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A novel cucumber gene associated with systemic acquired resistance

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Abstract

Several genes were isolated by differential display of mRNAs from cucumber leaves inoculated with the bacterium, *Pseudomonas syringae* pv. *lachrymans*. A full-length cDNA encoding a novel pathogen-induced gene, *Cupi4*, was cloned and characterized in detail. While *Cupi4* did not share evident homology with known sequences in the database at the nucleotide level, the predicted amino acid sequence of *Cupi4* shared homology with the pathogen-inducible proteins, pMB57-10G 5′ of *Brassica napus* (21%) and *CXc750*/*ESC1* of *Arabidopsis thaliana* (16%). *Cupi4* transcripts accumulated after 12 h in leaves inoculated with *P. s. lachrymans* and after 48 h in the systemic upper leaves of the inoculated plants. Treatment with the chemical inducers of systemic acquired resistance (SAR), salicylic acid, 2,6-dichloroisonicotinic acid and benzothiadiazole as well as inoculation with different pathogens, *P. s. syringae*, *Colletotrichum lagenarium* and tobacco necrosis virus also led to the accumulation of *Cupi4* transcripts. The increase of *Cupi4* transcripts in both the inoculated first leaf and in systemic upper leaves suggested that the *Cupi4* gene product is associated with systemic acquired resistance in cucumber. Induced expression of CUPI4 in different host strains of a bacterium, *Escherichia coli*, led to death of bacterial host cells, suggesting that CUPI4 might have antibacterial properties.

Keywords: Class III chitinase; Cucumber; *Cupi4*; *Pseudomonas syringae* pv. *lachrymans*; Pathogen-induced proteins; Systemic acquired resistance

1. Introduction

Application of a necrotizing pathogen or a chemical inducer to plants can result in a defense mechanism called systemic acquired resistance (SAR). It is a form of resistance that involves a local, hypersensitive response, as well as in protection of tissues distant from the site of first inoculation to a subsequent infection by the same and/or different pathogens. SAR usually exhibits a broad range resistance and provides long lasting protection [1–4]. Activation of inducible defenses depends upon recognition of the invading pathogen via a number of signal transduction pathways. Several signal molecules, including salicylic acid (SA), jasmonic acid (JA) and ethylene, have been identified. In Arabidopsis, a regulatory protein, *NPR1* has been implicated as a requirement for both SAR and induced systemic resistance. It mediates cross-talk between salicylic acid and jasmonic acid signaling pathways [5–8]. A number of mutants compromised in their ability to be induced to the SAR state have been documented in Arabidopsis [1,9]. During SAR, new proteins called pathogenesis-related (PR) proteins accumulate in infected tissues as well as uninfected systemic tissues [1–4,10]. Several chemicals including salicylic acid (SA), arachidonic acid, 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothionic acid *S*-methyl ester (BTH) are known as inducible substances for SAR, and their application leads to the expression of the same set of SAR genes as inoculation with pathogens [11–14].

Tobacco (*Nicotiana tabacum*), cucumber (*Cucumis sativus*) and Arabidopsis have been used as models for understanding host–pathogen interactions of the SAR [1–4]. In cucumber, induction of SAR by different pathogens as well as by SA, INA or BTH has been reported [15–20]. Infection of young cucumber plants with different pathogens can lead to broad spectrum SAR to at least 13 diseases and can protect plants from several pathogens for 4–6 weeks [21]. An increased level of chitinase, peroxidase, β-1,3-glucanase and lipoxygenase both in local and systemic tissues after inoculation with *Colletotrichum lagenarium*, tobacco necrosis virus (TNV),

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Pseudomonas syringae pv. *syringae* or *P. s. lachrymans* has been documented [19,22–26]. Among these proteins, cucumber class III chitinase has been used as a marker for SAR in cucumber because its expression is very low or undetectable in control tissues and is increased strongly in inoculated as well as systemic tissues after inoculation with TNV, and after treatment with SA, INA or BTH [23,27–29]. In this work, we used mRNA differential display to isolate novel genes that are expressed strongly during acquired resistance in cucumber induced by *P. s. lachrymans*. We isolated several novel cDNAs and one of them, cucumber pathogen-induced 4 (*Cupi4*), was characterized. *Cupi4* transcripts accumulated locally and systemically after inoculation with several pathogens and after treatment with SA, INA or BTH. These results support the hypothesis that *Cupi4* is associated with SAR in cucumber.

2. Materials and methods

2.1. Plant materials, treatment with different pathogens, chemical inducers of SAR and wounding

Cucumber plants (*C. sativus* L., cv. Wisconsin SMR-58) were grown in a greenhouse with a 14-h photoperiod. The first leaves were inoculated at 10 sites per leaf with *P. s. lachrymans* or *P. s. syringae* at a concentration of 2×10^8 cells/ml (A260 = 0.075) using a needleless syringe. Control plants were treated with water. Five microliters droplets of the spore suspension (2×10^5 spores/ml) of *C. lagenarium* were applied on the first leaves of cucumber plants. Plants treated with water were used as controls. For TNV treatment, one TNV-inoculated cotyledon of cucumber was ground in 2 ml of water, the homogenate was filtered through two layers of Miracloth and 10 mg of celite was added. The first leaves of cucumber plants were gently rubbed with the suspension of TNV particles and celite. Plants treated with the mixture of celite and water were used as controls.

One millimolar of SA, INA and BTH were applied by soil-drench application. As a control for SA, or INA and BTH treatment, plants were treated, respectively, with water or wetting powder. For wounding, the first leaves were squeezed with flat-bladed pliers at six different sites.

2.2. RNA extraction and mRNA differential display

The first leaves of cucumber plants grown in the greenhouse were inoculated on the border with the bacterial pathogen, *P. s. lachrymans* as described above and the inoculation with water was used as control. RNA from the middle non-inoculated area was isolated from duplicates of control and inoculated leaves 48 h after inoculation. For RNA isolation, 0.5–3 g of leaf material was ground in liquid nitrogen with a mortar and pestle. The ground powder was added to 8 ml of a 1:1 (v/v) mixture of phenol-chloroform-isoamyl alcohol and 2× NETS buffer (200 mM NaCl, 2 mM Na_2EDTA, 20 mM Tris–HCl pH 7.5, 1% (w/v) SDS). The slurry was vortexed vigorously, centrifuged and the upper phase was extracted twice with 4 ml chloroform. The RNA was precipitated by mixing the upper aqueous phase with an equal volume of 6 M LiCl for 16 h at 4 °C. After centrifugation at 10,000 × *g*, the RNA pellet was resuspended by adding 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volume of ethanol. After centrifugation, the RNA pellet was resuspended in TE buffer pH 8 (10 mM Tris–Cl, 1 mM EDTA), and was quantified by UV spectrophotometer and the solution was treated with RNase-free DNaseI (Boehringer) following manufacturer's instructions. mRNA differential display was performed according to Liang et al. [30] and Liang and Pardee [31]. The differentially expressed cDNAs were cloned in pBluescript vector (Stratagene) and were analyzed further.

2.3. RNA gel blot analysis

RNA samples (10 μg/lane) were separated on a 1% agarose gel containing formaldehyde [32] and were transferred for 16 h to a Hybond-N nylon membrane (Amersham) according to the manufacturer's instructions. The membrane was then air-dried and the RNA was cross-linked to the membrane with UV light (312 nm) for 3 min. Prehybridization was performed in a prehybridization solution (0.5 M phosphate buffer pH 7.2/7% SDS/1% bovine serum albumin) for 2 h at 65 °C, followed by hybridization in the same buffer overnight at 65 °C. The membrane was washed twice in 0.2× SSC (0.15 M NaCl/0.015 M Na_3 citrate)/0.1% SDS at 65 °C and exposed to an X-OMAT AR film (Kodak). The probes were radiolabelled with α-^{32}P-dATP using the RadPrime DNA labeling system (Gibco-BRL).

2.4. Screening of a cDNA library from infected plants

The single-stranded cDNA isolated by differential display was used as a probe to isolate the full-length cDNA in a λZAP cDNA library [32,33] made from systemic second leaves of cucumber plants which were infected on the first leaf with *P. s. syringae* (kindly provided by Dr. Ray Hammerschmidt, Michigan State University, East Lansing, MI, USA). Three plaque lifts per plate were performed on reinforced nylon membranes (Schleicher & Schuell, Germany). Prehybridization and hybridization were carried out under high stringency at 65 °C, under the same conditions as described for the RNA gel blot analysis. The Exassist helper phage (Stratagene) was used for in vivo excision of cDNAs from phage to pBluescript plasmid, following the manufacturer's instructions.

2.5. Genomic DNA gel blot analysis

Genomic DNA was extracted from leaves of cucumber and Arabidopsis plants as described [34]. A 10-μg portion of DNA was digested with the restriction enzymes, *Bam*HI, *Hin*dIII, *Eco*RI, or *Eco*RV. Digested genomic DNA was subjected to electrophoresis on a 1% agarose gel. The gel was then depurinated in 0.25N HCl for 15 min, denatured in 0.5N NaOH/1.5 M NaCl for 30 min, neutralized in 0.5 M Tris–HCl (pH 8.0)/1.5 M NaCl for 30 min, and blotted for 16 h to a Hybond-N nylon membrane (Amersham) according to the manufacturer's instructions. The DNA was cross-linked to the membrane with UV light (312 nm) for 3 min. The α-^{32}P-dATP radiolabelled

probe was generated using the RadPrime DNA labeling system (Gibco-BRL). Hybridization was carried out overnight at both high (65 °C) and low (50 °C) stringency. Washing steps were done twice with 0.2× SSC/0.1% SDS, at 65 and 50 °C for high and low stringency, respectively. The membrane was exposed to an X-OMAT AR film (Kodak).

2.6. Overexpression of Cupi4 cDNA in Escherichia coli

To limit potential toxic effects of *Cupi4* in *E. coli*, the sequence encoding for mature *Cupi4* without signal peptide was cloned into an isopropylthiogalactoside (IPTG)-inducible expression plasmid, pQE30 (Qiagen, CA). The 5′forward primer was designed with an in-frame *Bam*HI site (underlined), GTGGGATCCCGGCCTTATTACTTG and the 3′reverse primer incorporated a PstI site (underlined), CGCTGCAGGACGACAACACACC. PCR conditions were as follows: 30 cycles with the following steps: 94 °C for 30 s, 50 °C for 2 min, 72 °C for 2 min and ended with an additional extension step at 72 °C for 5 min. The PCR product was subcloned into the same restriction sites in the *E. coli* expression vector pQE30, to generate the amino-terminal six-histidine (6xHis)-tag recombinant protein. The resulting plasmid, pQE30-Cupi4 was selected and transformed into moderate expression *E. coli* host strains M15 (Qiagen, CA) or BL21(DE3) (Novagen, WI), or into a high-stringency expression host strain BL21(DE3)pLysS (Novagen, WI) via electroporation according to Sambrook et al. [32]. The purified plasmid pQE30-Cupi4 was sequenced to confirm an in-frame insertion. Expression and purification of the recombinant protein were performed according to the manufacturer's instructions (Qiagen, CA). Briefly, the *E. coli* strains carrying plasmid pQE30-Cupi4 were grown in 500 ml of LB medium containing 100 mg/ml ampicillin at 30 or 37 °C on a platform shaker rotating at 220 rpm. When the absorbance at 600 nm for each culture reached 0.5–0.7, the cultures were induced with 0.5 or 1 mM IPTG and grown for an additional 8 h. The growth rate of the bacteria was measured with a spectrophotometer at the absorbance of 600 nm before induction and every hour after induction for an additional 8 h.

3. Results

3.1. Identification and analysis of pathogen-induced cDNA fragments isolated by mRNA differential display

mRNA differential display was used to compare the expression of genes in the center of leaves inoculated on the border with *P. s. lachrymans* and in water-treated leaves. Fourteen bands that were differentially expressed were excised and amplified by polymerase chain reaction (PCR). The PCR products were cloned in the pBluescript plasmid. Twelve clones were collected for each band giving 168 clones in total [29]. The 168 cDNAs were used as probes for RNA gel blot analysis to confirm the differential expression of mRNAs after pathogen inoculation. Fourteen clones showed differential expression in leaves inoculated with *P. s. lachrymans* (Fig. 1). These 14 cDNA clones named Did-1 to Did-14 were sequenced and analyzed with the Basic Local Alignment Research Tool (BLAST) accessed through Internet at the site of the National Center for Biotechnology Information, NCBI (http://www.ncbi.nih.gov/BLAST/) [35] and with the Pedro's Bio-Molecular Research Tools (http://www.public.iastate.edu/~pedro/research_tools.html). Marro [29] discovered that the Did-2 sequence (136 bp) was identical to cucumber class III chitinase CUSCHI (GenBank accession no. AAA33120) [23]. The sequence of Did-3 (147 bp) [29], Did-5 (237 bp), Did-6 (393 bp) and Did-7 (312 bp) were identical to a cucumber ethylene-induced peroxidase, CuPer 2 (GenBank accession no. AAA33121) [36]. The Did-8 sequence (343 bp) was 66% homologous to an Arabidopsis peroxidase, prxr5 (GenBank accession no. CAA66961) [37]. The sequence of Did-13 (189 bp) showed 45% identity to the F-box family protein of Arabidopsis (GenBank accession no. NM_120479). Several cDNAs including Did-1 (311 bp, found by Marro [29]), Did-4 (146 bp), Did-9 (94 bp), Did-10 (106 bp), Did-11 (102 bp), Did-12 (148 bp), and Did-14 (112 bp) did not share any significant similarity with known sequences in databases using BLAST programs. The partial cDNA called Did-1 and Did-4 were further characterized because these two cDNAs were strongly expressed and exhibited a unique hybridization pattern on the RNA gel blot (Fig. 1). In this work, we focus only on the expression of Did-4. Did-1 has been studied extensively by Marro [29].

3.2. Isolation of a full-length cDNA and sequence analysis of Cupi4

The Did-4 cDNA fragment was used as a probe to isolate the corresponding full-length cDNA from a cDNA library made from systemic leaves of cucumber plants infected with *P. s. syringae*. The full-length cDNA of 649 bp was obtained and called *Cupi4* (cucumber pathogen-induced 4). Analysis of the sequence of the *Cupi4* cDNA showed that it contains a 25-bp long untranslated leader sequence followed by an open reading frame coding for a 87-amino acid-long protein with a putative signal peptide. A consensus polyadenylation site (AATAAA [38]) is present at 36 bp downstream from the stop codon in the 3′untranslated region but the poly (A) tail is absent. To confirm that *Cupi4* is a full length cDNA with no poly (A) tail, rapid amplification of cDNA ends (RACE) of both the 5′ and 3′ ends was performed with a 5/3 RACE Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. No poly(A) was detected at the 3′ end of the *Cupi4* mRNA (data not shown). In addition, RNA gel blot analysis using total RNA from leaves inoculated with *P. s. lachrymans* was used to estimate the size of the *Cupi4*cDNA in comparison to the RNA ladder (Gibco-BRL) as a standard marker. The RNA blots were hybridized with either *Did4* (84–230 bp fragment of *Cupi4*cDNA) or full length *Cupi4* cDNA as a probe. The hybridized bands of both probes had a calculated size of approximately 652 bp (data not shown). These results suggested that *Cupi4* is a near full length cDNA and it does not contain poly (A) tail at 3′UTR.

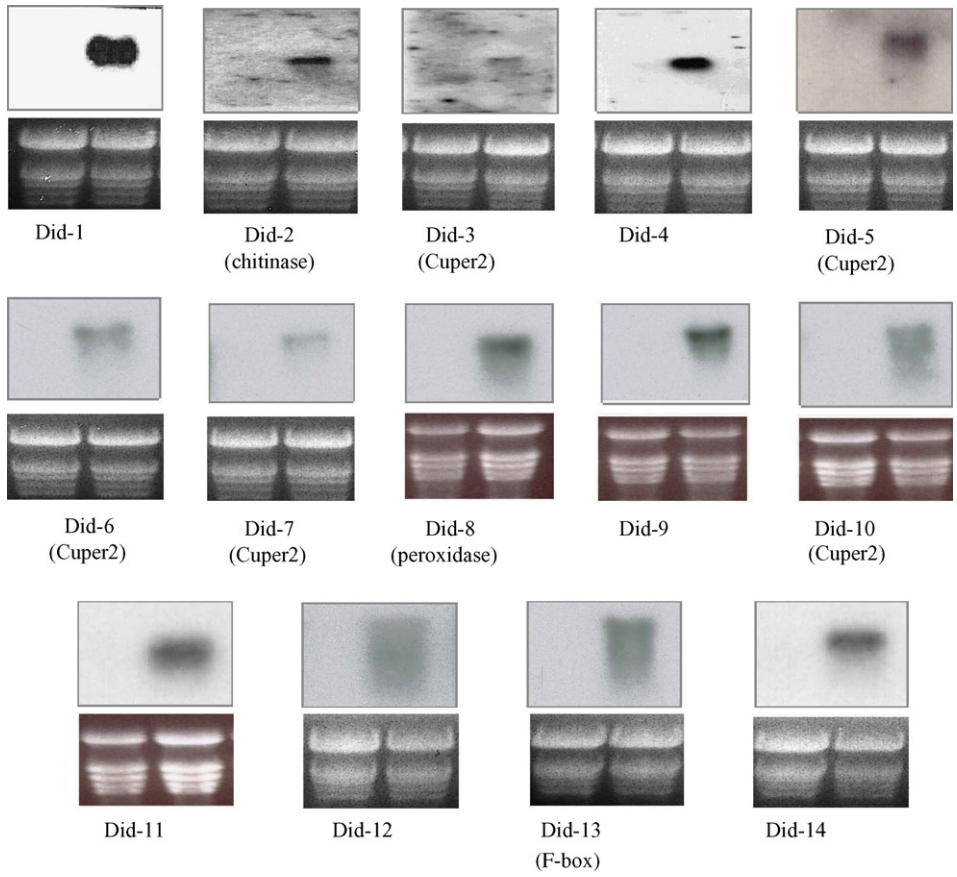

Fig. 1. RNA gel blot analysis of differential pathogen-induced mRNAs. Total RNA from control leaves treated with water (C) or inoculated leaves treated with *P. s. lachrymans* (I) was isolated 48 h after inoculation. Each lane contains 10 μg of RNA and was hybridized with the indicated radiolabelled cDNA probes isolated from PCR mRNA differential display. Ethidium bromide staining served as control for an equal loading.

The prediction of CUPI4 localization using PSORT (Predict Protein Sorting Signals Coded in Amino Acid Sequences, at GenomeNet, Japan, http://psort.ims.u-tokyo.ac.jp/form.html) and SignalP 3.0, using neural networks (NN) and hidden Markov models (HMM) trained on eukaryotes [39] shows that CUPI4 might be targeted either to the vacuole (83%) or outside the cells (82%). Hydropathy analysis of the amino acid sequence using the Kyte–Doolittle scale combined with

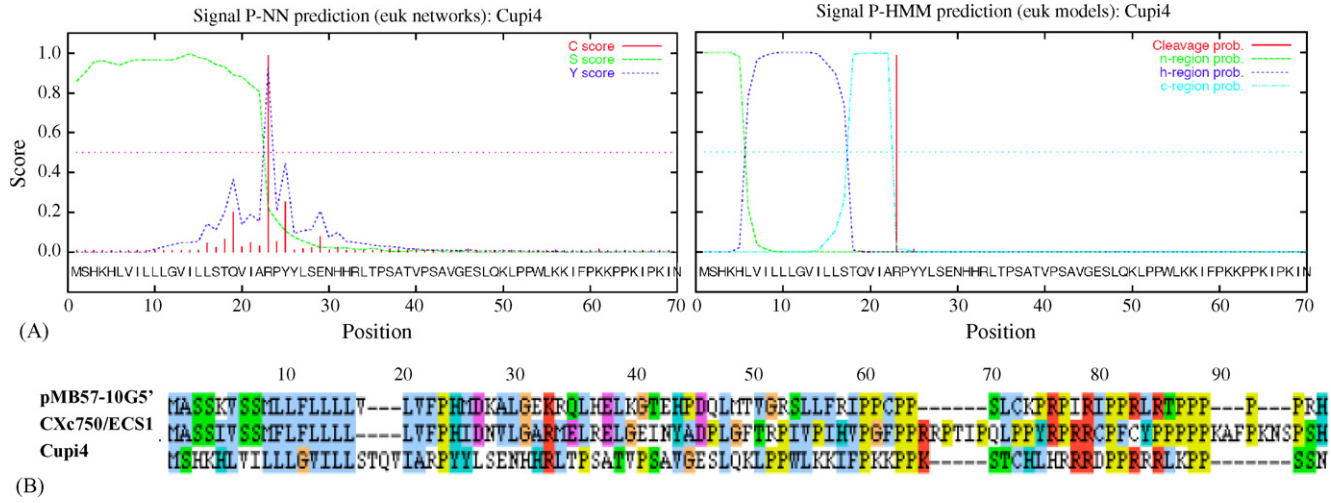

Fig. 2. (A) The graphical output for prediction of protein sorting signals and localization sites in amino acid sequences (PSORT) of CUPI4 by SignalP 3.0. The left panel derived from the neural network and the right panel derived from the hidden Markov model. In the left panel, C-score is the cleavage site score, S-score indicates the length of predicted signal peptide Y-score derived from the combination of C-score and S-score. In the right panel, a signal peptide is given by the position of the h-region, the cleavage site was assigned by the scores of the n-, h- and c-regions of the signal peptide. (B) Sequences alignment of the deduced amino acids of pMB57-10G 5′ (GenBank accession no. AI352744), CXc750/ECS1 5′ (GenBank accession no. X72022), and CUPI4 (GenBank accession no. DQ482461).

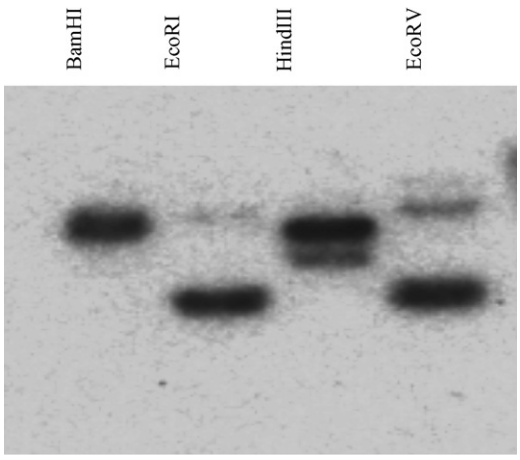

Fig. 3. Southern analysis of genomic DNA extracted from cucumber plants. DNA was digested with different restriction enzymes. Hybridization was performed under high stringency (65 °C) with the *Cupi4* cDNA as a probe.

hydrophobic moment and TMS prediction (http://www.tcdb.org/analyze.php) indicated that the protein is highly hydrophilic except for the N-terminal part that has the features of a potential signal sequence for translocation in the endoplasmic reticulum. The putative signal sequence comprises 23 amino acids and includes a positively charged amino terminal sequence followed by a central hydrophobic region and a more polar carboxyl terminal region. The predicted cleavage site of the signal peptide is between Ala-22 and Arg-23 (Fig. 2A). The mature form of the protein has a calculated molecular mass of 7391 Da and a calculated pI of 11.65. Analysis of the amino acid sequence of CUPI4 using ClustalW (http://www.ebi.ac.uk/clustalw/index.html [40]), showed that CUPI4 was 21% homologous and 44% identical to *Brassica napus* pMB57-10G 5′ (GenBank accession no. AI352744) [41] and was 16% homologous and 44% identical to Arabidopsis CXc750/ECS1 (GenBank accession no. X72022) [42,43]. The alignment of the sequences of these three proteins is shown in Fig. 2B.

3.3. Southern analysis of the cucumber genomic DNA

To estimate the number of *Cupi4*-related genes in cucumber, Southern blot analysis was performed with the *Cupi4* cDNA as a probe (Fig. 3). The enzymes used in this study were *Hin*dIII, which recognizes a single restriction site in the *Cupi4* cDNA at 326 bp, and *Bam*HI, *Eco*RI or *Eco*RV which do not cut within the sequence. When cucumber genomic DNA was digested with *Hin*dIII, *Cupi4* hybridized with two fragments. Only one or two fragments were detected when DNA was digested with *Bam*HI, *Eco*RI and *Eco*RV. This result indicates that the *Cupi4* gene is likely present in a low copy number in the cucumber genome. Southern blot analysis of Arabidopsis genomic DNA with the *Cupi4* cDNA as a probe did not show any hybridizing band either at low (50 °C) or high (65 °C) stringency (data not shown).

3.4. Expression of Cupi4 transcripts at different times after inoculation

To determine the time-course of accumulation of *Cupi4* transcripts in inoculated and uninoculated systemic leaves, total

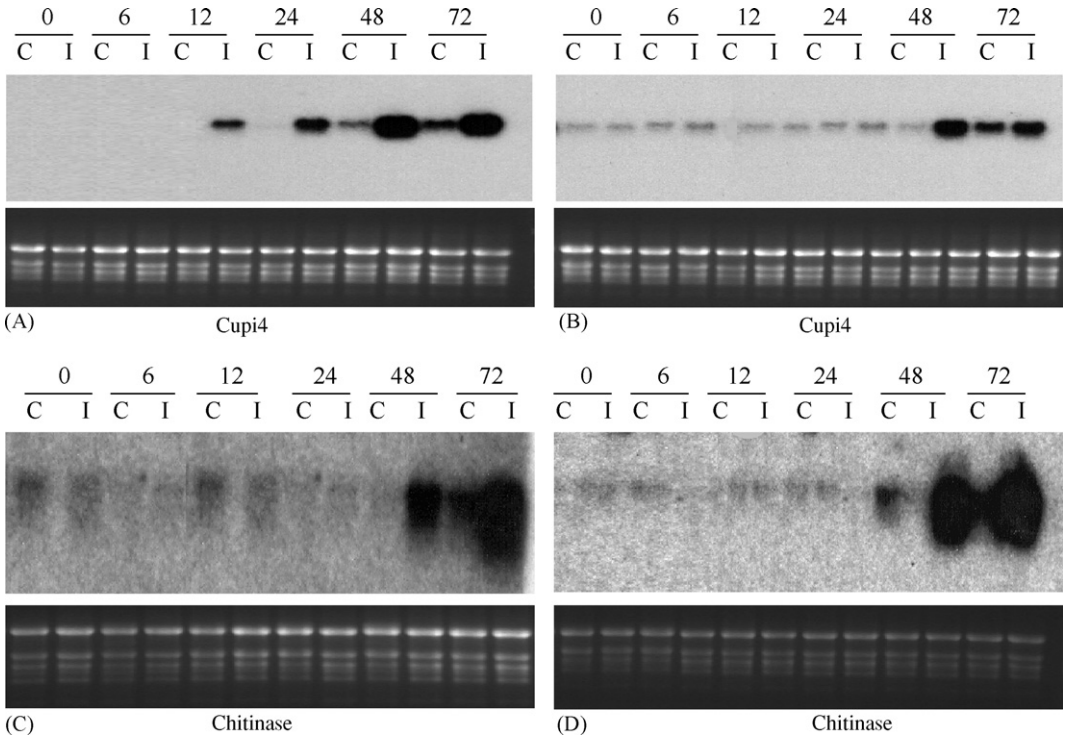

Fig. 4. Accumulation of *Cupi4* (A and B) and cucumber class III chitinase (C and D) transcripts at different times after inoculation with *P. s. lachrymans* analysed on RNA gel blots. First leaves were inoculated with *P. s. lachrymans* or water as control and RNA was isolated from inoculated first leaves (A and C) or systemic second leaves (B and D) from inoculated (I) or control (C) plants. The numbers indicate the time after inoculation in hours. Each lane contains 10 μg of RNA. Ethidium bromide staining served as control for an equal loading.

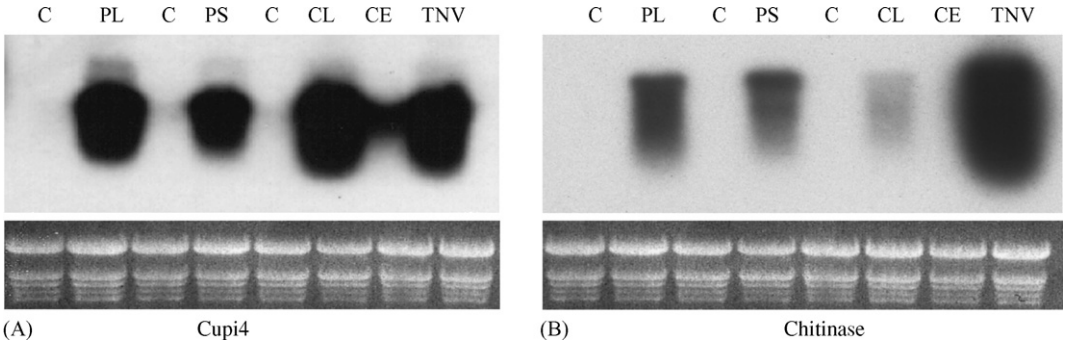

Fig. 5. Accumulation of *Cupi4* (A) and cucumber class III chitinase (B) transcripts after treatment with the different pathogens, *P. s. lachrymans* (PL), *P. s. syringae* (PS), *C. lagenarium* (CL), tobacco necrosis virus (TNV) or water (C) or celite (CE) as controls. Total RNA was extracted from the first leaves 3 days after inoculation. Each lane contains 10 μg of RNA. Ethidium bromide staining served as control for an equal loading.

RNA was isolated from first and second leaves of cucumber plants at different time points after inoculation of the first leaves with *P. s. lachrymans* or water as control. *Cupi4* transcripts began to accumulate 12 h post inoculation (hpi) in inoculated leaves and 48 hpi in systemic upper leaves and were present at a very low level or not detectable in the control leaves. We used cucumber class III chitinase as a marker for SAR in cucumber. In inoculated leaves, chitinase transcripts began to accumulate 24 hpi, which is later than the beginning of the expression of *Cupi4*. In systemic second leaves, the level of chitinase transcripts increased 48 hpi at the same time as the increase of *Cupi4* transcripts (Fig. 4).

3.5. Expression of Cupi4 transcripts in plants treated with pathogens, wounding and chemical inducers of SAR

To determine if the level of *Cupi4* transcripts increased after treatment with different pathogens that induce SAR in cucumber, the first leaf was inoculated with *P. s. lachrymans*, *P. s. syringae*, *C. lagenarium* or TNV. Total RNA was extracted from infected leaves 3 days after inoculation and analyzed by RNA blotting. *Cupi4* transcripts strongly accumulated after inoculation with these pathogens (Fig. 5A). Similarly, chitinase transcripts also accumulated (Fig. 5B).

The effects of a natural compound, SA, and the synthetic compounds, INA and BTH, which are known inducers of SAR in numerous plant species including cucumber were tested on the expression of *Cupi4* and chitinase. The levels of *Cupi4* and chitinase transcripts were increased after treatment with SA, INA or BTH compared to controls. The levels of both *Cupi4* and chitinase transcripts were higher in plants treated with BTH than with INA and SA (Fig. 6). Both *Cupi4* and cucumber class III chitinase transcripts were undetectable after wounding (data not shown).

3.6. Expression of Cupi4 transcripts in different plant tissues

To study the expression pattern of *Cupi4* and chitinase transcripts in different plant tissues, total RNA was isolated from leaves, roots (from plants grown in vitro), fruits, imperfect flowers and perfect flowers. The RNA gel blot showed that the level of *Cupi4* transcripts was high in fruits, imperfect flowers, perfect flowers and roots but undetectable in non-inoculated leaves (Fig. 7A). The level of chitinase transcripts was increased only in leaves inoculated with *P. s. lachrymans* (Fig. 7B).

3.7. In vitro expression of Cupi4

The growth of bacteria which carried a recombinant plasmid, pQE30-Cupi4 rapidly decreased within the first hour and stopped at 2 h after IPTG induction compared to the normal growth rate of bacteria from non-induced culture and from the

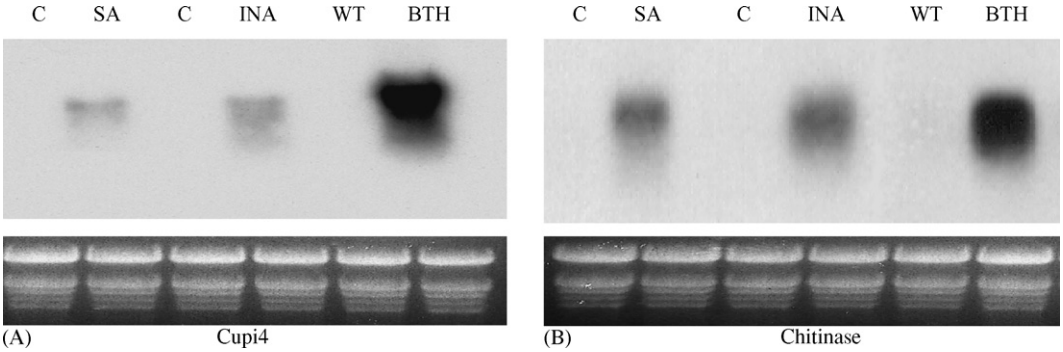

Fig. 6. Accumulation of *Cupi4* (A) and cucumber class III chitinase (B) transcripts after treatment with 1 mM of the SAR inducers; SA, and INA and BTH, or water (C) or wetting powder (WP), respectively, as controls. Total RNA was isolated from first leaves 48 h after soil drench application. Each lane contains 10 μg of RNA. Ethidium bromide staining served as control for an equal loading.

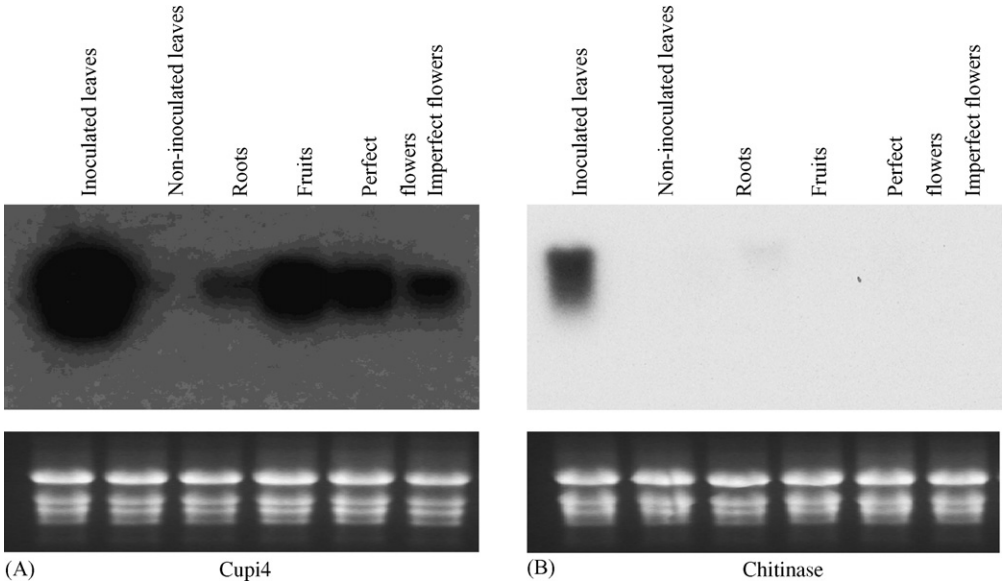

Fig. 7. Northern blot analysis of *Cupi4* (A) and cucumber class III chitinase (B) transcripts in different plant tissues. Total RNA was extracted from leaves inoculated with *P. s. lachrymans*, non-inoculated leaves, roots from plants grown in vitro, fruits, imperfect flowers and perfect flowers. Each lane contains 10 μg of RNA. Ethidium bromide staining served as control for an equal loading.

bacteria carrying a control plasmid, pQE30 (data not shown). The purification of 6xHis-Cupi4 was performed though a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen, CA). Samples were denatured and subjected to electrophoresis (90–125 V for 1–1.5 h) on a 20% polyacrylamide gel in the presence of SDS followed by Coomassie staining [32]. The expected product of mature CUPI4 (7391 Da) was not detected (data not shown). This data indicated that CUPI4 might be toxic to bacterial host cells.

3.8. *Effect of CUPI4 on the growth of E. coli host cells*

The result above indicated that the protein product from the expression of *Cupi4* cDNA might be toxic to the *E. coli* strains used in this experiment. To further clarify the toxicity of CUPI4, *E.coli* strains carrying pQE30 or pQE30-Cupi4 were cultured separately as described above. When the absorbance at 600 nm for each culture reached 0.5–0.7, the cultures with pQE30 and pQE30-Cupi4 were mixed and allowed to continue

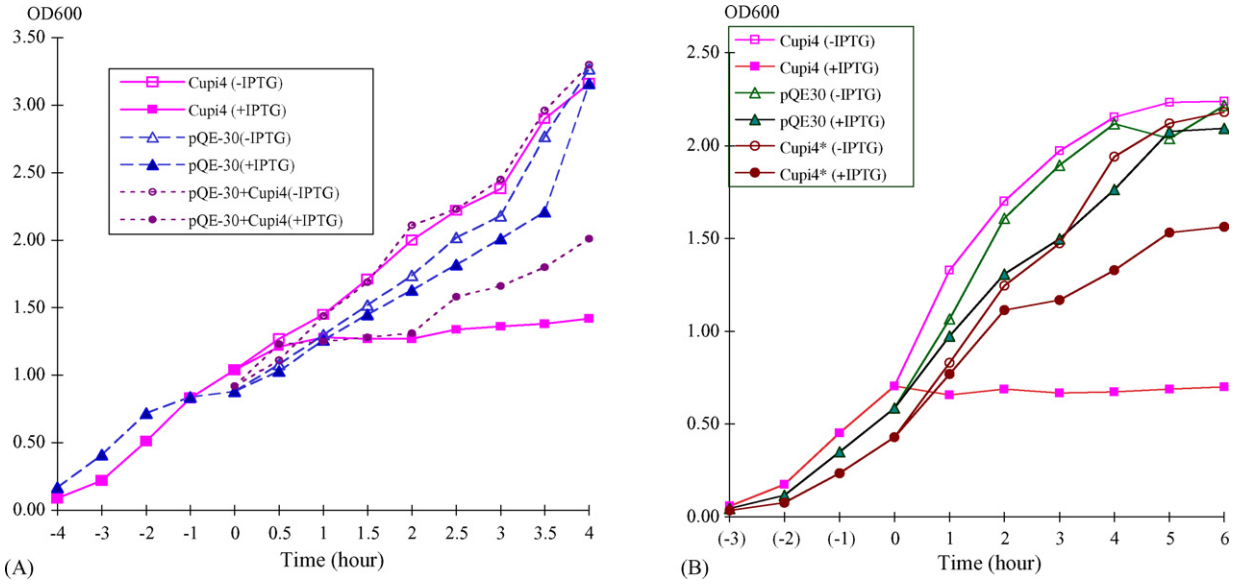

Fig. 8. Effect of CUPI4 on the growth of *E. coli* in LB medium. Growth curves were obtained by measuring the optical density at 600 nm. Protein expression was induced with 1 mM IPTG at time zero, −IPTG = no IPTG added, +IPTG = 1 mM IPTG added. (A) Growth curves of mixed cultures of *E. coli* in the presence (closed symbols) or absence (open symbols) of IPTG. Plasmid symbols are as follow; Cupi4 = pQE30-Cupi4, pQE-30 = control plasmid with no insert, pQE30 + Cupi4 = mixed culture of pQE-30 and pQE30-Cupi4. (B) Growth curves of individual cultures of *E. coli* in the presence (closed symbols) or absence (open symbols) of IPTG. Plasmid symbols are as follow; Cupi4 = pQE30-Cupi4, pQE-30 = control plasmid with no insert and Cupi4* = pQE30-modified-Cupi4*, with premature stop codon at amino acid position eight.

to grow for an additional 30 min, followed by an induction with 1 mM IPTG. Each individual culture was served as control. The growth rate of the individual and mixed cultures was measured every 30 min for 4 h before and after induction (Fig. 8A). The growth of bacteria carrying plasmid pQE30 alone increased similarly in both induced and non-induced cultures. The growth of bacteria carrying plasmid pQE30-Cupi4 alone slowed and then stopped within the first hour after induction (OD_{600} = 1.28) compared to the non-induced culture (OD_{600} = 1.45). In the mixed culture of bacteria carrying plasmids pQE30 and pQE30-Cupi4 (pQE30 + pQE30-Cupi4), the growth of the bacteria slowed for the first hour after induction with the OD_{600} of 1.25 compared to the non-induced-culture (OD_{600} = 1.69). However, in this same culture, growth of bacteria started to increase again slowly after 2.5 h (OD_{600} = 1.31) to 4 h (OD_{600} = 2.25). This result indicated that CUPI4 might be toxic to the host cells and it is probably an unstable protein. An alternative explanation is that the growth of bacteria resistant to CUPI4 took over after 2.5 h or the bacteria carrying pQE30-Cupi4 were all killed, hence no CUPI4 was produced. It should be noted, however that the resumption of growth in the mixed culture was much slower than growth from the individual cultures.

Additional evidence demonstrating the toxicity of CUPI4 to the host cells was obtained from a comparison of the growth of bacteria carrying plasmid pQE30, pQE30-Cupi4 or pQE30-modified-Cupi4*. The latest construct has a stop codon inserted at the eighth amino acid, 6XHGS*RPYYLSEN** (Cupi4 sequence is underlined). The bacteria were grown separately, as described above. The growth of bacteria carrying plasmids pQE30 and pQE30-modified-Cupi4* increased similarly in both induced and non-induced cultures, while bacteria carrying plasmid pQE30-Cupi4 slowed and stopped after the first hour after induction (OD_{600} = 0.66) compared to the non-induced culture (OD_{600} = 1.33, Fig. 8B).

4. Discussion

mRNA differential display was performed to isolate genes whose expression is induced in cucumber tissues expressing SAR after a first inoculation with *P. s. lachrymans*. Fourteen pathogen-induced mRNAs were detected. Marro [29] has shown that the partial sequence of a cDNA called Did-2 was identical to cucumber class III chitinase, CUSCHI [21]. This chitinase is induced after inoculation with different pathogens as well as the chemical inducers of SAR, SA, INA or BTH [8,23,28]. The isolation of the chitinase cDNA indicates that the differential display is an appropriate method to isolate genes that are induced during SAR because the level of chitinase is extremely low in non-infected leaves and increases strongly (60–2000-fold) in infected leaves [18]. Four cDNAs called Did-3, Did-5, Did-6 and Did-7 were identical to a cucumber ethylene-induced peroxidase, CuPer 2 [36]. Wounding or infection by several pathogens can lead to ethylene production and can induce some PR proteins such as glucanase and chitinase (reviewed in [44]). It is possible that *P. s. lachrymans* induced CuPer2 expression via the production of ethylene either by the plant or the bacteria. Did-8 was 66% identical to the Arabidopsis peroxidase, prxr5 [37]. The others, Did-1, Did-4, Did-9, Did-10, Did-11, Did-12, Did-13 and Did-14 did not share any significant similarity with any known sequences in databases using BLAST programs. The partial cDNA Did-4 was chosen for further characterization because it was strongly expressed and exhibited a unique hybridization pattern on the RNA gel blot. The analysis of Did-1 is reported elsewhere [29].

A 649-bp long cDNA, *CuPi4*, corresponding to the Did-4 fragment was isolated, sequenced and analyzed. This cDNA is called *Cupi4*. It contains an open reading frame of 87 amino acids with a polyadenylation site (AAUAAA) 36 bp downstream from the stop codon in the 3′-untranslated region. No poly (A) tail is present, possibly because of an artifact in cDNA synthesis or the instability of the mRNA during the library construction [38,45]. The sequence analysis of *Cupi4* showed that it contains a putative signal peptide and might be targeted either to the vacuole (83%) or outside the cells (82%). The sequence of CUPI4 is rich in proline (19.31%), leucine (14.77%), and lysine (10.22%) residues. It is not known whether the proline residues are hydroxylated in the mature protein. At the translated amino acid level, CUPI4 showed homology to two pathogen-inducible proteins; pMB57-10 G 5′, from canola (*B. napus*) [41] and CXc750/ECS1 from Arabidopsis [42,43]. The function of these two proteins is unknown. The expression of the pMB57-10G 5′ and of the *CXc750/ECS1* genes was induced by *Leptosphaeria maculans* and *Xanthomonas campestris* pv. *campestris*, respectively. The three proteins share several characters, such as encode small basic proline-rich proteins with potential signal peptide and are predicted to enter the secretory pathway. In addition, *CXc750/ECS1* mRNA transcripts were only detected in ecotypes which showed a resistant phenotype against *X. c. campestris* race 750. However, overexpression of *CXc750/ECS1* in *X. c. campestris* race 750-sensitive ecotype did not lead to resistance against *X. c. campestris* race 750 suggesting that *CXc750/ECS1* is not a resistance gene. Subcellular localization of the CXc750/ECS1 protein indicates that it is associated with the plant cell wall [42]. In humans, it has been reported that small proline-rich proteins are increased when there is damage in genomic DNA and during keratinocyte development. Keratinocytes are found in the human epidermis and function in protecting the skin from the damaging effect of external agents, such as ultraviolet (UV) light [46]. CUPI4 protein might be a reinforcing cell wall compound or have an antimicrobial activity.

Interestingly, *Cupi4* transcripts accumulated more rapidly than chitinase transcripts in first leaves inoculated with *P. s. lachrymans* but their accumulation occurred simultaneously in systemic second leaves. The expression of *Cupi4* both in inoculated first leaves and in systemic non-inoculated second leaves of cucumber plants inoculated on the first leaves with *P. s. lachrymans* and after inoculation with several other pathogens suggests that *Cupi4* is associated with SAR.

Inoculation of plants with pathogens provokes the accumulation of SA, JA or ethylene, suggesting their roles as signaling compounds that leads to SAR [47–51]. In cucumber, SA mediated SAR has been reported [52–54]. Exogenous application of BTH leads to induced resistance against *Cladosporium*

cucumerinum, moreover, chitinase accumulates more rapidly in plants treated with BTH than in plants treated with SA or water [28]. Similar results were observed in this study; *Cupi4* and cucumber class III chitinase transcripts levels, were higher in cucumber plants treated with BTH than in plants treated with SA or INA. The induction of *Cupi4* transcripts in cucumber plants treated with SA, INA or BTH suggests that *Cupi4* functions in SAR via a SA-dependent pathway. *Cupi4* was constitutively expressed in fruits, imperfect flowers, perfect flowers and in vitro grown roots, suggesting a potential role of CUPI4 protein during cucumber development. The expression of *Cupi4* in leaves was detected only when plants were inoculated with pathogens as expected, corresponding to the fact that cucumber leaves are more susceptible to infection than the other part of the plants. Overexpression of the CUPI4 in bacteria was attempted, but the protein product seems to be toxic to the host cells even when several conditions were performed. These included three different *E. coli* host strains, two different temperatures (30 and 37 °C) for bacterial culture and decreased concentration of IPTG from 1 to 0.5 mM. In any cases, we were not able to detect His/CUPI4. While, the role of *Cupi4* in SAR is still not clear and needs to be characterized, this present study clearly shows that *CuPi4* is a novel SAR gene in cucumber plant.

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