PNRSV-infected cucumber, and a partial cDNA from MNSV-infected melon leaves. The deduced aminoacid sequence revealed a notable degree of identity with homologous C4H enzymes from other plant species. In agreement with the induction of the phenylpropanoids presently described, it is reported that in cucumber and melon leaves, both viral infections studied induced C4H mRNA expression. A similar induction was observed for the first phenylpropanoid biosynthetic enzyme, phyenylalanine ammonia-lyase (PAL, EC 4.3.1.5), and for chitinase and peroxidase defence-related genes.

*Keywords*: *Cucumis sativus*, *Cucumis melo*, Cinnamic acid 4-hydroxylase, Compatible interactions, Phenylpropanoids

## 1. Introduction

Plants are continuously exposed to a great diversity of biotic (viroids, viruses, fungi or bacteria) and abiotic environmental stresses, and their lack of mobility render them unable to escape from these potentially damaging agents. This fact has led them to develop very efficient and polyvalent defence mechanisms [1] and, as a consequence, disease is the exception rather than the norm [2, 3]. This multi-component defence response includes the synthesis of antimicrobial, low-molecular-weight natural products of phenylpropanoid metabolism [4, 5]. Ample evidence of their importance in plant defence responses and the establishment of the systemic acquired resistance have been obtained from tobacco and alfalfa transgenic plants with altered expression of phenylpropanoid genes or modified levels of phenylpropanoid metabolites [6, 7]. Cucumber has been used as a model to study biochemical and physiological aspects of plant defence reactions against invasive pathogens [8]. Previously, we reported the accumulation of the phenolic gentisic acid (2,5-dihyroxybenzoic acid), a metabolic derivative of salicylic acid [9], in cucumber plants infected with prunus necrotic ringspot virus [10, 11]. This metabolite is proposed to act as a signal for the activation of plant defences in cucumber. Two types of interactions have been used in this study: *Cucumis sativus* /Prunus necrotic ringspot virus (PNRSV) and *Cucumis melo* /Melon necrotic spot virus (MNSV). PNRSV is an economically important virus infecting a wide range of *Prunus* species and can be mechanically transmitted to cucumber [12], producing systemic chlorotic local lesions. MNSV infects a narrow range of host plants, being largely restricted to species belonging to the *Cucurbitaceae* family. In melon plants, the virus causes necrotic brown lesions in the inoculated cotyledons and, as the

infection progresses, the virus systemically infects the first leaf, also producing necrotic spots or large lesions in the leaves, and necrosis in the stem.

It is well known that, in plant tissues, phenolic compounds such as hydroxycinnamic acids can be found as glucosylated forms (esters or glycosides) of the glucose anomeric hydroxyl group. In addition of glucosylated forms, large amounts of esters of hydroxycinnamic acids also accumulate in many plant tissues. Among them, chlorogenic acid can reach important levels in plant tissues, and shiquimate and quinate esters play a major role in the phenylpropanoid pathway [13, 14]. Cinnamte 4-hydroxylase (C4H, EC 1.14.13.11) is a pivotal regulatory enzyme which catalyses the hydroxylation of cinnamic acid to *p*-coumaric acid in the phenylpropanoid pathway. In this work, we report the structural characterization and induction of hydroxycinnamic acids in cucumber and melon plants infected by PNRSV and MNSV, respectively, and the cloning of a full-length C4H cDNA from cucumber and a partial sequence from melon. C4H gene induction after virus infection has been studied in both cucumber and melon, and compared with the expression patterns of the genes encoding defence-related proteins PAL, chitinase, and peroxidase.

## 2. Material and methods

### *2.1. Plant material and inoculations*

*Cucumis sativus* L. cv. Wisconsin SMR-58 or *Cucumis melo* L. cv. Galia plants (one per pot) were grown in a greenhouse from seeds in 10-cm-diameter pots containing a mixture of peat and vermiculite (1:1) at 22 °C/18 °C (day/night), and a relative humidity ranging from 50 to 70%. Experimental blocks of 20 plants from either

cucumber and melon plants of uniform morphological and physiological conditions were prepared. Plants were subirrigated twice a day with Hoagland solution. Infection of ten-day-old-cucumber with PNRSV (isolate NCM1) [15] was done with a viral extract obtained from the first leaf of infected plants, which was homogenized in 30 mM phosphate buffer (pH 8) supplemented with 0.1% 2-mercaptoethanol using 3 ml of buffer per gram of fresh weight of infected tissue. Before inoculation, plants were kept in the dark during 12 hours. One-week after sowing, fully expanded cotyledons were dusted with Carborundum, and buffer (mock-inoculated control) or viral extract was applied by gently rubbing the upper face of the two cotyledons. The cotyledons were washed with distilled water 5 min later. Ten-day-old melon plants were mechanically inoculated by rubbing fully expanded cotyledons with MNSV transcripts following the protocol previously described [16].

*2.2. Extraction and quantification of phenylpropanoids*

Levels of free and β-glucosidase or esterase-hydrolysable phenylpropanoids were compared in the first leaf from control and infected cucumber and melon plants at different days after inoculation. For each enzymatic assay, three control and three infected leaves from cucumber or melon plants were used and the assay was repeated twice. The control and infected first leaves (about 0.5 g) were ground to a fine powder in a mortar using liquid nitrogen and homogenized in 1.5 mL 100% methanol. The extracts were then sonicated for 10 min and centrifuged in Eppendorf tubes with a microfuge for 15 min. The supernatant corresponding to each analysis was divided into two equal portions and dried at room temperature. The two dried residues were resuspended in 900 µl of the buffer corresponding to each enzymatic digestion: 50 mM

sodium acetate (pH 4.5) for β-glucosidase (EC 3.2.1.21), and 50 mM Tris-HCl (pH 8) for esterase (EC 3.1.1.1) digestions. Then, 10 U of almond β-glucosidase (14.3 U/mg, Fluka), or porcine esterase (250 U/mg, Sigma), dissolved in 100 µl of water were added to each enzymatic reaction and 100 µl of water without enzymes were added to the controls. The reactions were incubated overnight at 37 °C and then stopped by adding 75 µl of 70% perchloric acid to the incubation mixtures (final concentration about of 5% perchloric acid, v/v), and free and conjugated phenylpropanoids were extracted with a mixture of cyclopentane/ethyl acetate (1:1, v/v). The organic phase was removed, dried under nitrogen at room temperature, and the residue dissolved in 200 µl of methanol. Samples were filtered through 0.45 µm nylon filters (Waters) prior to HPLC analysis. Forty µl were injected with a Waters 717 autosampler into a reverse-phase X-Terra MS C18 5 µm (3.9 x 150 mm) column maintained in an oven at 30 °C. A 20-min linear gradient of 1% (v/v) acetic acid in water to 100% methanol at a flow rate of 1 ml/min was applied with a 600E Waters HPLC pump. Hydroxycinnamic acids were photometrically detected (320 nm) with a Waters 460 tunable absorbance detector, and quantified with the Waters Millennium[32] software, using synthetic standards. Data were corrected for losses in the extraction procedure, and recovery of metabolites ranged between 50 and 80%.

*2.3. Identification of phenylpropanoids*

To identify the structure of the major peaks from β-glucosidase-digested methanolic extracts from PNRSV-infected cucumber or MNSV-infected melon leaves, samples were analyzed by ESI-MS using a 1515 Waters HPLC binary pump, a 996 Waters photodiode detector (range of maxplot between 240 and 400 nm, spectral

resolution of 1.2 nm), and a ZMD Waters single quadrupole mass spectrometer equipped with an electrospray ionization ion source. The source parameters of the mass spectrometer for ESI in negative mode were the following: capillary voltage 2500 V, cone voltage 20 V, extractor 5 V, RF Lens 0.5 V, source block temperature 100 °C and desolvation gas temperature 300 °C. The desolvation and cone gas used was nitrogen at a flow of 400 l and 60 l per min, respectively. Other mass spectrometer conditions were: low mass resolution 13.5, high mass resolution 13.5, ion energy 0.5, multiplier 650. ESI data was acquired using the conditions of a full scan range from mass-to-charge ratio (*m/z*) 100 to 700 at 1 s per scan. Samples (20 µl) from β-glucosidase digested methanolic extracts were injected at room temperature into a reverse-phase X-Terra MS C18 5 µm (3.9 x 150 mm) column. A 20-min linear gradient of 1% (v/v) acetic acid (J. T. Baker) in Milli Q water to 100% methanol (J. T. Baker) at a flow rate of 1 ml/min was applied. A post-column split delivered approximately 25% of the flow to the mass spectrometer ant the rest to the Waters 996 photodiode array detector. Mass and UV-absorption spectra of the unknown and synthetic standard peaks were performed using the Masslynx Waters software. Samples (20 µl) from undigested and β-glucosidase digested methanolic extracts from cucumber and melon were subjected to selected ion recording (SIR) analysis with the following parameters: dwell 0.5 s, cone voltage 20 V, inter-channel delay 0.05, and span1 in ESI negative mode. The HPLC conditions were the same to those described above.

### *2.4. Preparation and analysis of RNA*

RNA from control or virus infected cucumber and melon leaves were prepared using the TRIzol reagent (Gibco BRL), according to the manufacturer's instructions.

For Northern analyses, 15 µg of total RNA were electrophoresed in 1.2% agarose gels in the presence of formaldehyde, then transferred to Nytran membranes and hybridized using standard procedures. DNA probes for cucumber PAL, peroxidase and chitinase were prepared by RT-PCR of RNA from virus-infected cucumber leaves. cDNA synthesis of 10 µl total RNA was accomplished by using 100 U of M-MLV reverse transcriptase (Promega), using an 18-mer oligo(dT) as a primer as previously described [10], then subjected to PCR amplifications with the suitable primers. To obtain the cucumber PAL probe [17], the following primers sequences were used: forward 5'-AGTTGCATGAAATGGATCCTC-3' and reverse 5'-TTATGTTGCTCGGCACTTTG-3'. For cucumber peroxidase [18], the primer sequences were: forward 5'-CGACGTATCCAACATTGTGC-3' and reverse 5'-CGGTGTTGGACTGAAGGTTT-3'. The cucumber chitinase probe [19] was prepared as previously described [10]. Radioactive DNA probes were prepared using the RediPrime II labelling kit (GE Healthcare). Hybridizations and high stringency washes were performed as described by Church and Gilbert [20].

*2.5. Construction of the complete C4H cDNA sequence from cucumber*

Degenerate primers were designed based on alignments of different plant C4H sequences (accession numbers **AY065145**, **Y09447**, **L07634**, **AF548370** and **AF255014**). Forward (5'-AAGGGAGAATCAACGA-3') and reverse (5'-CGTAA(CT)TG(CT)CC(AT)CCTTTCTC-3') oligonucleotides were used to amplify the reverse-transcribed cDNAs from PNRSV-infected cucumber leaves as a template. A 600-bp sequence was obtained, and the DNA band was extracted from agarose gels by using the QIAquick gel extraction kit (QIAGEN), cloned in pGEM-T-Easy (Promega)

and sequenced on both strands. To obtain the unknown 3' end, a RACE procedure was performed according to Frohman et al. [21]. Reverse transcription of RNAs from virus-infected cucumber leaves was primed with a $(dT)_{17}$-adaptor oligonucleotide (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3'), followed by a PCR amplification using the same $(dT)_{17}$-adaptor as reverse primer, and a specific oligonucleotide deduced from the cloned cucumber C4H sequence (5'-AGATAATGTGCTTTACATTG-3') as the forward primer. Ten µl of this reaction were re-amplified using the same forward primer, but only the adaptor sequence (5'-GACTCGAGTCGACATCGA-3') as a reverse primer. This second amplification yielded an 870-bp DNA fragment, which was cloned and sequenced as described above. The 5' end of cucumber C4H was obtained by PCR amplification of the reverse transcription reaction previously described, using an internal, specific sequence of cucumber C4H as a reverse primer, and a direct, degenerate primer based on the N-terminal region MDLLLLEK, which is highly conserved among plant C4H enzymes. Forward (5'-ATGGA(CT)CT(CT)CT(AC)(CT)T(CG)(CT)T(AG)GA(AG)AA-3') and reverse (5'-CATAATGTTTCAATTGCTGC-3'), amplified a 950-bp DNA fragment which was cloned and sequenced as indicated. Finally, a specific set of primers was used to amplify the complete cucumber C4H cDNA, and to confirm the sequence data obtained in the previous steps. The EMBL accession number for this sequence is **AM284167**. A 600-bp DNA fragment was also obtained from reverse-transcribed MNRSV-infected melon leaves, using the forward (5'-AAGGGAGAATCAACGA-3') and reverse (5'-CGTAA(CT)TG(CT)CC(AT)CCTTTCTC-3') degenerate primers described above for cucumber. The EMBL accession number for the melon C4H sequence is **AM284168**.

## 3. Results and discussion

### *3.1. Structural characterization of phenylpropanoids accumulated in virus-infected cucumber and melon plants*

Methanolic extracts from PNRSV-infected cucumber and MNSV-infected melon leaves, showing strong symptoms of the disease, were digested with β-glucosidase or esterase. Figure 1 depicts representative HPLC chromatograms of β-glucosidase-hydrolysable methanolic extracts from both PNRSV-infected cucumber (left), and MNSV-infected melon (right) with the corresponding control mock-inoculated plants at day 14 after inoculation. Similar HPLC elution profiles were obtained when analysing methanolic extracts hydrolysed with esterase (data not shown). To survey the metabolites of the phenylpropanoid pathway, the 320 nm wavelength was selected after studying the absorbance spectra recorded with the diode array detector by scanning from 240 to 400 nm. As observed, two (9.46- and 9.82-min relative retention time) and three (6.01- 9.46-, and 9.82-min relative retention times) peaks were found in extracts from the first leaf of PNRSV-infected cucumber and MNSV-infected melon plants, respectively. These peaks were essentially absent in equivalent first leaves from mock-inoculated cucumber or melon plants. To precisely identify these unknown compounds, an HPLC-MS analysis combined with electrospray ionization (ESI) of soluble-methanol phenolic fraction from cucumber and melon infected leaves was performed. The mass spectra from total ion current chromatograms showed deprotonated fragment ions of *m/z* 163 and 193 $[M-H]^-$ for cucumber extracts which coincided with the molecular weight of *p*-coumaric (4-hydroxycinnamic, *M*r = 164) (the possibility that the hydroxycinnamic was *o*-coumaric was excluded because its retention

time was clearly different from that of *p*-coumaric), and ferulic (4-hydroxy-3-methoxycinnamic, *M*r = 194) acids, respectively. Similarly, deprotonated fragment ions of *m/z* 163, 179 and 193 $[M-H]^-$ were obtained for melon extracts coinciding with the molecular weight of *p*-coumaric, caffeic (3,4-dihydroxycinnamic, *M*r = 180), and ferulic acids, respectively. The molecular fragmentation of these compounds gave the anticipated results for this type of structures (Table 1). In addition, retention times corresponding to single ion chromatograms of *m*/*z* species of 163, 179, and 193 (Fig. 1), matched well with those of UV-absorbing peaks corresponding to 6.01-, 9.46- and 9.82-min retention times, respectively, and their absorbance maxima (Table 1) coincided with those of authentic *p*-coumaric, caffeic and ferulic acids. On the other hand, β-glucosidase is considered to be specific for β-glucosidic bounds, but not for glucose. Interestingly, esterase can digest both ester-glucosides and other esters, such as shikimate and quinate esters or chlorogenic acid, which are major forms of free phenolics in plants involved in stress responses in many plants [13, 14]. Taking this into account, to verify the nature of the molecule conjugated to the hydroxycinnamic acids, we used the sensitivity of the selecting ion recording (SIR) technique. Using this analysis in the negative mode, we analyzed the non digested extracts for the presence of the ion fragments indicated in Table 2, which corresponded to β-glucosides of hydroxycinnamic acids, shikimate and quinate esters. Figure 2A shows a SIR chromatogram of *m/z* species of 325 (retention time 5.39 min), and 355 (retention time 5.95 min) in undigested methanolic extracts from control and PNRSV-infected cucumber which correspond to β- glucosides of *p*-coumaraic and ferulic acids. Figure 2B shows a SIR chromatogram of *m/z* species of 325 (retention time 5.34 min), 341 (retention time 4.08 min), and 355 (retention time 5.54 min) in undigested methanolic extracts from control and MNSV-infected melon corresponding to β-glucosides of *p*-

coumaraic, caffeic, and ferulic acids. These signals disappeared when methanolic extracts were hydrolyzed with β-glucosidase (Figs. 3A and 3B), and also with esterase. Interestingly, β-glucosidase hydrolysis of PNRSV-infected cucumber extracts also gave rise to the *m/z* 163 and 193 fragment ions, which correspond to those of *p*-coumaric and ferulic acids, respectively (Fig. 3A). β-glucosidase hydrolysis of MNSV-infected melon leaves gave rise to the *m/z* 163, 179, and 193 fragment ions, which match those of *p*-coumaric, caffeic and ferulic acids, respectively (Fig. 3B). The retention times of the signals coincided with those of authentic standards, thus suggesting that *p*-coumaric, caffeic and ferulic acids are conjugated to glucose. In addition, hydrolysis of methanolic extracts with esterase produced the same results (data not shown). These results strongly indicate that *p*-coumaric, caffeic, and ferulic acids are ester-linked to the anomeric carbon atom of the glucose through a β-glucosidic linkage, thus forming 1-*O*-coumaroyl-β-glucose, 1-*O*-caffeoyl-β-glucose, and 1-*O*-feruloyl-β-glucose, respectively. Accumulation of two of these metabolites, 1-*O*-(4-coumaroyl)-β-glucose and 1-*O*-feruloyl-β-glucose, was also observed during the growth of cell suspension cultures of *Chenopodium rubrum* [22]. Because esterase can also hydrolyse shikimate and quinate esters, and therefore, the hydrolysis of these esters may explain the amount of free hydroxycinnamic acids obtained when esterase are used, we looked for the presence of fragment ions of *m/z* 319, 337, 335, 353, 349, and 367 corresponding to shikimate and quinatae esters of *p*-coumaric, caffeic and ferulic acids, respectively (Table 2). No detectable fragment ions of such *m/z* species were found (data not shown).

### *3.2. Induction of phenylpropanoids in PNRSV-infected cucumber and MNSV-infected melon plants.*

In order to study the accumulation profile of these compounds, time-course studies were performed on both PNRSV-infected cucumber and MNSV-infected melon leaf extracts after hydrolysis with β-glucosidase or esterase. The first systemically infected leaf from both cucumber and melon was used for these studies because a strong accumulation of the viruses was observed there [12, 23]. Induction kinetics of the metabolites after virus inoculation was very similar in both infections. HPLC analysis indicates that practically no detectable levels of free hydroxycinnamic acids were found in soluble methanol extracts from PNRSV-infected cucumber and MNSV-infected melon leaves during symptom development (Figs. 4 and 5). However, when β-glucosidase or esterase-hydrolysable hydroxycinnamic glucosides were analysed, an increment of *p*-coumaric and ferulic acids in infected cucumber (Fig. 4), and *p*-coumaric, caffeic, and ferulic acids in infected melon plants (Fig. 5), was observed. Similar induction kinetic patterns of these metabolites, although in lower amounts were observed in virus infected melon plants. These metabolites started to accumulate the first week after inoculation, and they progressively reached considerable levels (5-10 μg/g FW) at days 14 and 21 after cotyledon inoculation (Figs. 4 and 5). Moreover, the increment of phenylpropanoids paralleled the development and severity of symptoms. These results seem to indicate that free hydroxycinnamic acids are rapidly conjugated after their synthesis, which is in agreement with what is generally found in plants, i.e.: free forms of phenolics are, normally, much less abundant than their conjugated counterparts [4, 24]. The results presented here extend the findings of Daayf et al. [25], which demonstrated an increase of *p*-coumaric, caffeic, and ferulic acids in leaves from cucumber treated with powdery mildew. These authors suggest that these phenylpropanoids could be instrumental in repressing powdery mildew infection. It is tempting to speculate that the accumulation of hydroxycinnamic acids is also involved

in virus resistance, as suggested by our results. Future studies will be aimed at elucidating this important point, as this would represent a general mechanism of plant defence against disparate biotic stresses.

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*3.3. Cloning of virus-induced cucumber cinnamate 4-hydroxylase*

Cinnamic acid 4-hydroxylase catalyses the hydroxylation of cinnamic acid to produce *p*-coumaric acid [26, 27], and successive hydroxylation and methylation
320 reactions give rise to caffeic and ferulic acids, respectively [4]. Since we have found a notable increment in the phenylpropanoid metabolites *p*-coumaric, caffeic and ferulic in infected cucumber and melon plants, we investigated whether the infection were also accompanied by an increase in the expression of C4H enzyme. For this purpose, we isolated and characterised a cucumber cDNA clone based on sequence alignments. A set
325 of degenerate primers was designed to amplify a conserved C4H sequence using RNA isolated from virus-infected cucumber leaves as a template. Based on this core sequence, we obtained the 3' end of the cDNA by RACE [20], whereas the 5' region was amplified using degenerate primers corresponding to the highly conserved N-terminal sequence of different plant C4H enzymes. The complete cucumber cDNA
330 spans a 1695 nucleotide sequence which displays striking homologies to other plant C4Hs. The deduced amino acid sequence (Fig. 6) indicates the presence of a short N-terminal signal peptide, most probably corresponding to a signal-anchor sequence [28], which is a common feature of P450 proteins [29]. This domain is followed by the sequence PPGPLPVP, which has been proposed to be involved in the correct orientation
335 of P450s in the ER membranes [30, 31]. Near the carboxyl terminus is the sequence PFGAGRRSCPG, corresponding to the conserved heme-binding domain of Group A

plant P450s [32]. Using the above mentioned degenerate oligonucleotides, we were also able to amplify a 569-bp DNA using virus-infected melon mRNAs as a template. This nucleotide sequence is 96% identical to the cucumber C4H cDNA, and its deduced amino acid sequence also includes the conserved heme-binding domain (not shown). GenBank/EMBL accession numbers for the sequences are **AM284167** and **AM284168** for cucumber and melon C4H, respectively. C4Hs from several plant species have been described as being encoded by single genes, or by a very low copy number set of genes [33-35]. Southern analyses in our laboratory, using cucumber and melon C4H cDNAs probes, indicate that the same occurs in *Cucurbitaceae* (data not shown).

*3.4. Virus induction of cinnamate 4-hydroxylase and phenylalanine ammonia-lyase*

To correlate the elevated levels of phenylpropanoids found in virus-infected leaves with the expression pattern of C4H, RNAs from control and infected cucumber and melon leaves were subjected to Northern analysis, using the respective C4H sequences as probes. Figure 7 shows the time course of accumulation of C4H transcripts after PNRSV and MNSV infection of cucumber and melon plants, respectively. C4H transcript levels were increased 5 days after inoculation of the plants. This increment coincided with the first appearance of the symptoms of the disease and preceded the induction of phenylpropanoids which was evident 7 days after inoculation of the plants.. A steady level of C4H transcripts was maintained during the 17 day experiment. A basal level of C4H was observed in control samples, which is consistent with the fact that C4H is a key enzyme for phenylpropanoid metabolism. A similar induction pattern of C4H was observed in melon plants inoculated with MNSV (Fig. 7). The increase of C4H expression levels appears to be coordinated with the induction of the PAL gene,

encoding the preceding enzyme in the phenylpropanoid pathway. PAL transcripts also increase in a similar manner upon PNRSV and MNSV infection of cucumber and melon plants, respectively, whereas only a slight basal level can be detected in control plants, as is also the case for C4H. This result is in accordance with that observed for cucumber tissues infected with *Pseudomonas syringae,* in which an increment of PAL enzymatic activity was observed after inoculation of the pathogen [36].

### *3.5. Virus induction of defence proteins*

Recently, it has been reported [10] that PNRSV-infected cucumber leaves presented elevated total peroxidase enzyme activity and increased expression of the gene encoding the defensive 28 kDa cucumber chitinase [19]. Thus, we have studied the accumulation of these two defence genes in both melon and cucumber infected plants. Chitinase mRNA started to accumulate 5 days after inoculation of cotyledons from cucumber and melon with PNRSV or MNSV, respectively and reached a maximum between the days 11 and 14 after inoculation of the plants (Fig. 7). A similar pattern of induction, although to a somewhat lesser extent, of transcripts for chitinase was observed in melon plants infected with MNSV (Fig. 7). Levels of peroxidase mRNA started to increase 5 days after inoculation of both cucumber and melon cotyledons and progressively accumulated during the 17 day experiment experiment. MNSV infection of melon plants produced, although to a lower level, a similar pattern of induction of peroxidase transcripts (Fig. 7). This observation is in accordance with the results obtained in cucumber treated with ethylene [18], or infected with tobacco necrosis virus [19]. An increased level of peroxidase and chitinase in cucumber tissues after inoculation with different pathogens has been documented [37-39]. The possibility that

chitinase could be involved in enhancing the resistance of transgenic cucumber to the fungus *Botrytis cinerea* has been speculated [40]. In this and our previous work [10], we have observed elevated levels of chitinase in infected cucumber. The elucidation whether chitinase could play a role in cucumber defence to virus infection needs further investigation. The results obtained in this work indicate that activation of phenylpropanoid pathway occurs as a component of the response of cucumber and melon to viruses producing systemic infections, thus extending previous results obtained in plants infected with fungi to cucumber and melon infected by virus. Induction of C4H mRNA levels supports the role of this enzyme for the increased accumulation of phenylpropanoids in PNRSV-infected cucumber and MNSV-infected melon and is in accordance with previous suggestions [41-42] on its possible role in plant-pathogen interactions.

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Fig. 1. A) Representative absorbance chromatograms (recorded at 320 nm) from β-glucosidase-hydrolysable methanol leaf extracts of control and PNRSV-infected cucumber (upper chromatograms), and control and MNSV-infected melon (lower chromatograms) at day 14 after inoculation of the cotyledons. Absorbance was monitored by a photodiode array detector with a range of maxplot between 240 and 400 nm, and a spectral resolution of 1.2 nm. GA and SA represent peaks corresponding to gentisic and salicylic acids. The arrows show the unknown phenolic compounds with a retention time of 6.01, 9.46, and 9.82 min. B) Reconstructed single ion chromatograms from total ion current of *m*/*z* species of 163, and 193 (left), and of *m*/*z* species of 163, 179 and 193 (right).

Fig. 2. Representative selecting ion recording (SIR) chromatograms from undigested methanolic extracts of PNRSV-infected cucumber, and MNSV-infected melon plants, and their non-infected corresponding controls. A) SIR chromatograms of *m/z* species of 325 (retention time 5.39 min), and 355 (retention time 5.95) from undigested samples of PNRSV-infected cucumber plants. B) SIR chromatograms of *m/z* species of 325 (retention time 5.34 min), 341 (retention time 4.08 min), and 355 (retention time 5.54 min) from undigested samples of MNSV-infected melon plants.

Fig. 3. Representative selecting in recording (SIR) chromatograms from digested methanolic extracts of PNRSV-infected cucumber, and MNSV-infected melon plants and their undigested corresponding controls. A) SIR chromatograms of *m/z* species of 163 (retention time 9.42 min), and 193 (retention time 9.80 min) from β-glucosidase-hydrolysable extracts of PNRSV-infected cucumber plants. B) SIR chromatograms of *m/z* species of 163 (retention time 9.48 min), 179 (retention time 6.04), and 193

(retention time 9.86) from β-glucosidase-hydrolysable extracts of MNSV-infected melon plants.

Fig. 4. Time courses for the accumulation of *p*-coumaric and ferulic acids in PNRSV-infected cucumber leaves digested with β-glucosidase and esterase. White bars, *p*-coumaric and ferulic acids in control plants; gray bars, *p*-coumaric and ferulic acids in β-glucosidase-hydrolysable extracts of PNRSV-infected cucumber; hatched bars, *p*-coumaric and ferulic acids in esterase-hydrolysable extracts of PNRSV-infected cucumber. Results are the mean ± SE from three replicates.

Fig. 5. Time courses for the accumulation of *p*-coumaric, caffeic and ferulic acids in MNSV-infected melon leaves digested with β-glucosidase and esterase. White bars, *p*-coumaric, caffeic, and ferulic acids in control plants; gray bars, *p*-coumaric, caffeic, and ferulic acids in β-glucosidase-hydrolysable extracts of MNSV-infected melon; hatched bars, *p*-coumaric, caffeic, and ferulic acids in esterase-hydrolysable extracts of MNSV-infected melon. Results are the mean ± SE from three replicates.

Fig. 6. Comparison of deduced amino acid sequence for cucumber C4H protein with other representative C4H sequences: *Arabidopsis thaliana* **D78956**; *Medicago sativa* **L11046**; *Helianthus tuberosus* **Z17369**; *Ammi majus* **AY219918**. Putative N-terminal ER membrane anchor sequences, and heme-binding domains are indicated by brackets.

Fig. 7. Expression of cucumber and melon C4H, PAL, chitinase and peroxidase transcripts in PNRSV-infected cucumber, and MNSV-melon plants. Fifteen micrograms of total RNA isolated from cucumber (left), and melon (right) were separated on

formaldehyde gels, transferred to nylon membranes, and probed for the presence of the transcripts of C4H, PAL, chitinase, and peroxidase in both control and infected plants. Samples were collected at the indicated days after virus inoculation. A duplicate gel was stained with ethidium bromide as a control for RNA loading.

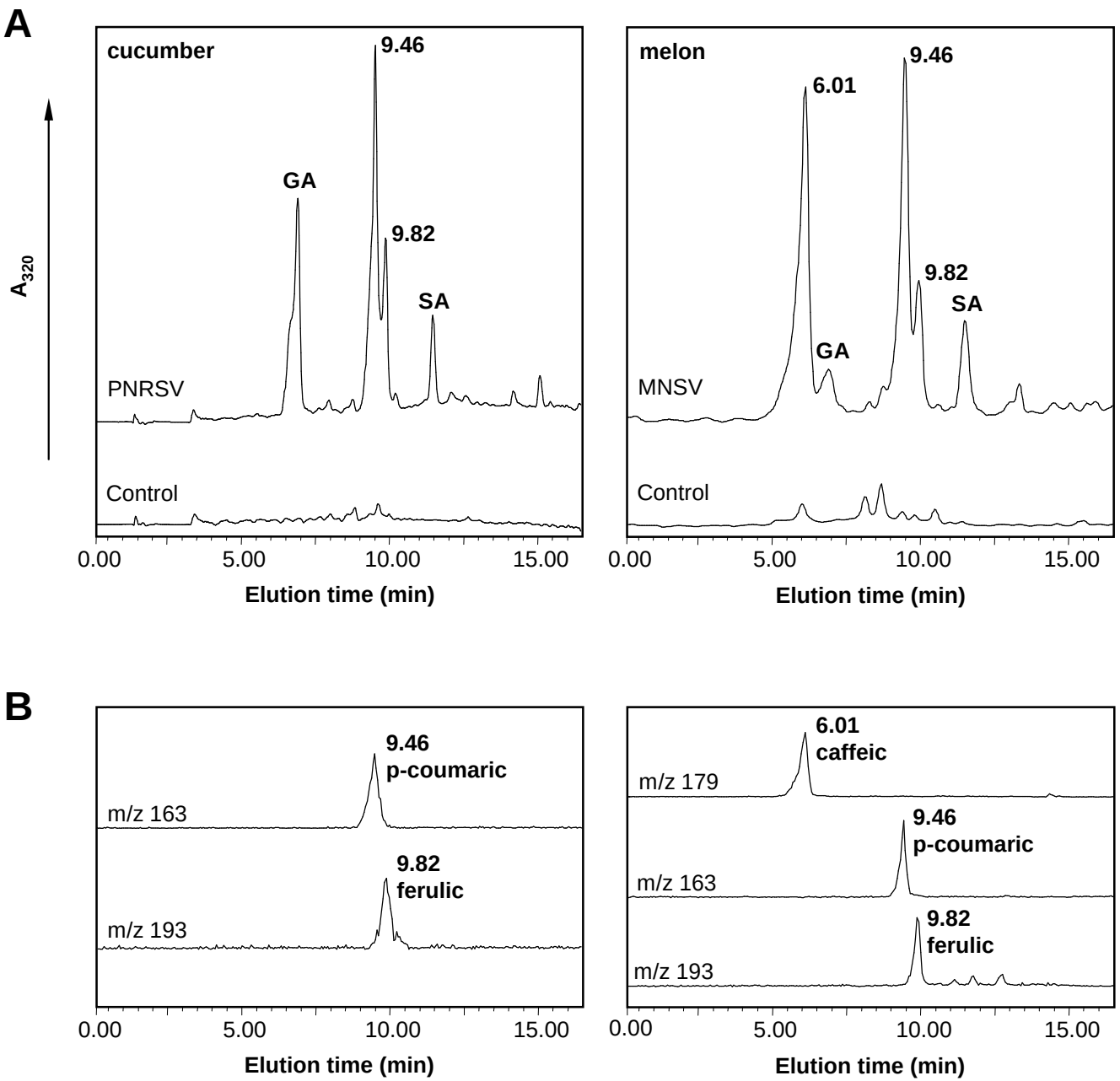
Table 1. ESI mass spectra in negative ion detection mode [*m/z* (%)], and absorbance maxima (nm) of compounds eluting at 6.01-, 9.46- and 9.82-min retention times.

| | Metabolites (retention time, min) | | |
|---|---|---|---|
| | 6.01 min | 9.46 min | 9.82 min |
| Negative Ions | | | |
| [M – H] | 179 (100%) | 163 (56%) | 193 (100%) |
| [M – H – $CH_3$] | | | 178 (7%) |
| [M – $CO_2H$] | 135 (15%) | 119 (100%) | 149 (10%) |
| [M – $CO_2H$ – $CH_3$] | | | 134 (15%) |
| $\lambda_{max}$ | 325 nm | 310 nm | 322 nm |

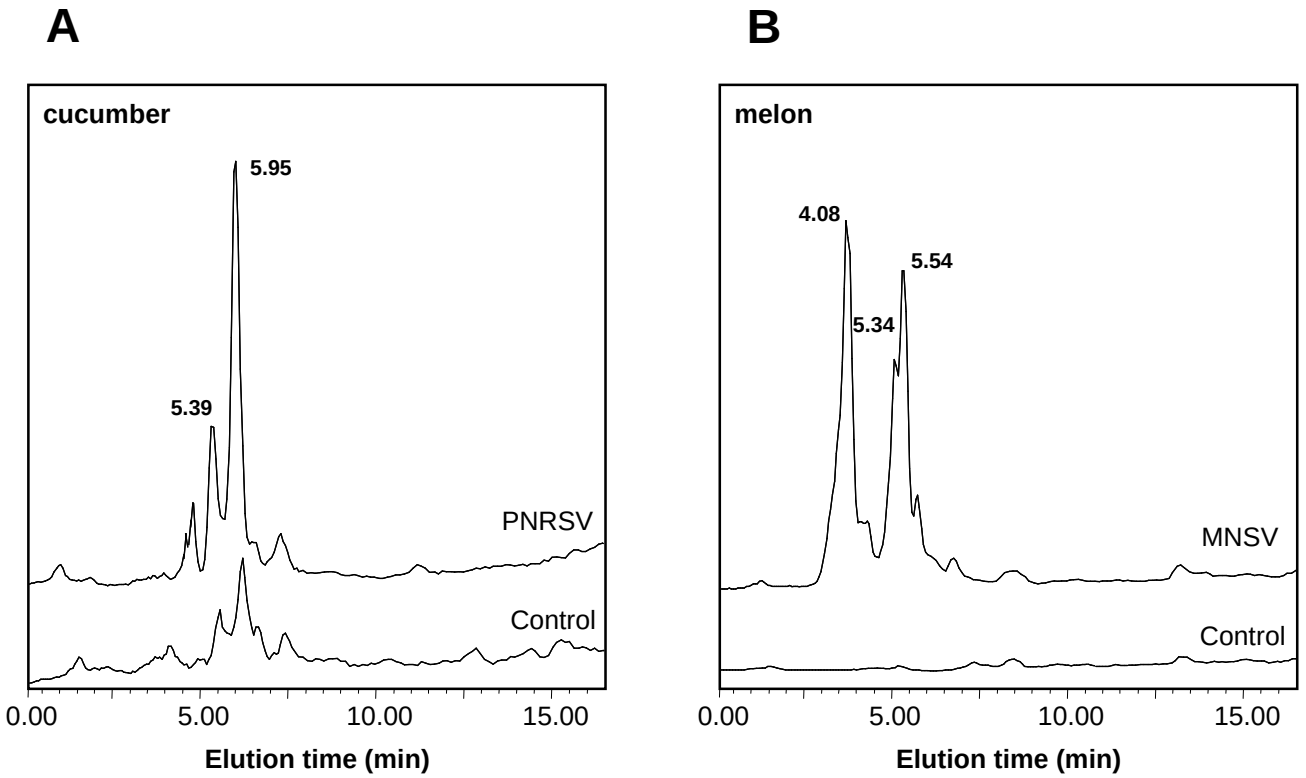
Table 2. Fragment ions searched by ESI-MS using the negative ion detection mode $[M-H]^-$ in total undigested methanolic extracts from PNRSV and MNSV infected cucumber and melon plants respectively.

| *m/z* | Fragment ion $[M-H]^-$ |
|---|---|
| 325 | 1-*O*-coumaroyl-β-glucose |
| 341 | 1-*O*-caffeoyl-β-glucose |
| 355 | 1-*O*-feruloyl-β-glucose |
| 319 | *p*-coumaroyl-shikimate |
| 337 | *p*-coumaroyl-quinate |
| 335 | caffeoyl-shikimate |
| 353 | caffeoyl-quinate |
| 349 | feruloyl-shikimate |
| 367 | feruloyl-quinate |

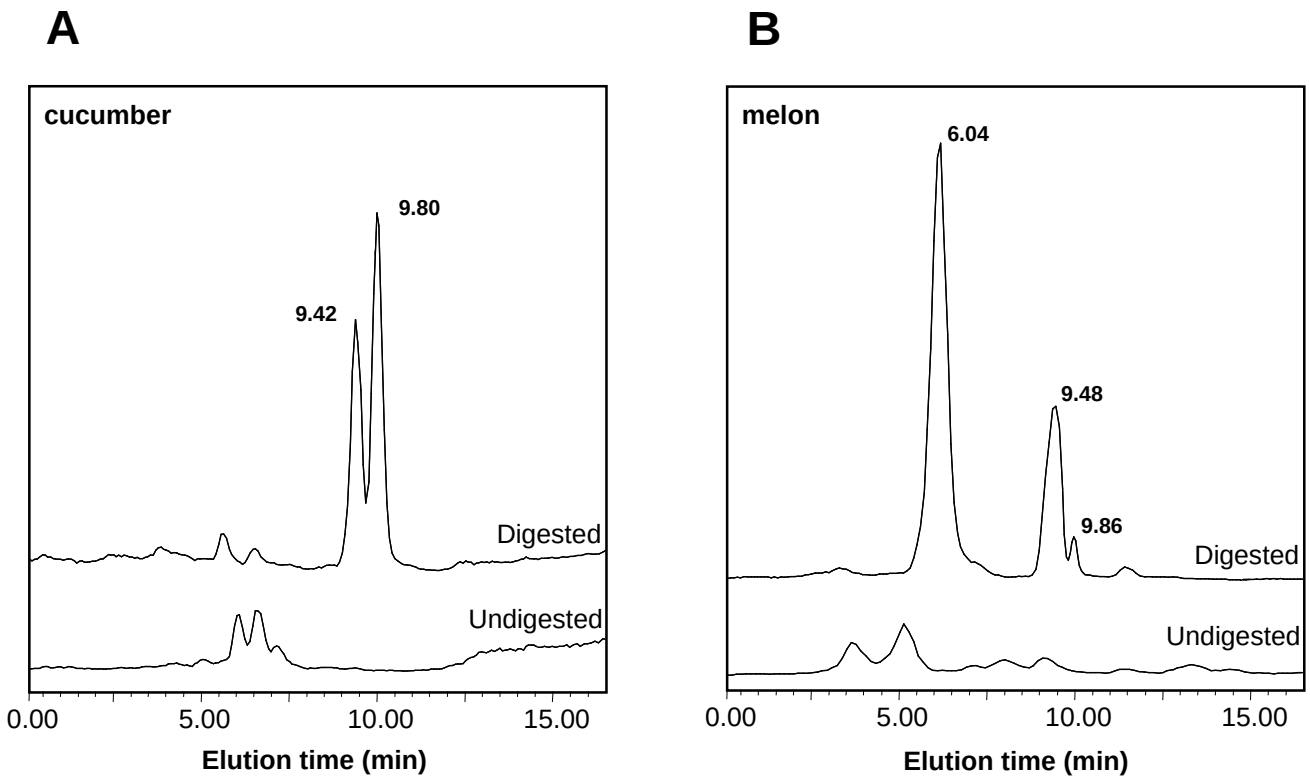
# Figure 1


A
cucumber
9.46
GA
9.82
SA
PNRSV
Control
A320
0.00
5.00
10.00
15.00
Elution time (min)
melon
9.46
6.01
9.82
SA
GA
MNSV
Control
0.00
5.00
10.00
15.00
Elution time (min)
B
9.46
p-coumaric
m/z 163
9.82
ferulic
m/z 193
0.00
5.00
10.00
15.00
Elution time (min)
6.01
caffeic
m/z 179
9.46
p-coumaric
m/z 163
9.82
ferulic
m/z 193
0.00
5.00
10.00
15.00
Elution time (min)

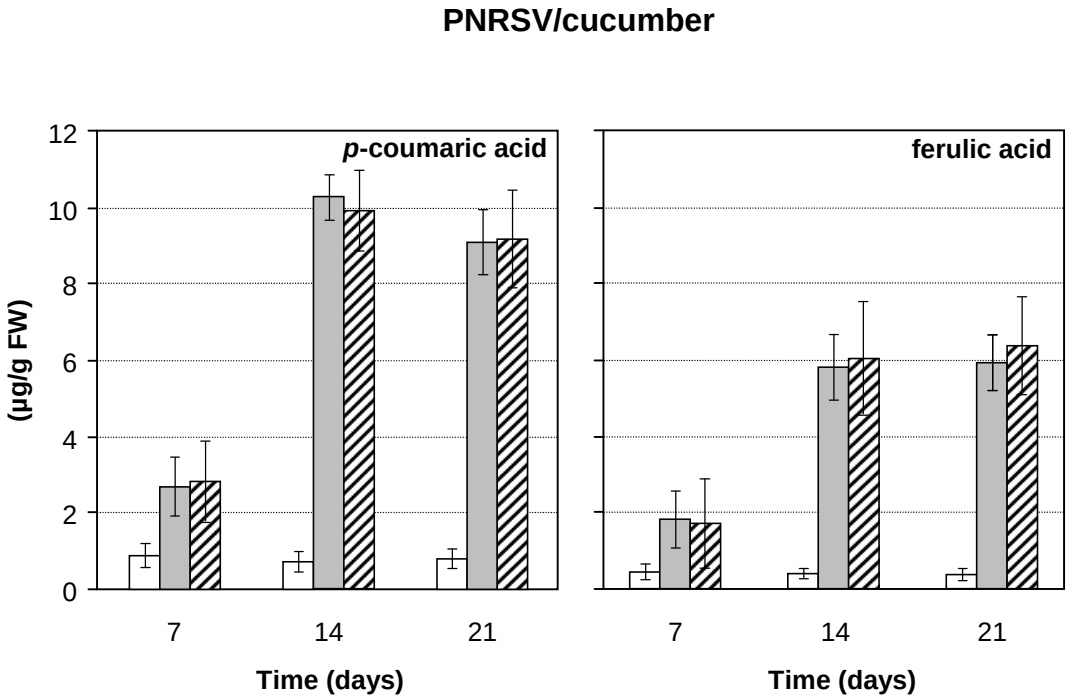
# Figure 2


A
cucumber
5.95
5.39
PNRSV
Control
0.00
5.00
10.00
15.00
Elution time (min)
B
melon
4.08
5.54
5.34
MNSV
Control
0.00
5.00
10.00
15.00
Elution time (min)

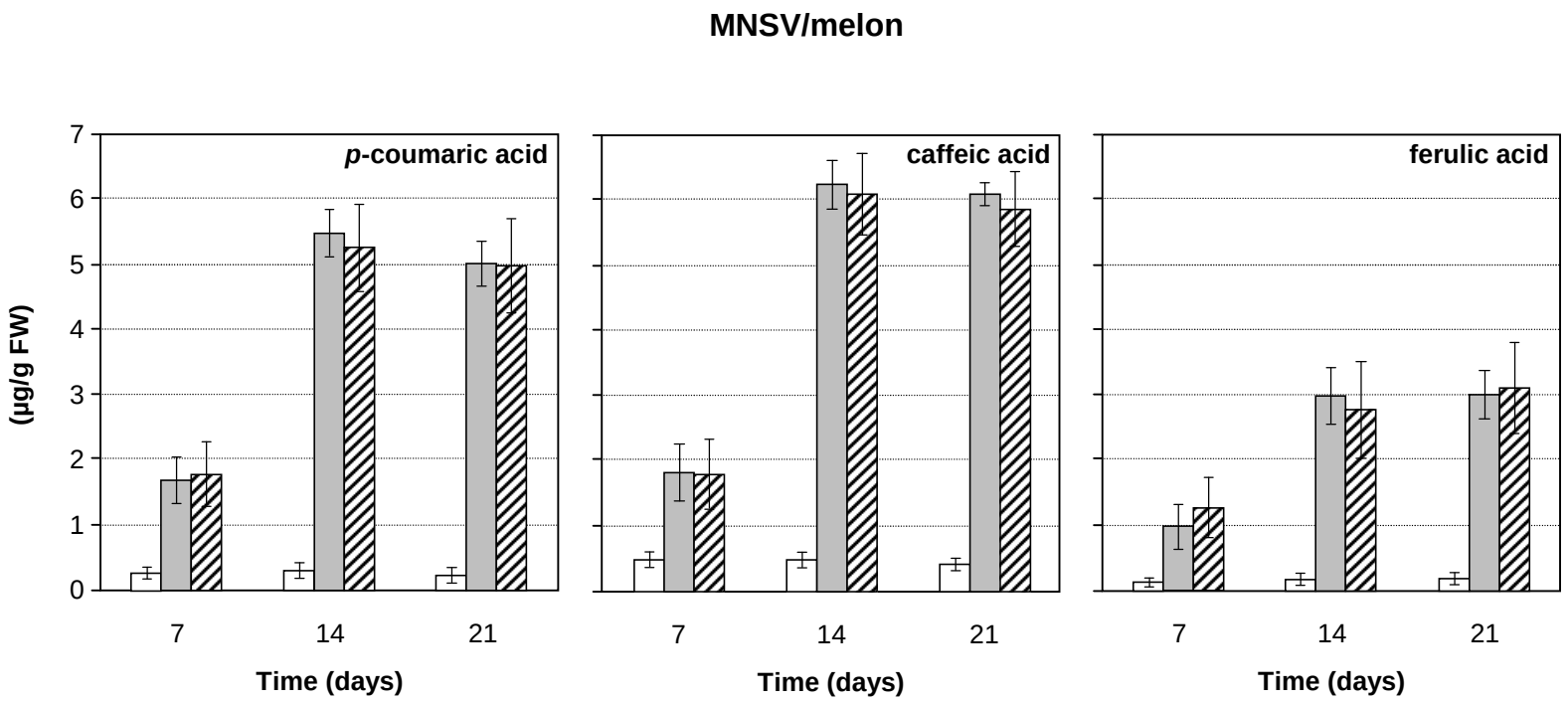
# Figure 3


A
cucumber
9.80
9.42
Digested
Undigested
0.00
5.00
10.00
15.00
Elution time (min)
B
melon
6.04
9.48
9.86
Digested
Undigested
0.00
5.00
10.00
15.00
Elution time (min)

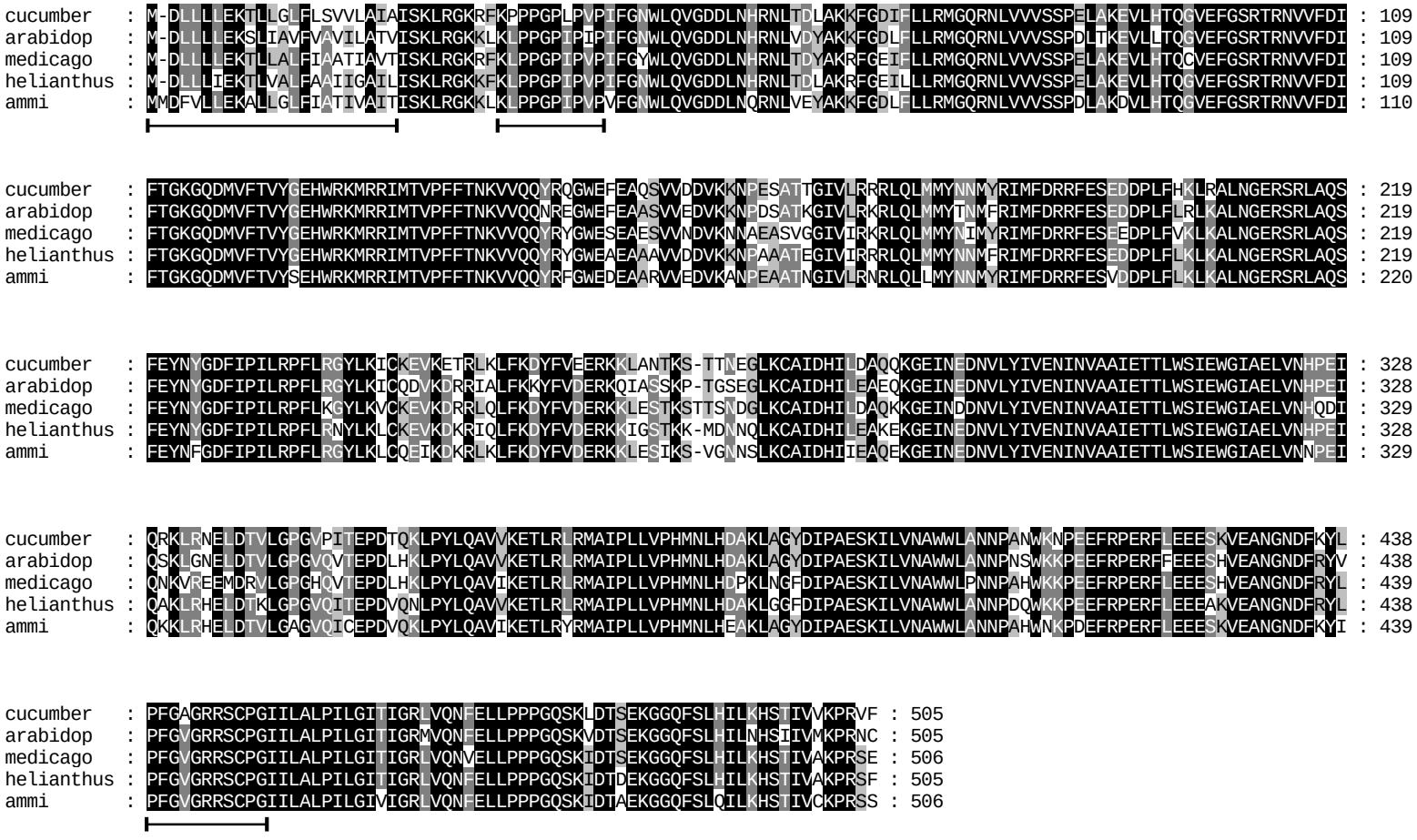
# Figure 4


PNRSV/cucumber
p-coumaric acid
ferulic acid
(µg/g FW)
0
2
4
6
8
10
12
7
14
21
Time (days)
7
14
21
Time (days)

# Figure 5


MNSV/melon
p-coumaric acid
caffeic acid
ferulic acid
(µg/g FW)
0
1
2
3
4
5
6
7
7
14
21
Time (days)

# Figure 6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arabidop : M-DLLLLEKSLIAVFVAVILATVISKLRGKKLKLPPGPIPIPIFGNWLQVGDDLNHRNLVDYAKKFGDLFLLRMGQRNLVVVSSPDLTKEVLLTQGVEFGSRTRNVVFDI : 109
medicago : M-DLLLLEKTLLALFIAATIAVTISKLRGKRFKLPPGPIPVPIFGYWLQVGDDLNHRNLTDYAKRFGEIFLLRMGQRNLVVVSSPELAKEVLHTQCVEFGSRTRNVVFDI : 109
helianthus : M-DLLLIEKTLVALFAAIIGAILISKLRGKKFKLPPGPIPVPIFGNWLQVGDDLNHRNLTDLAKRFGEILLLRMGQRNLVVVSSPELAKEVLHTQGVEFGSRTRNVVFDI : 109
ammi : MMDFVLLEKALLGLFIATIVAITISKLRGKKLKLPPGPIPVPVFGNWLQVGDDLNQRNLVEYAKKFGDLFLLRMGQRNLVVVSSPDLAKDVLHTQGVEFGSRTRNVVFDI : 110
cucumber : FTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRQGWEFEAQSVVDDVKKNPESATTGIVLRRRLQLMMYNNMYRIMFDRRFESEDDPLFHKLRALNGERSRLAQS : 219
arabidop : FTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQNREGWEFEAASVVEDVKKNPDSATKGIVLRKRLQLMMYTNMFRIMFDRRFESEDDPLFLRLKALNGERSRLAQS : 219
medicago : FTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRYGWESEAESVVNDVKNNAEASVGGIVIRKRLQLMMYNIMYRIMFDRRFESEEDPLFVKLKALNGERSRLAQS : 219
helianthus : FTGKGQDMVFTVYGEHWRKMRRIMTVPFFTNKVVQQYRYGWEAEAAAVVDDVKKNPAAATEGIVIRRRLQLMMYNNMFRIMFDRRFESEDDPLFLKLKALNGERSRLAQS : 219
ammi : FTGKGQDMVFTVYSEHWRKMRRIMTVPFFTNKVVQQYRFGWEDEAARVVEDVKANPEAATNGIVLRNRLQLLMYNNMYRIMFDRRFESVDDPLFLKLKALNGERSRLAQS : 220
cucumber : FEYNYGDFIPILRPFLRGYLKICKEVKETRLKLFKDYFVEERKKLANTKS-TTNEGLKCAIDHILDAQQKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEI : 328
arabidop : FEYNYGDFIPILRPFLRGYLKICQDVKDRRIALFKKYFVDERKQIASSKP-TGSECLKCAIDHILEAEQKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNHPEI : 328
medicago : FEYNYGDFIPILRPFLKGYLKVCKEVKDRRLQLFKDYFVDERKKLESTKSTTSNDGLKCAIDHILDAQKKGEINDDNVLYIVENINVAAIETTLWSIEWGIAELVNHQDI : 329
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ammi : FEYNFGDFIPILRPFLRGYLKLCQEIKDKRLKLFKDYFVDERKKLESIKS-VGNNSLKCAIDHIIEAQEKGEINEDNVLYIVENINVAAIETTLWSIEWGIAELVNNPEI : 329
cucumber : QRKLRNELDTVLGPGVPITEPDTQKLPYLQAVVKETLRLRMAIPLLVPHMNLHDAKLAGYDIPAESKILVNAWWLANNPANWKNPEEFRPERFLEEESKVEANGNDFKYL : 438
arabidop : QSKLGNELDTVLGPGVQVTEPDLHKLPYLQAVVKETLRLRMAIPLLVPHMNLHDAKLAGYDIPAESKILVNAWWLANNPNSWKKPEEFRPERFFEEESHVEANGNDFRYV : 438
medicago : QNKVREEMDRVLGPGHQVTEPDLHKLPYLQAVIKETLRLRMAIPLLVPHMNLHDPKLNGFDIPAESKILVNAWWLPNNPAHWKKPEEFRPERFLEEESHVEANGNDFRYL : 439
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cucumber : PFGAGRRSCPGIILALPILGITIGRLVQNFELLPPPGQSKLDTSEKGGQFSLHILKHSTIVVKPRVF : 505
arabidop : PFGVGRRSCPGIILALPILGITIGRMVQNFELLPPPGQSKVDTSEKGGQFSLHILNHSIIVMKPRNC : 505
medicago : PFGVGRRSCPGIILALPILGITIGRLVQNVELLPPPGQSKIDTSEKGGQFSLHILKHSTIVAKPRSE : 506
helianthus : PFGVGRRSCPGIILALPILGITIGRLVQNFELLPPPGQSKIDTDEKGGQFSLHILKHSTIVAKPRSF : 505
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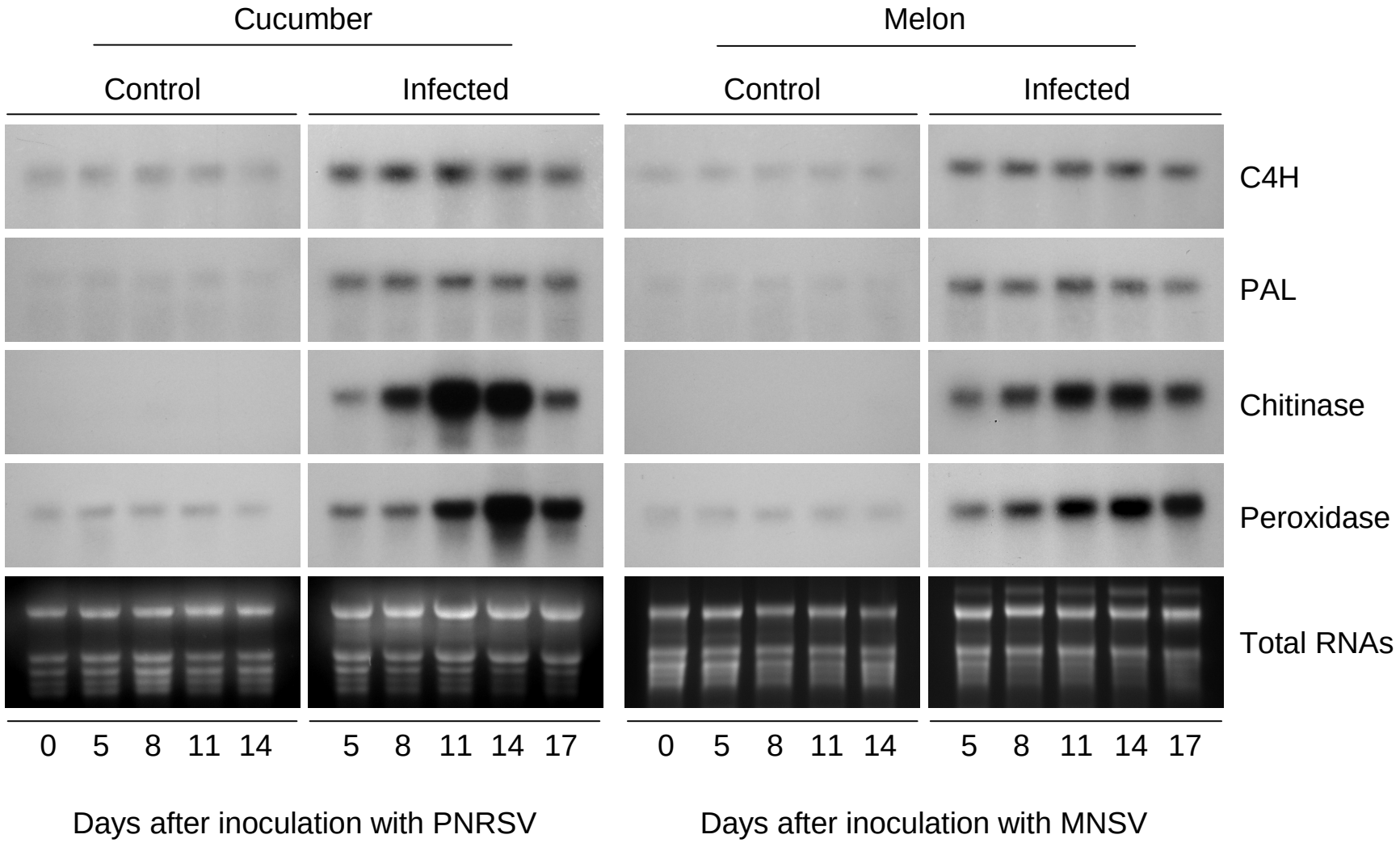
**Figure 7**


Cucumber
Melon
Control
Infected
Control
Infected
C4H
PAL
Chitinase
Peroxidase
Total RNAs
0 5 8 11 14
5 8 11 14 17
0 5 8 11 14
5 8 11 14 17
Days after inoculation with PNRSV
Days after inoculation with MNSV