The Corky Root Rot Pathogen *Pyrenochaeta lycopersici* Secretes a Proteinaceous Inducer of Cell Death Affecting Host Plants Differentially

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ABSTRACT

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Pathogenic isolates of *Pyrenochaeta lycopersici*, the causal agent of corky root rot of tomato, secrete cell death in tomato 1 (CDiT1), a homodimeric protein of 35 kDa inducing cell death after infiltration into the leaf apoplast of tomato. CDiT1 was purified by fast protein liquid chromatography, characterized by mass spectrometry and cDNA cloning. Its activity was confirmed after infiltration of an affinity-purified recombinant fusion of the protein with a C-terminal polyhistidine tag. *CDiT1* is highly expressed during tomato root infection compared with axenic

culture, and has a putative ortholog in other pathogenic Pleosporales species producing proteinaceous toxins that contribute to virulence. Infiltration of CDiT1 into leaves of other plants susceptible to *P. lycopersici* revealed that the protein affects them differentially. All varieties of cultivated tomato (*Solanum lycopersicum*) tested were more sensitive to CDiT1 than those of currant tomato (*S. pimpinellifolium*). Root infection assays showed that varieties of currant tomato are also significantly less prone to intracellular colonization of their root cells by hyphae of *P. lycopersici* than varieties of cultivated tomato. Therefore, secretion of this novel type of inducer of cell death during penetration of the fungus inside root cells might favor infection of host species that are highly sensitive to this molecule.

Pyrenochaeta lycopersici is the filamentous ascomycete responsible for corky root rot (CRR), a debilitating and ubiquitous disease of tomato (31). Infection by this pathogen leads the cortex of mature roots to develop a corky texture with time, and small feeder roots to rot (25). As a result, circulation of water and nutrients is disturbed, causing stunt and important fruit yield reduction (16). Isolates of this fungus are not only pathogenic to several species of the family Solanaceae, including pepper, tobacco, and eggplant, but also to other species of Eudicotyledones, such as cucumber, melon, safflower, and spinach (17,26,34,35,39).

Control of CRR is problematic, because all methods available are hindered by either prohibitive cost, adverse effect on the environment, low efficiency, or restrictive conditions of use (11). Resistance breeding still stands as the best compromise between efficiency, flexibility of use, and sustainability. Nevertheless, although sources of partial genetic resistances do exist (18,21,33,40), introduction of resistance genes into commercially interesting cultivars of tomato has rarely been implemented, because of tedious, costly, and unreliable procedures of screening (10).

Genetic discoveries made recently in pathogens taxonomically related to *P. lycopersici* are opening promising perspectives of improvement of resistance screening procedures. The genus *Pyrenochaeta* belongs to order Pleosporales, like several other

necrotrophic and hemibiotrophic plant pathogens, such as *Alternaria*, *Cochliobolus*, *Pyrenophora*, *Phaeosphaeria*, *Leptosphaeria*, *Pleospora*, *Phoma*, and *Didymella* spp. (42). Virulence of these pathogens often depends on their capacity to produce host-selective toxins (HSTs), molecules both promoting infection and determining host specificity (23). *Cochliobolus* spp. and pathogenic subspecies of *Alternaria alternata* secrete phytotoxic secondary metabolites required to cause disease on specific genotypes of their hosts (23). The three pathogens of cereals *Stagonospora nodorum* (anamorph of *Phaeosphaeria nodorum*, responsible of wheat glume blotch), *Pyrenophora tritici-repentis* (wheat tan spot), and *P. teres* (barley net blotch) have been shown to produce proteins contributing to virulence (14,28). Wheat resistance against *S. nodorum* has a complex and polygenic determinism with additive effects (32) and seemed difficult to unravel, until demonstration was made that *S. nodorum* secretes proteinaceous HSTs contributing quantitatively to virulence, and that a gene of sensitivity (*S*) of wheat is corresponding to each gene encoding an HST of the pathogen (14). Additive effects resulting from several such pairwise genetic interactions determine glume blotch severity, partial resistance of wheat being due to HST-insensitive alleles of *S* genes, which are usually recessive (13). Because they are major virulence determinants interacting with a limited number of specific host genes that condition resistance, characterization of putative HSTs from phytopathogenic Pleosporales species is a research priority. To our knowledge, occurrence of HSTs has never been investigated in *Pyrenochaeta lycopersici* or in any other root pathogen.

Secretion of phytotoxic molecules by *P. lycopersici* has been reported but it is still unclear whether or not such compounds could contribute both quantitatively and in a host-specific manner to virulence. Their identification might help at clarifying their role and at unraveling the genetics of resistance against CRR in to-

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mato. Estimation of molecular weight of some active compounds secreted in liquid culture and determination of their heat sensitivity suggest a proteinaceous nature (22). Heat-sensitive macromolecules of 10 to 15 kDa secreted by *P. lycopersici* have also been shown to cause necrosis specifically in the apical part of the root of tomato (7). Tolerance of tomato seedlings to phytotoxicity of *P. lycopersici* culture filtrates was proposed as a test for selecting cultivars tolerant to CRR, based on a correlation between presence of toxins in fungal culture filtrates, host genotype, and disease severity (12). Preliminary experiments carried out in our laboratory have shown that a heat-sensitive macromolecule present in culture filtrates of several isolates of *P. lycopersici* induces cell death in the mesophyll of tomato leaves. Using this robust assay, we aimed to purify this molecule and to evaluate its contribution to CRR.

MATERIALS AND METHODS

Plant and fungal material. *P. lycopersici* pathogenic isolate DSM 62931 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Isolates ER824, ER876, and ER931 showing variable virulence levels on the roots of tomato 'Corbarino' (19), were kindly provided by A. Infantino (ISPAVE, Rome). Isolates were routinely cultivated on oatmeal-agar medium as previously described (7). For production of culture filtrates, mycelium was grown for 3 weeks in 500-ml Erlenmeyer flasks containing 100 ml of MS-20 liquid medium at room temperature (19 to 23°C) and without shaking. MS-20 was prepared with research grade sucrose (SERVA electrophoresis GmbH, Heidelberg, Germany) at

20 g/liter and Murashige and Skoog (MS) medium, including Gamborg's B5 vitamins (15,24) (Duchefa Biochemie BV, Haarlem, The Netherlands) at 4.4 g/liter. After cultivation, the medium was drained through cheesecloth and filtered at 0.45 µm.

Seed of all plant cultivars used in this study (Table 1) were germinated on moistened filter paper in an incubator at 28°C. Seedlings were transferred into circular pots (height = 11 cm and diameter = 13 cm) filled with a derivative of Cornell peat-lite mix (150 liters of peat moss, 85 liters of medium to coarse vermiculite, 0.6 kg of dolomitic limestone, 140 g of 10-10-15 fertilizer [NPK rating: 10% of total weight in elemental N, 4.4% in elemental P, and 12.45% in elemental K], and 0.6 kg of 17-6-12 fertilizer) immediately after root emergence and cultivated in the greenhouse. Light conditions were 150 to 300 µmol photons m⁻² s⁻¹. Temperature was set to 22°C during the daily light cycle of 13 h and to 19°C during the daily dark cycle of 11 h. Relative humidity was not regulated.

Leaf infiltration assays. Syringes (1 ml) were used to inject 30 to 100 µl of fungal culture filtrate into the apoplast of the first or second leaves of three plants for each species tested. The epidermis of the abaxial side of the leaves was gently pricked with a needle before infiltration. Plants were cultivated in the greenhouse in the conditions described for preparation of plant material. Leaves of all plant cultivars tested at the same time were infiltrated with the same batch of filtrate for comparison. Infiltration of sterile MS-20 medium was used as negative control. Surface of infiltrated area was proportional to the volume of liquid infiltrated and was delimited with a marker immediately after infiltration. Cell death-inducing activity was monitored after 24 to 72 h of incubation in the greenhouse.

TABLE 1. Plant cultivars used in this study

Species name	Cultivar	Seed provided by or purchased from
<i>Solanum lycopersicum</i>	Money Maker	Department of Plant Pathology, Cornell University, Ithaca, NY
	Moboglan	ISPAVE, Rome
	Mogeor	ISPAVE, Rome
	Corbarino	ISPAVE, Rome
	Pearson	Department of Plant Pathology, Cornell University, Ithaca, NY
	PI 260397 96GI	USDA-ARS, Cornell University, Geneva, NY
<i>S. pimpinellifolium</i>	Castlemart	Department of Plant Pathology, Cornell University, Ithaca, NY
	Culebras	TGRC, University of California, Davis
	Babahoyo	TGRC, University of California, Davis
	Chota to El Angel	TGRC, University of California, Davis
	Viv	USDA-ARS, Cornell University, Geneva, NY
	Pichilingue	TGRC, University of California, Davis
<i>S. lycopersicum</i> × <i>S. habrochaites</i>	F1 Brigeor	INRA, Montfavet, France
<i>S. neorickii</i>	...	TGRC, University of California, Davis
<i>Nicotiana benthamiana</i>	...	Department of Plant Pathology, Cornell University, Ithaca, NY
<i>N. tabaccum</i>	W38	Botaniska Institutionen, Stockholms Universitet, Sweden
<i>Capsicum annuum</i>	Maor	Department of Plant Pathology, Cornell University, Ithaca, NY
	Thai Hot	Department of Plant Pathology, Cornell University, Ithaca, NY
<i>C. frutescens</i>	2814.6	Department of Plant Pathology, Cornell University, Ithaca, NY
<i>C. chinese</i>	Habanero	Department of Plant Pathology, Cornell University, Ithaca, NY
	Pi 159234	Department of Plant Pathology, Cornell University, Ithaca, NY
<i>S. melongena</i>	Tsakoniki	Department of Plant Pathology, Cornell University, Ithaca, NY
	Langada	NAGREF, Thermi, Greece
	PA4	NAGREF, Thermi, Greece
	V-S-11	Universidad Politécnica de Valencia, Valencia, Spain
	C-S-16	Universidad Politécnica de Valencia, Valencia, Spain
	...	Universidad Politécnica de Valencia, Valencia, Spain
<i>Datura stramonium</i>	...	Bergianska Trädgården, Stockholm, Sweden
<i>Cucumis sativus</i>	Rhensk Druv	Weibulls-Econova Garden AB, Åby, Sweden
	F1 Futura	Weibulls-Econova Garden AB, Åby, Sweden
<i>Carthamus tinctorius</i>	Orange Grenade	Bröderna Nelsons Frö AB, Tingsryd, Sweden
<i>Cucumis melo</i>	Boule d'Or	INRA, Montfavet, France
	Delicious 51	INRA, Montfavet, France
	Doublon	INRA, Montfavet, France
	Ginsen Makuwa	INRA, Montfavet, France
	Iran A	INRA, Montfavet, France
	Ogon 9	INRA, Montfavet, France
	F1 Stellio	Weibulls-Econova Garden AB, Åby, Sweden
	F1 Diamant	Weibulls-Econova Garden AB, Åby, Sweden
	Kaempfmelon	Weibulls-Econova Garden AB, Åby, Sweden
	...	...
<i>Cucurbita pepo</i>	...	...
<i>C. maxima</i>	...	...

Characterization of *P. lycopersici* culture filtrates. Culture filtrates of the four isolates of *P. lycopersici* used in this study (DSM 62931, ER824, ER876, and ER931) were tested for their capacity to induce cell death after infiltration into *Solanum lycopersicum* 'Money Maker' (MM) leaves. Heat sensitivity of DSM 62931 filtrates was assessed by keeping an aliquot of 4 ml in a boiling water bath for 15 min before infiltration into MM leaves. In order to determine whether induction of cell death by DSM 62931 filtrates could depend on the exposition of MM leaves to the light, plants were kept overnight in the dark before infiltration, and incubated 48 h longer in the dark after infiltration. Molecular weight of the cell-death-inducing compound present in DSM 629131 filtrates was evaluated by loading 4-ml aliquots into Amicon Ultra 4 centrifugal filter devices with nominal molecular weight limits (NMWL) of 100, 50, 30, and 10 (Millipore, Billerica, MA). Concentrates resulting from ultrafiltration were diluted to their initial volume of 4 ml with fresh and sterile culture medium before infiltration into MM leaves. Filtrates were infiltrated without dilution. When needed, concentration of protein in the solutions infiltrated into the leaves was determined by the method of Bradford (5), using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA).

Purification and characterization of cell death in tomato 1. Purification of the cell death inducer from culture filtrates of DSM 62931 was carried out by fast protein liquid chromatography (FPLC) using a Pharmacia Äkta Prime apparatus (GE Healthcare, Uppsala, Sweden). Cell-death-inducing activity of each chromatography fraction was determined by infiltration into MM leaves after buffer exchange using Econo-Pac 10 DG disposable chromatography columns (Bio-Rad Laboratories AB, Sundbyberg, Sweden) equilibrated with 20 mM Tris, pH 8.0. Infiltration of the same buffer was used as negative control. For anion exchange chromatography, filtrates were injected in a 1-ml HiTrap Q-sepharose XL column (GE Healthcare) equilibrated in 20 mM Tris, pH 8.0. Proteins bound to the anion exchanger were eluted with an NaCl gradient (0 to 1.2 M in 10 min) at flow rate of 1 ml/min. For size-exclusion chromatography in native conditions, proteins recovered from the anion exchanger were injected in a Pharmacia HiPrep 16/60 Sephacryl S200 HR gel-filtration column (GE Healthcare) equilibrated with 20 mM Tris (pH 8.0) and 150 mM NaCl at a flow rate of 0.5 ml/min. Size-exclusion chromatography under denaturing conditions was carried out in the same column equilibrated with 6 M Guanidine-HCl, 20 mM Tris (pH 8.0), and 150 mM NaCl at flow rate of 0.3 ml/min. Internal standards used for size-exclusion experiments were Blue Dextran, carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) (Sigma-Aldrich GmbH, Buchs, Switzerland). Stock solutions were prepared in appropriate gel-filtration buffer at a concentration of 1 mg/ml and stored at 4°C.

Aliquots of the fractions resulting from size-exclusion chromatography and inducing cell death in MM leaves were concentrated 13- to 14-fold using Amicon Ultra 0.5 centrifugal filter

devices with a NMWL of 10. In all, 10 µl was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-Tricine buffer system (29). The best candidate for the cell death inducer was searched in fractions showing a strong correlation between (i) a peak of UV absorbance on the chromatogram of size exclusion, (ii) the highest residual cell-death-inducing activity after 3- and 10-fold dilutions with 20 mM Tris (pH 8.0), and (iii) the presence of a single band after SDS-PAGE.

A protein sample of the best candidate for cell death in tomato 1 (CDiT1) after separation by SDS-PAGE was cut out from the gel with a blade and sent to Alphalyse A/S (Odense, Denmark) for mass spectrometry (MS) analysis. The protein was reduced and alkylated with iodoacetamide, and digested with trypsin (3). Resulting peptides were concentrated using a ZipTip micropurification column (Millipore) and eluted onto an anchorchip target for analysis with an Autoflex III matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF) instrument (Bruker Daltonik GmbH, Bremen, Germany). The peptide

Fig. 1. Typical mesophyll cell death induced after infiltration of an aliquot of a culture filtrate of *Pyrenochaeta lycopersici* (isolate DMS 62931) into the leaf apoplast of tomato (*Solanum lycopersicum* 'Money Maker'). Photograph was made 48 h postinfiltration.

TABLE 2. Primers used in this study

Name	Sequence
Dg7+	5'-GTNACNGGNGTNGCNTC-3'
Dg3-	5'-ACRTCNCGNACRTGRTT-3'
Ra5	5'-GGAGACTCTCCCTTCGTGAACCACTTAGCCTGGCAG-3'
Ra5n	5'-GGATCCAGGACGTCCCGAGGGAGTATTGGAGGCCACC-3'
Ra3	5'-GGTGGCCTCCAATACTCCCTCGGGACGTCCTGGATCC-3'
Ra3n	5'-CTGCCAGGCTAAGTGGTTCACGAAGGGAGAGTCTCC-3'
Pr1+	5'-GCGTGGCCTCCAATACTCCC-3'
Pr2-	5'-CCACGTGATTGAACCTTGAGC-3'
Hi1+	5'-CTCCCTCGAGCAATTGACAC-3'
Hi2-	5'-CTACTAGTGATGATGATGATGATGATGGACCTTCGTAGGAGTAATC-3'
Act1+	5'-ATCAACCCCAAGTCCAACC-3'
Act2-	5'-GGAGCAATGATCTTGACCTTC-3'

mixture was analyzed in positive reflector mode for accurate peptide mass determination. Two peptides were selected for further analysis by tandem MS (MS/MS) fragmentation and de novo sequencing using Bruker Biotoools software. MS and MS/MS spectra were combined to make a search using Mascot software (www.matrixscience.com) and the AlphaLysate A/S in-house protein database, a nonredundant protein sequence database regularly updated with sequence files downloaded from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/protein>). Matching proteins were selected on the basis of their number of matching peptide masses and peptide fragment masses, and identification made according to a probability-scoring algorithm. Mascot was used for mapping of MALDI MS peptides with CDi1 sequence as well.

Cloning of CDi1. Total RNA samples were prepared from mycelium of DSM 62931 cultivated in MS-20 medium during 4 days as described previously (37). First-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen,

Carlsbad, CA), including pretreatment of RNA sample with DNaseI. Initial polymerase chain reaction (PCR) amplification of a fragment of CDi1 cDNA was performed with degenerated primers designed after translation of the sequences of the two peptides identified after CDi1 MS analysis, Dg7+ and Dg3– (Table 2), and using a touchdown PCR program: annealing temperature was set to decrease at constant rate from 53 to 45°C over 40 cycles, and extension time set to 1 min. Amplification products were cloned and analyzed by sequencing. Rapid amplification of cDNA ends (RACE) experiments were performed using SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA) with primers Ra5 and Ra5n for 5' RACE, and Ra3 and Ra3n (Table 2) for 3' RACE. Reverse-transcription (RT)-PCR amplification of CDi1 full coding sequence in isolates DMS 62931, ER824, ER876, and ER931 was achieved using primers Fc1+ and Fc2– (Table 2). Orthologs of CDi1 were searched in NCBI public nucleotide and protein sequence databases (<http://www.ncbi.nlm.nih.gov>) using BLASTP and BLASTN

Fig. 2. Purification of the cell death inducer of *Pyrenochaeta lycopersici* by fast protein liquid chromatography. **A**, Chromatogram of gel filtration. Active material bound the anion exchange column during the first step of purification was eluted with a NaCl gradient (data not shown), then separated by size exclusion and resolved in two peaks (numbers 1 and 2). **B**, Determination of fractions containing the cell death inducer. Aliquots of fractions collected during gel filtration, as well as their dilutions to the 3rd and to the 10th, were infiltrated into the leaves of 'MM' plants. Activity was ranked in four levels depending on the surface of the infiltrated area that was showing cell death after 48 h, from dark green = no activity (fraction number 9 diluted to the 3rd and to the 10th on the leaf photograph) to red = cell death in the entire infiltrated area (fractions 15 and 17 on the leaf photograph). The peak of activity (fractions 17 and 18) was shown this way to correspond to peak of UV absorbance number 2. **C**, Analysis of protein content by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the fractions inducing cell death. Aliquots of fractions collected during gel filtration were concentrated (nominal molecular weight limit = 10) and separated by SDS-PAGE (12% acrylamide). Peak of UV absorbance number 2 was shown to correspond to a polypeptide of 17 to 18 kDa (black arrow).

algorithms (1). Alignment of protein sequences was made using the ClustalW program (36). SignalP 3.0 algorithm (4) was used to detect a putative N-terminal signal peptide for secretion in the amino acid sequence of CDiT1. Putative functional domains were searched using algorithms of protein sequence analysis available at ExPASy (www.expasy.org).

Genomic DNA of *P. lycopersici* was extracted from mycelium of DSM 62931 grown in Iwasaki liquid medium (9) for 7 days. Extraction and Southern blot were performed as previously described (27). Genomic restriction fragments were transferred onto Nylon N membrane (GE Healthcare) after electrophoresis in a 0.7% agarose gel. Radioactive probes were synthesized using Amersham Rediprime II Random Prime Labelling System (GE

Healthcare), α - ^{32}P dCTP (Perkin-Elmer, Zaventem, Belgium), and PCR product resulting from *CDiT1* amplification with primers Pr1+ and Pr2- (Table 2) as template. For the construction of a genomic mini-library, 5- to 7-kb *SpeI*-*SacI* genomic restriction fragments were separated by electrophoresis in a 0.7% agarose gel and ligated to vector pBluescript SK- (Stratagene, Cedar Creek, TX). Ligation mix was introduced by transformation into competent cells of *Escherichia coli* XL1-blue strain (Stratagene). In all, 1,008 ampicillin-resistant colonies were transferred onto 82-mm-diameter discs of Nylon N membrane (GE Healthcare) and screened with a *CDiT1* radioactive probe.

Production and purification of recombinant CDiT1 bearing a C-terminal polyhistidine tag (CDiT1::HIS). An expression and transformation vector for the secretion by *P. lycopersici* of a recombinant CDiT1 bearing a C-terminal HIS tag (CDiT1::HIS) was constructed as follows. The 4.4-kb *ClaI*-*SacI* genomic restriction fragment containing the *CDiT1* full genomic sequence (including the two exons, intron, putative promoter, and terminator) was inserted into the polylinker of plasmid pKHyg (39), to give pKWT. The same restriction fragment was inserted into the polylinker of a derivative of plasmid pBluescript SK- (in which the *XhoI* site of the polylinker had been previously destroyed by ligation to the *SalI* site), to give pSWT. An oligonucleotide encoding six histidine residues was introduced at the 3' end of the second exon of *CDiT1* by PCR amplification using primers Hi1+ and Hi2- (Table 2), Platinum *Pfx* DNA polymerase (Invitrogen), and the genomic 4.4-kb *ClaI*-*SacI* restriction fragment as template. The PCR product was digested with restriction enzymes *XhoI* and *SpeI* and inserted into plasmid pSWT, in place of the 0.71-kb *XhoI*-*XbaI* restriction fragment containing the wild-type sequence of *CDiT1*, to give pSHIS. Plasmid pSHIS was digested with restriction enzymes *ClaI* and *SacI*, and the 4.4-kb restriction fragment resulting from this double digestion was inserted into the polylinker of plasmid pKHyg, to give pKHIS.

Plasmids pKWT and pKHIS were introduced into the genome of DMS 62931 by protoplast transformation as previously described (7). Ten transformants for each plasmid were grown in 50 ml of MS-20 medium, and aliquots of the culture media were analyzed by SDS-PAGE after 3 weeks of incubation. Recombinant CDiT1::HIS protein was purified using MagneHis Protein Purification System (Promega Corp., Madison, WI). Large-scale purification of CDiT1::HIS was carried out with the filtrate of a culture of transformant KHIS5 in 500 ml of MS-20 medium. Filtrate was concentrated to a volume of 5 ml using Amicon Ultra 15 devices (NMWL 10) before affinity purification using FPLC, with a 1-ml HisTrap HP column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0. CDiT1::HIS was eluted with a gradient of imidazole (0 to 0.5 M in 30 min) at a flow rate of 1 ml/min. Imidazole was eliminated by buffer exchange before infiltration into MM leaves as described for the characterization of FPLC fractions. Aliquots of the elution buffers of both affinity purification systems were processed in the same way and used as negative controls for infiltrations into MM leaves.

Root infection assays. Assays were carried out with 12 seedlings of three cultivars of *S. lycopersicum* (MM, 'Mogor' and plant introduction [PI] 260397 96GI) and three cultivars of *S. pimpinellifolium* ('Culebras', 'Viv', and 'Pichilingue'). Seedlings were inoculated with mycelium of 1K4, a transformant of DSM 62931 carrying a fusion of the β -glucuronidase (GUS) gene *uidA* with the promoter of *Avr4* of *Cladosporium fulvum* (20), which is highly expressed during root infection only (7). Seedlings were germinated and inoculated in vitro as described previously (7), except for the following: the inoculum was cut from a mycelium culture of 1K4 with a cork borer (diameter = 5 mm) and applied directly on the top of the root, mycelium facing the root. Seedlings were incubated for 8 days at room temperature (19 to 23°C). Inoculum was then removed and all roots were stained for GUS expression. Extent of fungal infection was recorded using a

Fig. 3. Cell death in tomato 1 (CDiT1) is a homodimeric protein of 35 kDa. **A**, CDiT1 elutes before carbonic anhydrase during size exclusion in native conditions. Upper chromatogram shows result of a size-exclusion experiment with a mixture of macromolecular standards alone (BD, blue dextran; CA, carbonic anhydrase; CytC, cytochrome c). Lower chromatogram shows separation of a mixture of the standards with the active fraction resulting from anion exchange chromatography of a culture filtrate of *Pyrenochaeta lycopersici* (isolate DMS 62931). Cell-death-inducing activity was detected in a single peak (number 2), which eluted at the position of a protein of ≈ 35 kDa, just before carbonic anhydrase (29 kDa). Flow rate was set at 0.5 ml/min and paper speed at 1 mm/min (black bar: 2 cm). **B**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of fractions collected during size exclusion in native conditions. Fractions were concentrated (nominal molecular weight limit = 10) before analysis by SDS-PAGE. Fractions corresponding to peak number 2 showed that CDiT1 ran at molecular mass of 17.4 kDa in SDS-PAGE, lower than carbonic anhydrase, S: protein standards, carbonic anhydrase (29 kDa), and cytochrome c (12.5 kDa). **C**, CDiT1 elutes between carbonic anhydrase and cytochrome c during size exclusion in denaturing conditions. Upper chromatogram shows separation of a mixture of the protein standards in the same conditions of a mixture of the protein standards with the active fraction resulting from anion exchange chromatography of a culture filtrate of *P. lycopersici* (isolate DMS 62931). Compared with the same experiment in native conditions, the position of peak number 2, corresponding to CDiT1, shifted between carbonic anhydrase and cytochrome c, at a position expected for a polypeptide of 17.4 kDa. Flow rate was set at 0.3 ml/min and paper recording at 0.5 mm/min (black bar: 2 cm).

SMZ-U stereoscopic microscope (Nikon Corporation, Tokyo) as the length (mm) of the root showing a blue staining, indicating expression of GUS. Statistical analysis was carried out using *t* tests for pairwise comparisons between *S. lycopersicum* and *S. pimpinellifolium* cultivars ($P = 0.05$).

Expression of *CDiT1*. For the analysis of *CDiT1* expression in vitro, total RNA from mycelium of DSM 62931 grown in MS-20 medium were extracted as described (37). For the analysis of *CDiT1* expression during infection, 300 MM seedlings were germinated in vitro and inoculated with DSM 62931 as previously

Fig. 4. Mass spectrometry (MS) analysis of cell death in tomato 1 (*CDiT1*). **A**, Matrix-assisted laser desorption/ionization/MS spectrum of *CDiT1*. Peptide mixture resulting from reduction and alkylation of *CDiT1* with iodoacetamide and digestion with trypsin was analyzed in positive reflector mode for accurate mass determination. Peaks 1145.618 and 1447.757 (boxes) were analyzed by tandem MS (MS/MS). Peak 842.533 (underlined) corresponds to trypsin autolysis fragment. **B**, MS/MS spectra of two *CDiT1* tryptic peptides. Amino acid sequences were deduced after peptide fragmentation using Mascot software.

TABLE 3. Matrix-assisted laser desorption/ionization/mass spectrometry peptide masses of cell death in tomato 1 (CDiT1)

m/z ^a	SN ^b	Quality factor	Resolution	Intensity	Area ^c
634.2	347.1	...	6,559	94,139	10,785
715.341	884.1	...	6,227	239,771	33,556
729.356	199.9	...	6,993	54,218	7,085
811.499	3.6	58	8,089	1,155.01	209
825.518	4.3	54	7,230	1,418.17	287
827.522	4.9	336	3,139	1,611.87	860
832.337	19.3	685	7,492	6,358.76	1,288
842.533	972.7	67,871	6,688	320,999.1	75,985
843.545	181.2	138	13,435	59,731.47	6,491
856.549	192.2	229,437	7,867	63,781.38	12,797
870.564	696.5	107,834	7,507	234,749.7	51,413
882.564	3.2	55	7,797	1,074.29	219
892.544	4	129	7,804	1,316.1	266
919.641	6.1	456	7,916	1,839.42	383
932.509	5.2	395	9,838	1,513.77	259
938.487	4.1	121	10,874	1,132.01	194
942.483	44.4	30,395	8,271	12,042.76	2,603
956.503	7	688	6,221	1,795.49	506
958.475	4.6	208	8,830	1,168.24	246
964.481	12.3	1016	8,407	3,031.33	671
980.482	5.6	116	7,123	1,340.69	344
982.599	63.2	27,125	8,447	15,140.68	3,405
1004.602	22.7	1811	8,525	5,358.07	1,212
1022.641	3.8	175	10,336	,870.36	164
1045.591	289.1	251,589	8,583	65,145.58	16,276
1057.595	3.9	50	9,916	,879.27	188
1095.683	42.2	21,033	9,670	10,865.3	2,602
1101.592	3.8	347	10,288	999.42	231
1109.416	6.1	745	3,342	1,625.58	1,148
1117.687	16.2	2,270	8,437	4,426.82	1,206
1126.581	5.8	320	11,255	1,612.63	336
1127.615	4.4	296	9,929	1,252.03	289
1130.656	3.7	182	4,166	1,042.97	607
1145.618	775.4	229,340	8,576	217,306.5	65,290
1158.642	6.2	591	7,402	1,656.17	603
1167.598	5.2	133	9,740	1,349.63	336
1230.616	3.8	208	12,539	757.54	179
1242.889	3.8	468	1,274	741.64	1,669
1258.649	13.8	2,189	10,179	2,791.91	797
1277.691	18.5	3,071	11,098	3,888.43	1,033
1291.687	3.5	128	10,179	762.81	216
1305.673	5.4	413	11,341	1,228.30	348
1308.62	13.3	1,826	10,430	2,937.49	918
1314.795	10	2,120	10,015	2,229.11	697
1330.637	5.3	291	11,092	1,193.92	342
1334.716	27.8	11,190	10,343	6,017.86	1,856
1348.71	5.9	520	10,047	1,242.37	393
1460.722	7.4	245	10,228	1,514.81	566
1462.663	4.5	219	4,132	917.93	896
1477.757	361	195,998	11,848	75,670.85	25,820
1482.731	5	153	10,091	1,059.38	389
1499.759	109.2	182,404	11,634	24,507.14	8,422
1515.726	23.3	6405	11,643	5,315.39	1,845
1517.788	4.8	406	13,346	1,102.87	365
1521.735	3.5	463	13,291	827.27	242
1531.685	4.3	177	12,822	979.71	309
1537.701	4.2	272	13,092	956.67	308
1539.694	4.3	261	10,564	953.69	398
1794.833	45.5	61,583	15,222	8,349.82	3,185
1798.833	6.4	561	14,796	1,125.50	464
1810.822	3.6	112	11,933	649.37	362
1816.825	10.4	1508	14,904	1,866.72	746
2016.986	58.5	46,025	15,448	9,434.66	4,283
2038.98	60.8	82,374	16,321	9,974.88	4,234
2054.959	9.6	916	14,667	1,666.27	793
2067.058	15.5	2,262	16,130	2,611.57	1,136
2069.081	5	319	13,902	857.07	429
2126.091	3.4	186	20,763	624.55	204
2211.138	26.7	8,768	17,550	4,999.14	2,202
2217.052	30.9	6,577	15,986	5,693.92	2,844

(continued on next page)

^a Mass over charge.^b Signal to noise ratio.^c Area of m/z peak.

TABLE 3. (continued from preceding page)

m/z ^a	SN ^b	Quality factor	Resolution	Intensity	Area ^c
2225.152	5.3	672	17,196	985.61	419
2233.118	16	2,663	14,371	2,803.70	1,517
2239.045	65.8	12,725	12,315	11,060.29	7,577
2249.075	4.8	276	8,710	794.95	822
2255.027	20.6	5,302	14,173	3,283.51	1,907
2261.135	3.7	255	8,098	560.53	610
2271.004	4.1	267	9,079	605.21	618
2564.231	4.9	403	20,007	398.80	193
2599.357	4.5	302	15,807	357.75	209
2807.378	45.4	4,252	13,437	2,953.05	2,376
2921.421	5.6	488	13,789	333.58	279

Fig. 5. A, Cell death in tomato 1 (CDiT1) protein sequence (191 amino acids). Bold: putative signal peptide for secretion. Underlined: peptides deduced from de novo sequencing. Dashed lines: matrix-assisted laser desorption/ionization/ mass spectrometry tryptic peptides (peptide mapping). **B**, Alignment of the amino acid sequence of CDiT1 with those of orthologs in other pathogenic species of Pleosporales (Pter: *Pyrenophora teres* f. *teres*, accession number EFQ87858; Pnod: *Phaeosphaeria nodorum*, accession number EAT77054; Ptri: *Pyrenophora tritici-repentis*, accession number EDU49117). Alignment was performed using ClustalW software.

described (7). Three days after inoculation, fungal inoculum was removed and a root piece of 1 cm was cut around the infected area of each seedling. The root pieces were pooled and immediately ground in liquid nitrogen, and total RNA was extracted as previously described (37).

Reverse transcriptions were performed using Superscript III reverse transcriptase in a total volume of 25 µl, with 3 µg of total RNA. PCR amplification of *CDiT1* transcripts was carried out using 4 µl of cDNA template, primers Pr1+ and Pr2-, annealing temperature of 51°C, and extension time of 45 s. Amplification of

fungal *actin* transcripts was carried out in the same conditions using primers Act1+ and Act2- (Table 2) as a reference gene. To achieve amplification of fungal *actin* in planta, PCR parameters were modified: nucleotides concentration was raised to 0.3 mM, number of cycles was raised to 40, a larger amount of cDNA template was used (7 instead of 4 µl), and Platinum *Pfx* DNA polymerase was used instead of *Taq*.

RESULTS

P. lycopersici secretes heat-sensitive macromolecules inducing cell death in tomato leaves. In order to screen molecules secreted by *P. lycopersici* and inducing cell death in tomato (*S. lycopersicum*), the filtrate of a culture of DSM 62931 was

infiltrated into the apoplast of leaves of MM. Cell death in MM leaf mesophyll occurred within 24 h after infiltration. It was characterized by tissue collapse in the entire infiltrated area, which displayed a dry texture and copper color with time (Fig. 1). Three other pathogenic isolates of *P. lycopersici* (ER824, ER876, and ER931) were also tested. Their culture filtrates were all able to cause cell death in leaves of MM (data not shown). Induction of cell death was not light dependent but was abolished after boiling of the filtrate (data not shown). Molecular mass of the compound responsible for this activity was estimated at 30 to 50 kDa using centrifugal filter devices (data not shown).

P. lycopersici cell death inducer CDi1 is a homodimeric protein of 35 kDa. The inducer of cell death was purified from the filtrate of a culture of DMS 62931 using FPLC with an anion

Fig. 6. Southern blot analysis of cell death in tomato 1 (*CDi1*). **A**, Genomic DNA of *Pyrenochaeta lycopersici* was digested with restriction enzymes *Bam*HI (**B**), *Eco*RI (**E**), *Pst*I (**P**), *Sal*I (**SI**), and *Xba*I (**Xb**). Restriction fragments were hybridized with a 385-bp-long *CDi1* radioactive probe (**P**) at 65°C. **M**: molecular weight marker. **B**, Restriction map of *CDi1* locus. Additional restriction sites: *Spe*I (**Sp**), *Clal* (**C**), *Xho*I (**Xh**), and *Sac*I (**Sc**). **P**: probe used for Southern blot experiment (109 to 494); 5'R: 5' end of 5' rapid amplification of cDNA ends (RACE) product (–82); 3'R: 3' end of 3' RACE product (+784). Boxes: *CDi1* exons (exon 1: 1 to 258 and exon 2: 341 to 655). Thick line: *CDi1* intron (82 bp).

Fig. 7. Recombinant cell death in tomato 1 (*CDi1*::*HIS*) induces cell death in tomato leaves. **A**, Secretion and purification of *CDi1*::*HIS* from *Pyrenochaeta lycopersici* transformant KHIS5. Aliquots of crude filtrates (CFs) of cultures of wild type (WT), transformant KWT1, and transformant KHIS5 were concentrated (nominal molecular weight limit = 10). Remaining crude filtrates of WT, KWT1, and KHIS5 cultures were mixed with nickel particles, eluates (AP) concentrated as well, and all types of concentrates analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Oversecretion of a protein with *CDi1* molecular weight was detected in crude filtrates of KWT1 and KHIS5 cultures (lanes KWT1-CF and KHIS5-CF) but only the eluate of KHIS5 crude filtrate affinity purification contained recombinant *CDi1*::*HIS* (lane KHIS5-AP). **B**, Infiltration into 'MM' leaf apoplast of the eluate of KHIS5 crude filtrate affinity purification induced cell death of mesophyll cells within 24 h, whereas **C**, the eluate of KWT1 crude filtrate affinity purification did not.

TABLE 4. Matrix-assisted laser desorption/ionization/mass spectrometry peptide masses of cell death in tomato 1 (*CDi1*) used for mapping

Peptide number	Input	Found	Deviation	Missed cleavage	From-to ^a	Sequence ^b
1	715.341	714.366	0.032	0	76–82	GESPLGR
2	982.599	981.586	–0.006	0	162–170	NPVLITPTK
3	1,095.683	1,094.670	–0.006	1	162–171	NPVLITPTK ¹ L
4	1,145.618	1,144.584	–0.027	0	15–26	VTGVASNTPSGR
5	1,277.691	1,276.656	–0.027	1	72–82	WFT ¹ GESPLGR
6	1,308.620	1,307.603	–0.009	0	127–138	YTASFTAEGSFK
7	1,477.757	1,476.736	–0.014	0	114–126	FNHVADVLYQGSK
8	2,016.986	2,015.958	–0.020	0	52–71	SDGTDVIVPAGSQGINCQAK
9	2,217.052	2,216.010	–0.034	0	139–161	VGDNLAQCGSGVCGWGLAPGK

^a *CDi1* putative signal peptide was excluded for mapping. Amino acid residues of peptide, from residue number to residue number.

^b ¹ indicates missed trypsin cleavage.

exchange and a gel filtration column, successively (Fig. 2). Activity was detected in fractions corresponding to a fungal protein of ≈ 35 kDa after a size-exclusion experiment including standards with known molecular masses (Fig. 3A, peak 2). However, SDS-PAGE analysis of the fractions collected during this experiment showed that this fungal protein of 35 kDa is running as a single band of 17 to 18 kDa in these conditions of electrophoresis (Fig. 3B). To confirm that the discrepancy between the two estimations of the molecular mass of this protein was not resulting from an SDS-PAGE artifact, the size-exclusion experiment was repeated using the same internal standards but with a denaturing buffer. In these conditions, the fungal protein eluted at the position expected for a protein of 17 to 18 kDa (Fig. 3C, peak 2). This suggests that this protein is a homodimer made of two subunits of 17 to 18 kDa in native conditions. As the putative candidate for the cell death inducer secreted by *P. lycopersici*, this protein was named CDiT1.

CDiT1 has orthologs in other phytopathogenic fungi. To establish identity of CDiT1, a protein sample was analyzed by MS (Fig. 4A; Table 3). MALDI MS peptide mass fingerprinting did not identify any known homolog of CDiT1 in the AlphaLys A/S in-house protein database. However, de novo sequencing of two tryptic peptides after MS/MS fragmentation yielded sufficient sequence information (Fig. 4B) to design degenerated primers that were used for PCR amplification of a 399-bp piece of *CDiT1* cDNA. A full open reading frame (ORF) of 573 bp was deduced

from sequence assembly of this PCR product with products of 5' and 3' RACE experiments. An N-terminal signal peptide was detected in the polypeptide sequence encoded by this ORF, with high probability of cleavage between amino acids 20 and 21 (Fig. 5A). The predicted mature secreted protein has 171 residues and a theoretical mass of 17.8 kDa, close to the estimation of 17 to 18 kDa made for CDiT1 after SDS-PAGE. Moreover, mapping of MALDI MS tryptic peptides of CDiT1 with the mature protein sequence gave nine hits and residue coverage of 59% (Table 4; Fig. 5A), confirming that the cDNA cloned corresponded to *CDiT1*. The nucleotide sequence encoding CDiT1 was registered in the European Nucleotide Archive under accession number HE615138.

A Southern blot experiment showed that *CDiT1* is present in only one copy in the *P. lycopersici* genome (Fig. 6A). Cloning and sequencing of this genomic locus showed that *CDiT1* has two exons of 258 and 315 bp and one intron of 82 bp (Fig. 6B). The *CDiT1* full coding sequence was amplified by RT-PCR from mycelium of *P. lycopersici* isolates ER824, ER876, and ER931 grown in liquid medium. Cloning and sequencing of amplification products confirmed that *CDiT1* is expressed by these isolates as well, and identical in sequence to the one in DMS 62931 (data not shown).

Fungal orthologs of CDiT1 were searched in public sequence databases. This revealed that CDiT1 has one hypothetical ortholog in three plant pathogens belonging to the order Pleosporales

Latin name	Common name	Cultivar/Ecotype	Family	Crude filtrate	Pure CDiT1
<i>Solanum lycopersicum</i>	tomato	Money Maker	Solanaceae	active (1)	active (1)
<i>Solanum lycopersicum</i>	tomato	Moboglan	Solanaceae	active (1)	active (1)
<i>Solanum lycopersicum</i>	tomato	Pearson	Solanaceae	active (1)	active (1)
<i>Solanum lycopersicum</i>	tomato	Mogeor	Solanaceae	active (1)	active (1)
<i>Solanum lycopersicum</i>	tomato	Corbarino	Solanaceae	active (1)	active (1)
<i>Solanum lycopersicum</i>	tomato	Castlemart	Solanaceae	active (1)	active (1)
<i>Solanum lycopersicum</i>	tomato	P.I. 260397 96GI	Solanaceae	active (1)	active (1)
<i>S. lycopersicum</i> x <i>S. habrochaites</i>	F1 interspecific hybrid	Brigeor	Solanaceae	active (1)	active (1)
<i>Solanum pimpinellifolium</i>	currant tomato	Culebras	Solanaceae	active (2)	non active
<i>Solanum pimpinellifolium</i>	currant tomato	Viv	Solanaceae	active (2)	non active
<i>Solanum pimpinellifolium</i>	currant tomato	Pichilingue	Solanaceae	active (1)	non active
<i>Solanum pimpinellifolium</i>	currant tomato	Chota to El Angel	Solanaceae	active (1)	non active
<i>Solanum pimpinellifolium</i>	currant tomato	Babahoyo	Solanaceae	active (1)	non active
<i>Solanum neorickii</i>	wild tomato	unknown	Solanaceae	active (1)	active (1)
<i>Solanum melongena</i>	eggplant	V-S-11	Solanaceae	active (2)	non active
<i>Solanum melongena</i>	eggplant	PA 4	Solanaceae	active (2)	non active
<i>Solanum melongena</i>	eggplant	C-S-16	Solanaceae	active (2)	non active
<i>Solanum melongena</i>	eggplant	Tsakoniki	Solanaceae	active (2)	non active
<i>Solanum melongena</i>	eggplant	Langada	Solanaceae	active (2)	non active
<i>Nicotiana tabacum</i>	tobacco	W38	Solanaceae	active (2)	non active
<i>Nicotiana benthamiana</i>	wild tobacco	unknown	Solanaceae	active (3)	non active
<i>Datura stramonium</i>	jimson weed	Bergianska Trädgården 2009	Solanaceae	active (1)	active (2)
<i>Capsicum annuum</i>	sweet pepper	Maor	Solanaceae	active (2)	non active
<i>Capsicum annuum</i>	sweet pepper	Thai Hot	Solanaceae	active (2)	non active
<i>Capsicum chinense</i>	chili pepper	Pi 159254	Solanaceae	active (2)	non active
<i>Capsicum chinense</i>	chili pepper	Habanero	Solanaceae	active (2)	non active
<i>Capsicum frutescens</i>	wild pepper	2814.6	Solanaceae	active (2)	non active
<i>Carthamus tinctorius</i>	safflower	Orange Grenade	Asteraceae	active (2)	non active
<i>Cucumis sativus</i>	cucumber	Rhens Druv	Cucurbitaceae	active (1)	active (1)
<i>Cucumis sativus</i>	cucumber	Futura F1	Cucurbitaceae	active (1)	active (1)
<i>Cucumis melo</i>	melon	Stellio F1	Cucurbitaceae	active (1)	active (1)
<i>Cucumis melo</i>	melon	Iran A	Cucurbitaceae	active (2)	non active
<i>Cucumis melo</i>	melon	Ogon 9	Cucurbitaceae	active (1)	active (1)
<i>Cucumis melo</i>	melon	Boule d'Or 1	Cucurbitaceae	active (1)	active (1)
<i>Cucumis melo</i>	melon	Ginsen Makuwa	Cucurbitaceae	non active	non active
<i>Cucumis melo</i>	melon	Doublon	Cucurbitaceae	active (1)	active (1)
<i>Cucumis melo</i>	melon	Delicious 51	Cucurbitaceae	active (1)	active (1)
<i>Cucurbita maxima</i>	pumpkin	Kaempmelon	Cucurbitaceae	active (1)	non active
<i>Cucurbita pepo</i>	squash	Diamant F1	Cucurbitaceae	active (1)	non active

Fig. 8. Results of the infiltration of a crude filtrate of *Pyrenochaeta lycopersici* cultivation medium and of a cell death in tomato 1 (CDiT1) solution into the apoplast of the leaves of various host plants. A green background indicates that the infiltration had no effect. Symptoms were otherwise ranked from yellow (mild chlorosis) to red (cell death) according to their intensity. Symptoms were observed in the entire infiltrated area only where "active" is in bold characters. Number in brackets indicates the number of days required for the expression of the symptoms recorded, which did not evolve beyond that stage. Systemic symptoms outside of the infiltrated area (growth inhibition, or even death of immature leaves) were observed in some Cucurbitaceae species after infiltration with the crude filtrate, and are indicated with "active" in yellow characters.

(Fig. 5B): *Phaeosphaeria nodorum* (accession number: EAT77054), *Pyrenophora teres f. teres* (EFQ87858), and *P. tritici-repentis* (EDU49117). CDiT1 has also one more distant ortholog in two other filamentous fungi belonging to the order Sordariales: *Neurospora crassa* and *Chaetomium globosum* (data not shown). None of these orthologs of CDiT1 has been isolated yet but their existence has been inferred from genome or cDNA library sequencing. No protein with a sequence similar to CDiT1 was found in any other organism. No known functional domain was identified in the sequence of mature CDiT1.

Recombinant CDiT1 induces cell death in tomato. To confirm that CDiT1 is causing cell death in MM leaves, a secreted recombinant version of CDiT1 bearing a C-terminal polyhistidine tag (CDiT1::HIS) was produced in *Pyrenochaeta lycopersici*. A DNA fusion encoding CDiT1::HIS was cloned between *CDiT1* promoter and terminator (construction pKHIS) and introduced in the genome of DSM 62931 by protoplast transformation. Transformants bearing additional wild-type copies of *CDiT1* (construc-

tion pKWT) were used as controls. Two transformants, KWT1 and KHIS5, were selected for secreting in a large amount a protein having the molecular mass expected for wild-type CDiT1 and for CDiT1::HIS, respectively (Fig. 7A). Affinity purifications were carried out with culture filtrates of the wild type, KWT1, and KHIS5. Analysis of elution products by SDS-PAGE showed that a single protein with the molecular mass expected for CDiT1::HIS was detected in elution product of KHIS5 only (Fig. 7A). All elution products were infiltrated into MM leaves after buffer exchange but only KHIS5 induced cell death (Fig. 7B and C). Concentration of CDiT1::HIS secreted by KHIS5 was measured after affinity purification and buffer exchange. Serial dilutions of this solution and of a solution of wild-type CDiT1 purified by FPLC were prepared after determination of protein concentration and infiltrated into MM leaves. Activities of the wild-type protein and of CDiT1::HIS were not detectable at concentrations lower than 3 µg/ml (data not shown). The elution product of KHIS5 was also analyzed by size-exclusion chroma-

Fig. 9. Current tomato (*Solanum pimpinellifolium*) has lower sensitivity to cell death in tomato 1 (CDiT1) than cultivated tomato (*S. lycopersicum*). A culture filtrate (CF) of *Pyrenochaeta lycopersici* (isolate DMS 62931) and a solution of CDiT1 purified by fast protein liquid chromatography (FPLC) (CDiT1) were infiltrated into leaves of several cultivars of *S. lycopersicum* and *S. pimpinellifolium*. All were sensitive to CF but only cultivars of *S. lycopersicum* were sensitive to CDiT1 (C1: sterile MS-20 medium; C2: sterile solution of 20 mM Tris, pH 8.0). **A**, *S. lycopersicum* 'Pearson'. **B**, *S. pimpinellifolium* 'Pichilingue'. **C**, Solution of wild type CDiT1 purified by FPLC and concentrated five times with a centrifugal filter device (5X) can induce cell death in the leaves in *S. pimpinellifolium* Pichilingue (1X: wild-type CDiT1 solution [14 µg/ml]; C: sterile solution of 20 mM Tris, pH 8.0).

Fig. 10. **A**, Epicortical (e) and intraradicular (i) expression of β -glucuronidase (GUS) on the root of an 'MM' seedling infected with *Pyrenochaeta lycopersici* transformant 1K4. Observation was made 8 days post infiltration. Black bar: 1 mm. **B**, Sensitivity to epicortical root infection by *P. lycopersici* is not species dependent. Extent of epicortical expression of GUS (mean values $\pm$ standard error) varied with cultivars but did not correlate with the type of *Solanum* sp. they belong to. Black bars = *Solanum lycopersicum* cultivars: Bo, 'PI 260397 96GI'; Mo, 'Mogeor'; and MM, 'Money Maker'. White bars = *S. pimpinellifolium* cultivars: Vi, Viv; Cu, Culebras; and Pi, Pichilingue. **C**, Cultivars of *S. pimpinellifolium* are more resistant to root cell colonization by *P. lycopersici* than cultivars of *S. lycopersicum*. Extent of intraradicular expression of GUS (mean values $\pm$ standard error) was significantly lower in three cultivars of *S. pimpinellifolium* compared with three cultivars of *S. lycopersicum*.

tography in native conditions. CDi1::HIS eluted from the gel-filtration column before carbonic anhydrase, at the retention time corresponding to a protein of 35 kDa (data not shown).

CDi1 affects host plants of *P. lycopersici* differentially. In order to determine whether other host plants of *P. lycopersici* are also sensitive to CDi1, the filtrate of a culture of DMS 62931 and a solution of CDi1 purified by chromatography were infiltrated in the leaf apoplast of several Dicotyledones to which *P. lycopersici* is pathogenic. Intensity (from a mild chlorosis to complete cell death in the entire infiltrated area) and speed of apparition of symptoms (1 to 4 days postinfiltration) following leaf infiltrations with the crude filtrate varied from one species to the other but all of them showed symptoms when compared to control infiltrations of sterile culture medium (Fig. 8). In contrast, infiltration of CDi1 resulted in cell death in the infiltrated area in some of the plants tested but complete absence of symptoms in others. CDi1 caused fast cell death in the entire infiltrated area of leaves of seven cultivars of *S. lycopersicum* (MM, 'Moboglan', 'Pearson' [Fig. 9A, results of infiltrations], Mogeor, Corbarino, 'Castlemart', and PI 260397 96GI), interspecific F1 hybrid *S. lycopersicum* × *S. habrochaites* Brigeor, *S. neorickii*, *Datura stramonium*, and two cultivars of *Cucumis sativus* ('Rhensk Druv' and the F1 hybrid 'Futura'). CDi1 caused cell death in part of the infiltrated area of leaves of four cultivars of *C. melo* highly susceptible to *P. lycopersici* ('Boule d'Or 51', 'Doublon', 'Ogon 9', and F1 hybrid 'Stellio') but showed no activity in leaves of all other plants tested (*S. pimpinellifolium* Culebras, 'Chota to El Angel', Viv, 'Babahoyo' and Pichilingue [Fig. 9B, results of infiltrations]; *S. melongena* 'Tsakoniki', 'Langada', 'PA-4', 'C-S-16', and 'V-S-11'; *Capsicum annuum* 'Maor' and 'Thai Hot'; *C. chinense* 'Habanero' and 'PI 159254'; *C. frutescens* '2814.6'; *Nicotiana tabacum* 'W38'; *N. benthamiana*; *C. tinctorius* 'Orange Grenade'; *C. pepo* F1 hybrid 'Diamant'; *Cucurbita maxima* 'Kaempmelon'; and *C. melo* 'Iran A' and 'Ginsen Makuwa').

In order to determine whether cultivars of *S. pimpinellifolium* are insensitive to CDi1 or less sensitive than cultivars of *S. lycopersicum*, aliquots of solutions of wild-type CDi1 purified by FPLC (14 µg/ml) and of recombinant CDi1::HIS purified by affinity (11 µg/ml) were concentrated fivefold and infiltrated into the leaf apoplast of *S. pimpinellifolium* Pichilingue, Culebras, and Viv and *S. lycopersicum* MM. Both solutions were active at their two different concentrations in MM. In the three cultivars of *S. pimpinellifolium*, both solutions were inactive at their lowest concentrations but active at their highest (Fig. 9C, results of infiltrations of wild-type CDi1 in Pichilingue), showing that *S. pimpinellifolium* is also sensitive to CDi1 but less than *S. lycopersicum*.

Cultivars of *S. pimpinellifolium* are more resistant to root colonization by *P. lycopersici* than cultivars of *S. lycopersicum*. The root of seedlings of three cultivars of *S. lycopersicum* and three cultivars of *S. pimpinellifolium* was inoculated in vitro with 1K4, a transformant of *P. lycopersici* carrying a GUS reporter gene fusion that is expressed only during infection. Expression of GUS was monitored after 8 days as a marker of fungal infection.

Areas stained in blue were separated in two categories according to their intensity, which reflected a difference in extent of root colonization by the fungus. Epicortical GUS expression was observed as pale blue staining, due to fungal hyphae present at the surface of the root cortex (Fig. 10A, area e). Though clearly distinct from vegetative hyphae, which grow without showing GUS expression, epicortical hyphae were never observed inside root cells at higher magnification. On the contrary, intraradicular GUS expression appeared as intense blue staining, due to fungal hyphae growing deeper inside the root cylinder and invading root cells (Fig. 10A, area i). The six cultivars tested here showed differences in extent of epicortical blue staining after inoculation that depended more on specific properties of each cultivar rather than on the fact that they belonged to *S. lycopersicum* or *S. pimpinellifolium* species (Fig. 10B; Table 5). In contrast, differences in extent of intraradicular growth of the fungus between the six cultivars were species specific: roots of the three cultivars of *S. pimpinellifolium* tested were all significantly less prone to have their root cells colonized by hyphae of 1K4 than those of the three cultivars of *S. lycopersicum* (Fig. 10C; Table 6). This experiment was performed twice with similar results.

CDi1 is highly expressed during tomato root infection. Expression levels of *CDi1* during growth of *P. lycopersici* in vitro and during infection of tomato root were compared using semiquantitative RT-PCR. Expression level of fungal *actin* was used as reference. Amplification levels of *CDi1* cDNA and of *actin* cDNA in vitro were high and similar. In infected roots, *CDi1* cDNA amplification level was also high, whereas *actin* cDNA amplification was hardly detectable. The intensity of cDNA amplification signal ratio (*CDi1/actin*) was very high, showing clearly that *CDi1* is expressed at a much higher level during infection than during growth in artificial medium (Fig. 11).

DISCUSSION

The rationale guiding this study was that virulence factors secreted by *P. lycopersici* can be detected after infiltration into host leaves, due to their capacity to induce disease-related symptoms. Hence, our objectives were to purify fungal inducers of cell death and to characterize them by using FPLC, MS, and gene cloning. Here, we report the identification of CDi1, a novel type of homodimeric protein inducing cell death in tomato. The activity of this protein was confirmed after infiltrating recombinant, affinity-purified CDi1 into plant leaves. CDi1 affects host plants of *P. lycopersici* differentially, their sensitivity to the protein being variable. Currant tomato (*S. pimpinellifolium*) shows lower sensitivity to CDi1 and higher tolerance to root colonization by *P. lycopersici* compared with cultivated tomato (*S. lycopersicum*). Interestingly, expression of *CDi1* increases drastically during root colonization, when fungal hyphae can secrete the protein directly inside root cells. Together with the ultrastructural modifications observed previously in root cells of a variety of cultivated tomato during fungal penetration (8), our

TABLE 5. Pairwise statistical analysis of the extent of epicortical expression of β-glucuronidase in three cultivars of *Solanum lycopersicum* and three cultivars of *S. pimpinellifolium*

	<i>P</i> (t tests)		
	<i>S. pimpinellifolium</i>		
<i>S. lycopersicum</i>	Viv	Culebras	Pichilingue
PI 260397 96GI	0.102	0.351	0.262
Mogeor	0.01**	0.479	0.09
Money Maker	0.009**	0.076	0.021*

a Asterisks: * indicates *t* was significant at *P* < 0.05 and ** indicates *t* was very significant at *P* < 0.01.

TABLE 6. Pairwise statistical analysis of the extent of intraradicular expression of β-glucuronidase in three cultivars of *Solanum lycopersicum* and three cultivars of *S. pimpinellifolium*

	<i>P</i> (t tests)		
	<i>S. pimpinellifolium</i>		
<i>S. lycopersicum</i>	Viv	Culebras	Pichilingue
PI 260397 96GI	0.0175*	0.038*	0.018*
Mogeor	0.004**	0.007**	0.005**
Money Maker	0.006**	0.013**	0.006**

a Asterisks: * indicates *t* was significant at *P* < 0.05 and ** indicates *t* was very significant at *P* < 0.01.

results support the idea that CDiT1 is a virulence factor, effective against sensitive species during this specific stage of infection. Nevertheless, formal demonstration that CDiT1 is a virulence factor is still lacking, because it is not yet possible to perform functional analysis of a gene in *P. lycopersici* (P.-H. Clergeot, unpublished). The four isolates of *P. lycopersici* monitored in this study secrete an inducer of cell death in liquid culture medium, and their *CDiT1* transcripts have identical sequences. Therefore, differences in virulence observed among the three isolates (ER824, ER876, and ER931) on Corbarino tomato (19) must be explained by other factors. These three isolates have been collected in the same geographic area (Italy), and a larger survey would be required before concluding that the production of this protein is common to all pathogenic isolates of *P. lycopersici* worldwide. CDiT1 is the first protein of its kind to be characterized and its biochemical function is still unknown; however, other types of dimeric proteins secreted by fungi, such as cerato-platanins, have been shown to play a role as inducers of necrosis during interactions with plants (38,41).

The discovery that CDiT1 has differential activity on various hosts of *P. lycopersici* could contribute to explain the variety of disease symptoms observed on these plants, and outline a model for the molecular interactions between the pathogen and its hosts. Disease caused by *P. lycopersici* on different hosts is not due to different pathogenic subspecies but to different isolates of a single species with a broad host range: isolates pathogenic on tomato are usually virulent on other hosts, and the reverse is also true (17,35). In comparison with other pathogenic species of Pleosporales, there is no evidence that *P. lycopersici* secretes a metabolite required to infect a specific host genotype, ruling out the possibility that virulence could depend on a single major HST, as in *Cochliobolus* spp. and pathotypes of *A. alternata* (23). *P. lycopersici* affects hosts taxonomically more distantly related than those of other pathogenic species of Pleosporales secreting proteinaceous HSTs, such as *Stagonospora nodorum* or *P. tritici-repentis* (6,32). Therefore, the concept of HST does not seem relevant to describe the interaction between *P. lycopersici* and its hosts. Nevertheless, the picture emerging from published data about host specificity of *P. lycopersici* and severity of CRR (17,22,26,34,35) is that development of disease symptoms could be governed by a limited number of genetic interactions between the pathogen and its different hosts. Reports about host range have sometimes been contradictory, depending on where, with

which fungal isolates, and with which host cultivars infection assays have been made. Cucumber (*Cucumis sativus*), jimson weed (*D. stramonium*), and nightshade (*Solanum nigrum*) have alternatively been described as host or non-host plants (17,22,35). Squash (*Cucurbita pepo*) has been described as a host plant but one of three cultivars remained symptomless (17). The inoculation of several Solanaceae spp. with an isolate of *P. lycopersici* pathogenic to tomato showed a large range of responses, from complete resistance to a higher sensitivity than tomato, and susceptibility did not always correlate with the possibility of reisolating the fungus from inoculated roots. For example, the fungus could be reisolated from the roots of *Physoclaena orientalis*, a completely resistant species, whereas it was impossible from the roots of some species described as susceptible, such as pepper (17,35). Reported hosts did not always develop the same type of symptoms, although it was demonstrated that they were caused by *Pyrenochaeta lycopersici*. Corky roots were observed on tomato, melon (*C. melo*), and safflower (*Carthamus tinctorius*) but not on tobacco (*N. tabacum*), pepper (*Capsicum annuum*), or spinach (*Spinacia oleracea*), where root browning was observed instead (17,26,30,34). A possible interpretation of these data is that similar disease symptoms occurring on different hosts could be due to the same limited number of virulence factors of *P. lycopersici*, and to similar host *S* genes, in spite of the taxonomic distance existing between these plants. This would explain why rather distantly related hosts such as tomato and safflower (two Asterid species) and melon (a Rosid species) develop very similar disease symptoms (corky roots), whereas *Solanum hirsutum*, a wild tomato species fertile with cultivated tomato, is completely resistant. It would also explain why similar intermediate symptoms (root browning) are observed on more distantly related hosts, such as pepper (an Asterid species) and spinach (a Caryophyllale species). In this model, virulence factors of the pathogen affect host plants differentially: symptoms result from the combination of the multiple molecular interactions between the host plant and these factors, and disease severity depends on whether the host is more or less sensitive to each of them. This model establishes a link between host sensitivity to purified fungal molecules detected using laboratory assays and the development of specific disease symptoms. One of our main goals in the future is to test its validity with CDiT1 and with other inducers of cell death secreted by *P. lycopersici*. Response to CDiT1 was characterized by the same clear phenotype of cell death in all cultivars of all highly sensitive host species tested, whereas host species with a lower sensitivity to CDiT1 showed no symptoms. In currant tomato (*S. pimpinellifolium*), low sensitivity to CDiT1 correlated with a higher tolerance to root colonization by *P. lycopersici* compared with CDiT1-sensitive cultivated tomato. The genetic basis of sensitivity to CDiT1 has not been investigated yet, but the possibility exists, because cultivated tomato and *S. pimpinellifolium* cultivars can be crossed. Moreover, a genetic linkage map between the two species is now available (2), which could facilitate genetic localization.

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Fig. 11. Expression of cell death in tomato 1 (*CDiT1*) in *Pyrenochaeta lycopersici* (isolate DMS 62931). Expressions of *CDiT1* and *actin* (*Act*) during mycelium growth in liquid medium (*in vitro*) and during infection of the root of tomato seedlings (*in planta*) were compared by semiquantitative reverse-transcription polymerase chain reaction (PCR). Amplification of both *actin* and *CDiT1* cDNAs was clear *in vitro*. Amplification of *CDiT1* cDNA was strong *in planta* but amplification of *actin* was very weak, even after optimization of PCR conditions, showing that the expression level of *CDiT1* is much higher during infection than in liquid medium.

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