Successful search for a resistance gene in tomato targeted against a virulence factor of a fungal pathogen

Richard Laugé*, Matthieu H. A. J. Joosten*, Jair P. W. Haanstra†, Paul H. Goodwin*‡, Pim Lindhout†, and Pierre J. G. M. De Wit*§

*Department of Phytopathology, Binnenhaven 9, 6709 PD Wageningen, The Netherlands; and †Department of Plant Breeding, Lawickse allee 166, 6709 DB Wageningen, The Netherlands

ABSTRACT The interaction between tomato and its fungal pathogen *Cladosporium fulvum* complies with the gene-for-gene system, in which specific recognition of fungal proteins by plant genotypes with matching resistance genes results in host resistance. Two proteins, ECP1 and ECP2, secreted by *C. fulvum* during infection, are required for full virulence of the fungus on tomato. We chose the most important virulence factor, ECP2, for a targeted search for hypersensitive response (HR)-based resistance among a collection of tomato genotypes. By screening with recombinant potato virus X that expresses the *Ecp*2 gene, we identified four lines that respond with HR toward ECP2. The capacity to recognize ECP2 and induce HR is sufficient to confer resistance in tomato against *C. fulvum* producing ECP2. Resistance is based on a single dominant gene, which we have designated *Cf-ECP2*, for resistance to *C. fulvum* through recognition of ECP2. Accordingly, an *Ecp*2-minus strain created by gene replacement is pathogenic on *Cf-ECP2* plants. However, due to lack of ECP2 the mutant strain is only weakly virulent. All strains of a worldwide collection of *C. fulvum* strains that were tested were found to produce a HR-inducing ECP2 protein. Because the *Cf-ECP2* gene operates through recognition of an important virulence factor, we expect it will confer durable resistance against *C. fulvum*. A similar targeted approach should allow the discovery of new valuable resistance genes in other pathosystems.

Plant surfaces and intercellular spaces are subjected continuously to potential pathogens. However, individual host plants that exhibit genetic resistance to a particular pathogen often occur in nature. Plant breeders have exploited natural resistance genes extensively via introgression from wild species into high-yielding agronomic cultivars. Nevertheless, this strategy has become less successful as resistance sources are limited and most plant pathogens show remarkable genetic variation leading to the appearance of strains that overcome introgressed genetic resistances (1). Most of the recognition mechanisms in plants remain unrevealed, and the practice of resistance breeding is rather empirical (2). In animal systems, viral vectors are used routinely to deliver antigens to raise antibodies against important pathogens (3–5). Often these antigens are derived from virulence factors of those pathogens. Following a similar approach, plant viruses can be used to deliver virulence factors of plant pathogens to find plants responding with a hypersensitive response (HR). Such plants would likely exhibit a durable type of HR-resistance toward the pathogen from which the virulence factor is originating.

The *C. fulvum*–tomato (*Lycopersicon esculentum*) interaction is a well established model system that complies with the gene-for-gene relationship (6). It is also one of the few systems in which there is ample experimental evidence for the involvement of proteinaceous elicitors (avirulence factors) from the pathogen in the induction of active HR-related resistance (7). Many HR-resistant traits have been described in accessions of wild *Lycopersicon* species and several dominant resistance genes have been introgressed into modern tomato cultivars. HR-mediated resistance in tomato against *C. fulvum* manifests itself as death of the first cells that come into contact with the penetrating fungus. Further growth of the pathogen is prevented, and the interaction is incompatible. In contrast to resistant plants, susceptible plants do not exhibit HR during interaction with the fungus. Colonization by fungal hyphae occurs through the whole leaf tissue and the interaction is compatible (8). As the development of *C. fulvum* is restricted to the apoplast of the leaf mesophyll, the elicitors that are secreted by the fungus can be isolated from apoplastic washing fluids (AFs) (9). In this way, two proteinaceous avirulence factors, AVR9 and AVR4, responsible for fungal recognition by host genotypes carrying the matching resistance genes *Cf-9* and *Cf-4*, respectively, have been characterized (10, 11). Two additional fungal extracellular proteins, ECP1 and ECP2, occur abundantly in AF of plants infected by *C. fulvum* (12, 13). Analysis of mutants of the fungus, in which either the *Ecp*1 gene or the *Ecp*2 gene had been deleted, showed that both ECP proteins are virulence factors for *C. fulvum*. ECP2 is the most important of the two because *Ecp*2-lacking strains are only weakly pathogenic, exemplified by poor leaf colonization and conidiation (14). By using the potato virus X (PVX) expression system (15), we have identified tomato lines that display HR upon exposure to ECP2. The gene, designated *Cf-ECP2*, which is responsible for ECP2 recognition confers resistance against *C. fulvum* on tomato. Because of the crucial role of ECP2 in virulence of the fungus, *Cf-ECP2* may prove to be of increased durability and would therefore be valuable in breeding programs aimed at sustainable agriculture.

MATERIALS AND METHODS

Construction of Recombinant PVX::*Ecp*2 and PVX Inoculation Procedure. The chimeric construct for *Ecp*2 expression and extracellular targeting of the ECP2 protein was obtained by PCR-mediated cloning. The two pairs of oligonucleotides PR1ECP2F (5′-CTTGCCGTGCCCGGAACGCTGGCAA-CTCGCCC-3′) and ECP2CLA (5′-CGGAAGCTTATCGA-TCTAGTCATCGTTGGACGGGTTG-3′); and OX10 (5′-CAATCACAGTGTTGGCTTGC-3′) and PR1ECP2R (5′-G-TTGCCAGCGTTCCGGGCACGGCAAGAGTGGGATA-TTAC-3′) were used for PCR with the *Ecp*2 cDNA and the PVX::*Avr*4 construct as templates, respectively (16). After

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Abbreviations: HR, hypersensitive response; AF, apoplastic fluid; PVX, potato virus X.
§To whom reprint requests should be addressed. e-mail: Pierre.DeWit@medew.fyto.wau.nl.
‡Present address: Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

PCR overlap extension and cloning in the PVX vector (15), the final PVX construct carried a chimeric transgene consisting of the sequence coding for the PR-1a plant signal peptide fused to the *Ecp*2 cDNA encoding the mature protein. This fusion was placed under the control of the PVX coat protein promoter, enabling expression as a subgenomic messenger during virus spread throughout the whole plant. Full-length infectious transcripts were generated *in vitro* by using the T7 mMES-SAGE mMACHINE kit (Ambion, Austin, TX) according to the recommendations of the supplier. The transcripts were inoculated by rubbing onto the leaves of *Nicotiana clevelandii* plants in presence of carborundum. Ten days after inoculation, infected leaves of *N. clevelandii* were collected and ground in 50 mM potassium phosphate buffer, pH 7.7, to prepare sap, which contains recombinant virus particles. The sap was used subsequently for inoculation of tomato lines in a similar way as described for *N. clevelandii*.

C. fulvum Inoculation Procedure. Suspensions of conidia of *C. fulvum* were prepared as described (17). In brief, fungal strains were grown on potato dextrose agar plates and conidia were suspended in water by rubbing the sporulating colony. The suspension that was obtained was sprayed onto the lower side of the tomato leaves. Disease symptoms were scored 2–3 weeks after inoculation.

Isolation, Purification, and Immunodetection of ECP2. Isolation of AFs from susceptible tomato plants inoculated with various *C. fulvum* strains was performed as described (9). Two weeks after inoculation, infected leaves were collected and water infiltrated in a vacuum chamber, and AF-containing soluble apoplastic compounds was obtained after centrifugation at $3,000 \times g$ for 10 min. Purified ECP2 was obtained from AF of a compatible *C. fulvum*–tomato interaction by gel filtration on a Sephadex G-50 column (Pharmacia) followed by chromatography on a Resource Q column (Pharmacia) as described by Wubben *et al.* (13). ECP2-containing fractions were identified with immunodetection by using polyclonal antibodies raised against ECP2 (13). For immunodetection of ECP2, AF containing the extracellular proteins produced *in planta* by the various strains that were tested, was separated on 15%-polyacrylamide gels containing SDS. The separated prot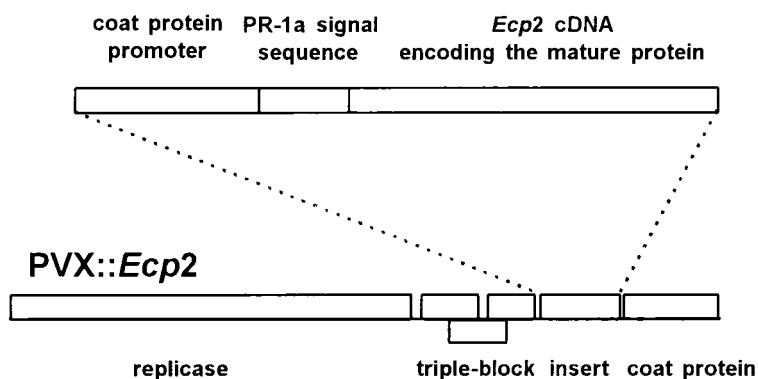eins were subsequently electro-transferred to Immobilon-P membrane (Millipore), and the blots were incubated with ECP2 polyclonal antibodies.

Crossings. To obtain three F_1 generations, lines 1 and 4 and their common ancestor were crossed to the nonresponsive Moneymaker cultivar, using the latter cultivar as the female parent. These F_1 generations were selfed to obtain the F_2 generations used in this study.

RESULTS

Identification of Tomato Genotypes that Display HR upon Exposure to ECP2. We have screened 21 lines that originated from early breeding programs for resistance against *C. fulvum*. They had been selected for resistance against the tester strain, race 2.3.4, which overcame all resistance genes, *Cf-2*, *Cf-3*, and *Cf-4*, that had been introgressed at that time (18, 19). The lines that should carry resistance genes different from these three genes were tested for the ability to respond with HR after exposure to ECP2. Screening was carried out by using PVX (15) for systemic production of the ECP2 protein, targeted to the apoplast of virus-infected plants (16), and results were verified with purified native ECP2 protein. PVX::*Ecp*2 recombinant virus was obtained by cloning the *Ecp*2 cDNA encoding the mature ECP2 protein, downstream of the sequence encoding the PR-1a plant signal peptide, under the control of the PVX coat protein promoter (Fig. 1*A*). Native ECP2 protein was obtained by sequential chromatographic separation of soluble proteins present in AF from a compatible *C. fulvum*–tomato interaction (Fig. 2*A*). Four of 21 tomato lines that were tested responded with HR after inoculation with PVX::*Ecp*2, as well as after injection with native ECP2. PVX::*Ecp*2 inoculation triggered the development of systemic chlorotic and necrotic lesions in leaves of the four lines (Fig. 1*B*). Upon injection of ECP2, the four lines developed chlorosis followed by necrosis in the center of the injected area within 3 days (Fig. 2*B*). Inoculation of these four lines with wild-type PVX caused normal mosaic symptoms. The other 17 lines and the control cultivar Moneymaker (lacking any known *C. fulvum* resistance gene) exhibited normal mosaic symptoms upon inoculation with either PVX::*Ecp*2 or wild-type PVX. Systemic HR never was observed in these plants. These results indicate that ECP2

A

coat protein promoter   PR-1a signal sequence   *Ecp*2 cDNA encoding the mature protein

PVX::*Ecp2*

replicase   triple-block insert   coat protein

B

FIG. 1.   Construction of the PVX::*Ecp*2 derivative and identification of lines responding with HR upon exposure to the ECP2 protein. (*A*) Schematic map of the PVX::*Ecp*2 derivative. (*B*) Specific HR upon ECP2 presentation. Four-week-old plants of the collection of tomato lines and the control cultivar Moneymaker (lacking any known *C. fulvum* resistance gene) were inoculated with sap containing PVX::*Ecp*2 (16) and symptoms were recorded 10–14 days after inoculation. Control plants of each line were inoculated with sap containing wild-type PVX. All plants gave normal systemic mosaic symptoms upon inoculation with wild-type PVX. Four lines gave systemic chlorosis and necrosis upon inoculation with PVX::*Ecp*2. The symptoms on one of these plants, line 1, are shown. Normal mosaic symptoms developed after inoculation with PVX::*Ecp*2 on all other plants.
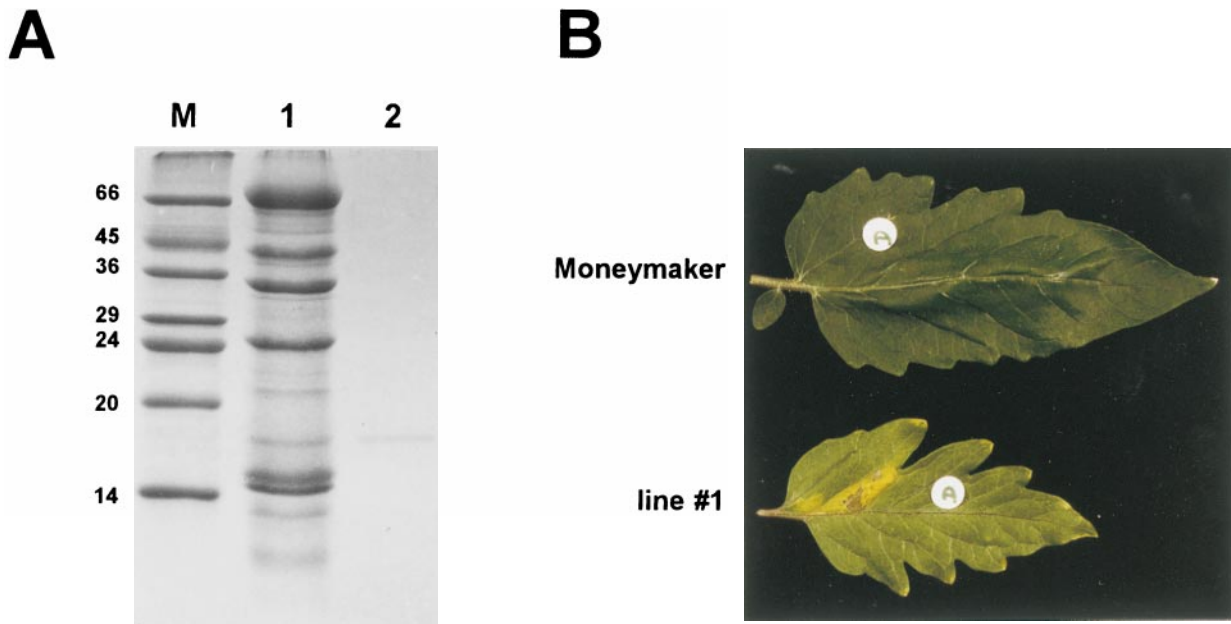
Fig. 2. HR induced after injection of purified ECP2 in a leaflet of line 1. (*A*) Purification of the ECP2 protein. Lane M contains molecular weight markers, lane 1 contains 50 μl of AF, and lane 2 contains the purified ECP2 protein. (*B*) HR induced after injection of ECP2 in the apoplast of a leaflet of a line 1 plant (*Lower*) and absence of HR after injection of ECP2 in the apoplast of a leaflet of the Moneymaker cultivar (*Upper*). Only the four lines identified by inoculation with PVX::*Ecp*2 responded by chlorosis, followed by necrosis in the injected area three days after injection of ECP2; all other lines showed no detectable response after ECP2 injection.

is both necessary and sufficient to induce HR on the four responding lines.

ECP2-Mediated HR Is Determined by a Single Dominant Gene. The four independent lines that recognize ECP2 have been reported to originate from the same *L. pimpinellifolium* ancestor and are likely to be four independent introgressions containing the same resistance factor (18, 19). We produced three F_1 progenies by crossing line 1, line 4, and their ancestor to the cultivar Moneymaker. F_2 progenies were generated to study the heritability of HR upon exposure to ECP2. We inoculated PVX::*Ecp*2 onto F_1 plants and three F_2 populations containing 93, 105, and 85 individuals, respectively, and determined the segregation ratio for HR. All F_1 individuals showed HR, and the three F_2 populations exhibited a 3:1 segregation for presence to absence of HR after inoculation with PVX::*Ecp*2 (Table 1). This demonstrates that one dominant gene determines the capacity to develop HR upon exposure to ECP2 in the ancestor and lines 1 and 4.

ECP2 Recognition Confers Resistance Against *C. fulvum*. We used isogenic strains of *C. fulvum*, either with or without the *Ecp*2 gene, to prove that resistance of these responding lines is solely caused by the fact that the fungus produces ECP2 during infection. We used line 1 as a representative of the four lines. The set of fungal strains tested comprised a wild-type, ECP2-producing strain (race 5), the corresponding isogenic *Ecp*2-lacking mutant in which the *Ecp*2 gene had been replaced

by an antibiotic resistance cassette, and an ECP2-overproducing strain obtained after retransformation of the *Ecp*2-lacking mutant with multiple copies of the *Ecp*2 gene (20). Whereas susceptible Moneymaker plants showed disease symptoms when inoculated with either of the three strains, only the *Ecp*2-lacking mutant caused disease on line 1 (Fig. 3). Plants of line 1 showed full resistance without any visible disease symptoms after inoculation with the wild-type ECP2-producing strain or the ECP2-overproducing near-isogenic strain. Microscopic examination of these resistant plants confirmed that fungal growth of both the wild-type and the ECP2-overproducing strains is arrested early after penetration of the tomato leaf. The *Ecp*2-lacking strain colonized to the same low extent the mesophyll of either plants of line 1 or Moneymaker plants (data not shown). The latter observation confirms the role of ECP2 as a virulence factor of *C. fulvum*

Table 1. Inheritance of HR induced by ECP2

	F_1		F_2		
	HR	Mosaic	HR	Mosaic	χ^2 Value
Line 1 × Moneymaker	4	0	77	16	1.13 ($P > 0.25$)
Line 4 × Moneymaker	5	0	82	23	0.27 ($P > 0.5$)
Ancestor ×					
Moneymaker	4	0	67	18	0.25 ($P > 0.5$)

Segregation of HR induction by ECP2 in F_1 and F_2 generations originating from a cross between the HR-displaying line 1, line 4, and their common ancestor with the non-HR-displaying cultivar Moneymaker. Plants were inoculated with PVX::*Ecp*2 and scored for systemic HR 2 weeks after inoculation.

ECP2-producing wild-type   ECP2-deficient mutant   ECP2-overproducing mutant
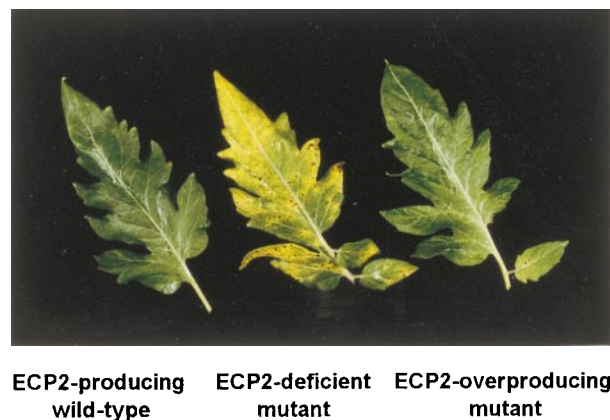
Fig. 3. Resistance of *Cf-ECP2* lines is solely dependent on the production of the ECP2 protein by *C. fulvum* during infection. Plants of line 1 were inoculated with the wild-type ECP2-producing strain (race 5) (*Left*), the isogenic *Ecp*2-lacking strain (*Center*), and the ECP2-overproducing strain (*Right*). Note that only the plants inoculated with the *Ecp*2-lacking strain showed symptoms of chlorosis on the upper side of the leaf 20 days after inoculation.

on tomato as described before (14). Thus, resistance toward *C. fulvum* of lines 1 to 4 is solely dependent on recognition of the ECP2 protein, as was expected from the results obtained with the PVX::*Ecp*2 experiments. We designate this resistance gene *Cf-ECP2* because this single dominant gene confers resistance toward *C. fulvum* through recognition of the ECP2 protein.

Strains of *C. fulvum* that Have Been Collected Worldwide Produce a HR-Inducing ECP2 Protein During Infection. To test the effectiveness of the *Cf-ECP2* gene, we analyzed AFs that were obtained from susceptible plants infected by a worldwide collection of 25 strains of *C. fulvum*, for the presence of the ECP2 protein and its HR-inducing activity. Western blot analysis with polyclonal antibodies raised against ECP2 confirmed the production of ECP2 by all strains (Fig. 4). Differences in accumulation levels of ECP2 are in agreement with accumulation levels of other *in planta*-secreted proteins of the various *C. fulvum* strains and reflect their relative aggressiveness. In addition, leaf injection of line 1 with AFs from all 25 strains resulted in the induction of HR, indicating that all strains produce an elicitor-active ECP2 protein (Fig. 4).

DISCUSSION

Resistance breeding programs are traditionally based on the identification of resistance to a pathogen in wild relatives, followed by several generations of backcrossing to introduce the resistance into elite breeding lines. Initially, inoculations with local and worldwide strains of the pathogen give insight into the effectiveness of the resistance (2). HR-based resistance can be induced by a variety of elicitors originating from the pathogen, of which the importance in pathogenicity or virulence is not known beforehand. If the elicitors are not important pathogenicity or virulence factors, it is likely that the pathogen will easily overcome the resistance either by mutating or losing the encoding gene. Here, we exploited biochemical and molecular data concerning a factor that is important for full virulence of the pathogen and followed a targeted search for plants showing a HR response to this factor.

Frequently, new races of *C. fulvum* appear that overcome known resistance genes such as *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9*. Molecular analysis of strains of *C. fulvum* that circumvent the *Cf-9* resistance gene revealed that complete deletion of the
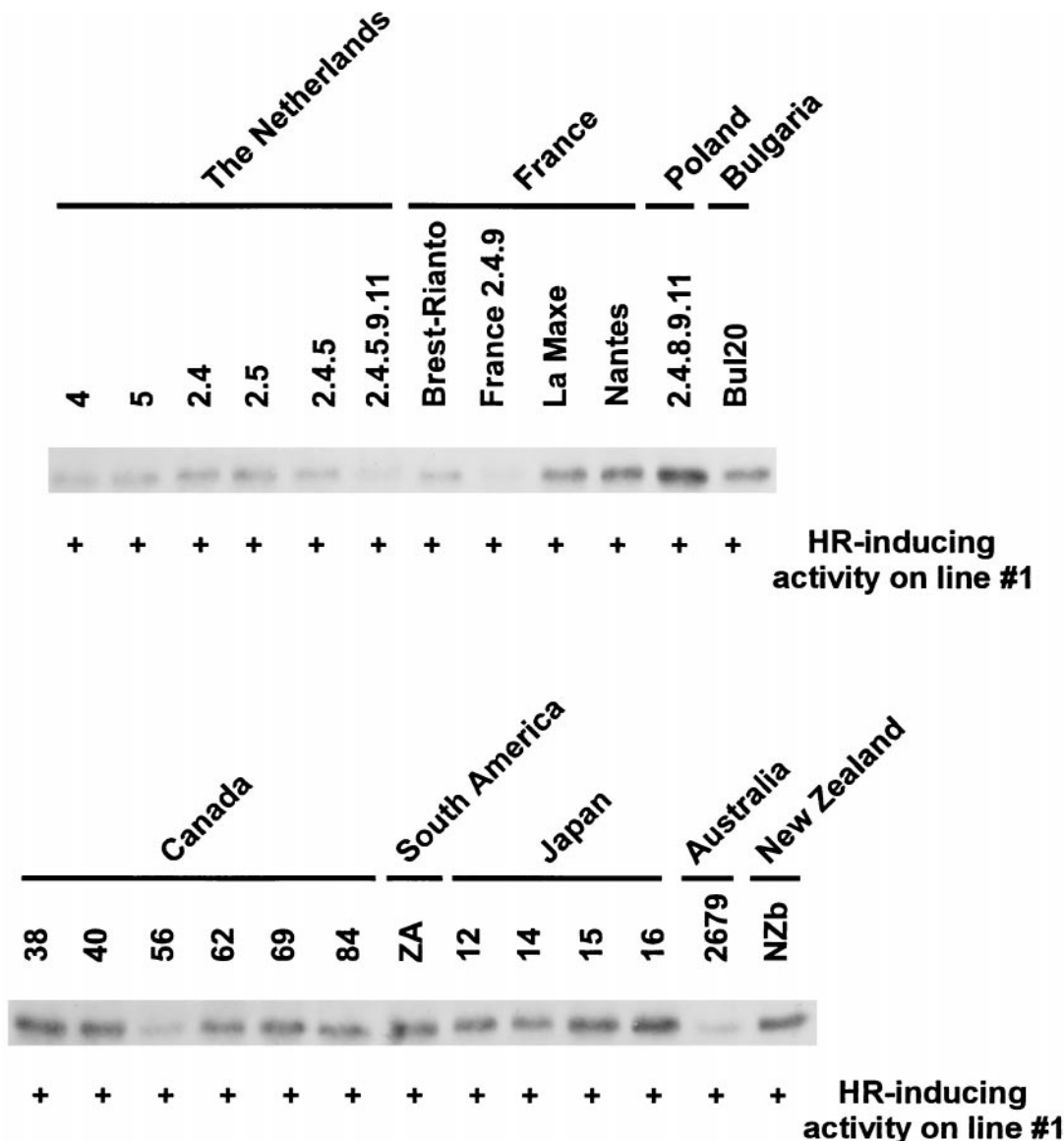

FIG. 4. Immunodetection of ECP2 in AFs isolated from cultivar Moneymaker inoculated with 25 strains of *C. fulvum* obtained from a worldwide collection. A volume of AF containing 4 μg of protein was separated on denaturing polyacrylamide gel, blotted, and incubated with polyclonal antibodies raised against ECP2. ECP2 is detected in each lane. HR-inducing activity of each AF sample was assayed by injecting 5–10 μl into a leaflet of line 1. HR activity visible 3 days after injection is indicated by "+."

*Avr*9 gene had occurred in the fungal genome, with the consequence that recognition is avoided as the AVR9 elicitor is no longer produced (21). *C. fulvum* strains that circumvent the *Cf-4* resistance gene exhibit a single point mutation in the *Avr*4 gene, which causes instability of the encoded product, because no proteins homologous to AVR4 are detected in AF of plants inoculated with these strains (11, 16). So far, no intrinsic biological function has been assigned to the *Avr*9 and *Avr*4 genes of *C. fulvum*. It seems that these genes can be lost without a detectable fitness penalty, so they appear not to encode important factors of pathogenicity or virulence for *C. fulvum*.

The *Cf-ECP2* resistance gene matches an important virulence factor of *C. fulvum* (14), which is present in all strains of *C. fulvum* that have been tested up till now. Overexpression of *Ecp*2 in plants lacking *Cf-ECP2* by using the PVX expression system does not give a phenotype, which might be explained by the fact that ECP2 rather suppresses activation of plant defense responses than being a toxic virulence factor. So far, four bacterial and one fungal avirulence gene (*avrA* and *avrE* from *Pseudomonas syringae* pv. *tomato*, *avrRPM1* from *Pseudomonas syringae* pv. *maculicola*, *avrBs2* from *Xanthomonas campestris* pv. *vesicatoria*, and *nip1* from *Rhynchosporium secalis*; refs. 22–25) have been reported to possess a function in virulence. However, these genes were not discovered and isolated based on a targeted search as described here. The matching resistance genes in the host plant are expected to confer durable protection against these pathogens. We do not know whether there are separate domains present in the mature ECP2 protein of 142 amino acids, which are important for its virulence function and for the HR-inducing activity. If there are no separate domains in the ECP2 protein, or if the two separate domains overlap, the fungus cannot circumvent the *Cf-ECP2* resistance by mutations in the *Ecp*2 gene, without a serious decrease in virulence. Altogether, we expect the *Cf-ECP2* resistance gene to be efficient and durable in protecting tomato crops against *C. fulvum*.

Using a viral expression system, such as the one based on PVX, has great potential. It ensures that the plant response is solely due to the protein of which the encoding cDNA has been inserted into the PVX vector. PVX screening can easily be performed with any protein. As in immunization assays used for mammals, the PVX vector presents the protein throughout the infected plant, increasing the amount of responding plant tissue from one single injection site to the whole plant, in which the virus systematically spreads (26). If the heterologous protein expressed by PVX triggers a quick HR response in the host, spread of the virus might be restricted to the inoculated area. In this case, compared with systemic mosaic symptoms caused by wild-type PVX, absence of mosaic symptoms combined with localized necrosis on the inoculated leaves may indicate recognition of the expressed protein. This method allows screening of large populations of wild plants or recombinants for rare individuals that exhibit specific recognition of the protein to which they have been exposed. We are currently testing large collections of wild *Lycopersicon* species for HR upon exposure to several additional potential virulence factors of *C. fulvum*. With the availability of several plant viral expression vectors for dicots and monocots, such as PVX, tobacco mosaic virus, cauliflower mosaic virus, tomato golden mosaic virus, cassava latent virus, brome mosaic virus, and barley stripe mosaic virus (15, 27–33), plants other than Solanaceae can be screened for HR-mediated resistance toward these viral vectors expressing pathogenicity or virulence factors produced by economically important plant pathogens.

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