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The *in-planta* induced *ecp2* gene of the tomato pathogen *Cladosporium fulvum* is not essential for pathogenicity

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Abstract. During the colonization of tomato leaves, the fungal pathogen *Cladosporium fulvum* excretes low-molecular-weight proteins in the intercellular spaces of the host tissue. These proteins are encoded by the *ecp* genes which are highly expressed in *C. fulvum* while growing *in planta* but are not, or are only weakly, expressed in *C. fulvum* grown in vitro. To investigate the function of the putative pathogenicity gene *ecp2*, encoding the 17-kDa protein ECP2, we performed two successive disruptions of the gene. In the first of these, the *ecp2* gene was interrupted by a hygromycin B resistance gene cassette. In the second gene disruption, the *ecp2* gene was completely deleted from the genome, and replaced by a phleomycin resistance gene cassette. Both disruption mutants were still pathogenic on tomato seedlings, indicating that the *C. fulvum* *ecp2* gene is not essential for pathogenicity in tomato.

Key words: *Cladosporium fulvum* - Extracellular protein – Gene disruption – Pathogenicity

Introduction

The fungus *Cladosporium fulvum*, the causal agent of tomato leaf mould, is a biotrophic pathogen; it penetrates the leaves through the stomata and grows between the mesophyll cells without causing structural damage (Lazarovits and Higgins 1976; De Wit 1977). At no stage of the infection cycle does the fungus develop specialized penetration or feeding structures (such as appressoria and haustoria, respectively). After an incubation period of about 10 days, conidiophores emerge through the stomata which become disfunctional resulting in chlorosis of the leaves. Analysis of the intercellular fluid (IF) of *C. fulvum*-infected tomato leaves (De Wit and Spikman 1982; De Wit et al. 1986) has led to the identification of several proteins of both plant and fungal origin which are specific in some cases of both compatible and incompatible interactions.

From the host plant, several classes of pathogenesis-related proteins have been characterized, purified (Joosten and De Wit 1989; Joosten et al. 1990), and their structural genes cloned (Van Kan et al. 1992; Danhash et al. 1993). Although these proteins are produced following the infection of the plant by *C. fulvum*, they also accumulate after infection by other pathogens or as a consequence of abiotic stress factors (Linthorst 1991).

The fungal proteins present in the IF can be divided into two classes. The first of these consists of the so-called race-specific elicitors which are the inducers of plant defence responses in resistant genotypes of tomato. These protein elicitors are encoded by avirulence genes of the pathogen and are thought to interact with receptors present in the resistant host plant. In races which are no longer recognized by the host plant the avirulence gene is either deleted, and as a consequence the race-specific elicitor is not produced anymore, as has been demonstrated for the *avr9* gene (Van Kan et al. 1991; Van den Ackerveken et al. 1992; Marmeisse et al. 1993), or is altered by a single amino acid in the protein, which is no longer recognized by the plant, as has been demonstrated for the *avr4* gene (Joosten et al. 1994). The second class of fungal proteins is produced by all races of *C. fulvum*; these proteins are considered to be putative pathogenicity factors that might be involved in the establishment and maintenance of basic compatibility in tomato. Three of those extracellular proteins (ECPs) have been purified and characterized: the 9-kDa ECP1 protein, previously named P1 (Joosten and De Wit 1988), the 17-kDa ECP2 protein (Wubben et al. 1994), and the 19-kDa ECP3 protein (De Wit et al., unpublished). The ECPs accumulate in the intercellular space of susceptible tomato plants infected with *C. fulvum* and are not produced by the fungus when grown in vitro. The available protein and

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DNA data indicate that ECP1, 2 and 3 are unique and are not structurally related (the respective antibodies do not cross-react). The structural gene encoding ECP2 has been cloned and sequenced (Van den Ackerveken et al. 1993). The *ecp*2 coding sequence of 495 bp (165 aa) is interrupted by one intron of 56 bp. The *ecp*2 gene is highly expressed in *C. fulvum* when growing *in planta* but hardly, or not at all, when growing in vitro (Van den Ackerveken et al. 1993). Immunocytological localization of ECP2 in *C. fulvum*-infected tomato leaves revealed that this protein is present in the extracellular matrix between fungal and plant cell walls and in low amounts in the fungal hyphae (Wubben et al. 1994). The nucleotide sequence of the *ecp*2 gene and its deduced amino-acid sequence do not share any significant homology to sequences present in any database.

In filamentous fungi, gene disruption by homologous recombination has proven to be a powerful technique for investigating the biological function of cloned genes. In *C. fulvum*, disruption of the avirulence gene *avr*9 resulted in virulence on a previously resistant plant, showing the power of this technique (Marmeisse et al. 1993). To investigate the significance of ECP2 in pathogenicity we have disrupted the *ecp*2 gene of *C. fulvum* by a gene-replacement strategy. Our results indicate that the *C. fulvum ecp*2 gene is not essential for pathogenicity on tomato.

Materials and methods

Fungal strains and culture conditions. Strains of *C. fulvum* Cooke [syn. *Fulvia fulva* (Cooke) Cif] used in this study are presented in Table 1. *C. fulvum* was grown at 22 °C on either Potato Dextrose Agar (Merck), Czapek Dox (Oxoid), or complete medium (Harling et al. 1988), depending on the type of experiment.

Plasmids and fungal transformation. pAN7-1 (Punt et al. 1987) and pAN8-1 (Mattern et al. 1988) contain bacterial antibiotic resistance genes fused to *Aspergillus nidulans gpd*A promoter and *trp*C terminator sequences and confer resistance to hygromycin B (*hph* gene) and phleomycin (*ble* gene), respectively. pCF170 (Van den Ackerveken et al. 1993) contains the *ecp*2 gene on a 4.3-kb *Eco*RI-*Bam*HI fragment cloned in pGEM-3Zf(+) (Promega). From pCF170, the plasmids pCF172 and pCF176 were constructed (see legend of Fig. 1) and used for disruption of the *ecp*2 gene by homologous recombination. For transformation of *C. fulvum* we followed the protocol of Oliver et al. (1987) with modifications as described by Marmeisse et al. (1993). One day after transformation hygromycin B-resistant transformants were selected on complete medium, at a hygromycin B (Sigma) concentration of 100 $\mu g.ml^{-1}$; phleomycin-resistant transformants were selected on Czapex Dox medium, at a phleomycin (Cayla, Toulouse) concentration of 25 $\mu g.ml^{-1}$.

DNA manipulations. Methods used in plasmid construction, propagation in *E. coli* DH5α, and isolation, were as described by Sambrook et al. (1989). *C. fulvum* genomic DNA was extracted from freeze-dried mycelium by the method of Van Kan et al. (1991). For Southern blot analysis, restriction enzyme-digested genomic DNA was separated in 0.8% agarose gels, transferred onto nylon membranes (Hybond N^+, Amersham) and hybridized to ^{32}P-labelled DNA fragments at 65 °C, as described by Marmeisse et al. (1993). Isolation of RNA and subsequent Northern analysis were performed as described by Van Kan et al. (1991).

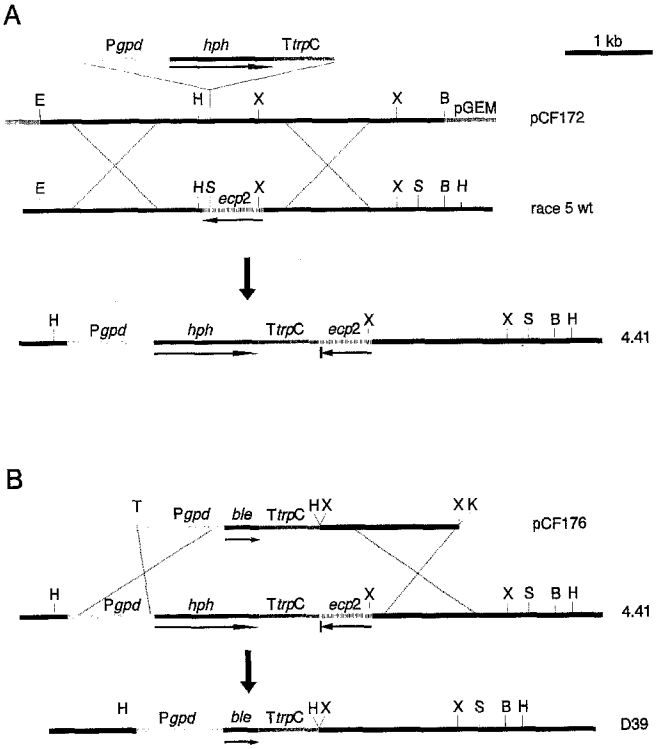

Fig. 1A, B. Plasmids used to disrupt the *ecp*2 gene in two sequential gene replacement steps. **A** construction of pCF172. *ecp*2 was cloned as a 4.3-kb *Eco*RI-*Bam*HI fragment (pCF170). Of the two *Sst*II sites present, the one upstream of the coding region was removed by T4 DNA polymerase on the linearised plasmid followed by religation. The *hph* gene cassette, obtained as a 2.8-kb *Sst*I-*Hin*dIII fragment from pAN7-1, was introduced in the remaining *Sst*II site of the *ecp*2 gene to give pCF172. Prior to transformation the plasmid was linearised with *Dra*I which cuts three times in the vector sequences. **B** construction of pCF176. The *Xho*I-*Xho*I fragment upstream of *ecp*2 was cloned into the *Xho*I site of pBluescript (Stratagene). The plasmid was then digested with *Sst*I and *Xba*I which both cut in the plasmid polylinker at one side of the insert. This allowed the insertion of the *ble* gene cassette, obtained as a 2.2-kb *Sst*I-*Xba*I fragment from pAN8-1, to give pCF176. Prior to transformation the plasmid was linearised by *Sst*I and *Kpn*I which both cut once in the plasmid polylinker. Restriction sites: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *K*, *Kpn*I; *S*, *Sst*II; *T*, *Sst*I; *X*, *Xho*I

Table 1. *C. fulvum* strains used in this study

Strain	Description[a]
Race 5	Hygromycin B^s, phleomycin^s; wild-type strain virulent on tomato genotype *Cf5*, used as recipient in the transformation experiments
4.41	Hygromycin B^r, phleomycin^s; *ecp*2::*hph* insertion disruption mutant
4.41E	Hygromycin B^r, phleomycin^r; strain 4.41 containing extra copies of the wild-type *ecp*2 gene introduced by co-transformation with pAN8-1; overproduces the ECP2 protein
D39	Hygromycin B^s, phleomycin^r;Δ*ecp*2::*ble*, *ecp*2 gene deleted and replaced by the *ble* gene cassette

[a] The superscripts ^{r} and ^{s} indicate resistance or sensitivity to the antibiotics. The bacterial resistance genes to phleomycin and hygromycin B with control sequences from *A. nidulans* are named *ble* and *hph*, respectively

Pathogenicity tests and analysis of intercellular fluids. Inoculations of 14-day-old tomato seedlings of the susceptible cultivar Moneymaker with conidial suspensions of *C. fulvum* were performed as described by Talbot et al. (1988). Intercellular fluids (IFs) were collected 14 days after inoculation as described by De Wit and Spikman (1982). The proteins in the IF were precipitated at –20 °C by adding acetone to a final concentration of 80% (v/v). Following centrifugation (10 min, 5 000 g) the pellet was dissolved in water in one-fifth of the original volume. The concentrated IF was analysed by polyacrylamide-gel electrophoresis as described by Joosten and De Wit (1988). One gel was stained with Coomassie brilliant blue and two gels were electro-blotted onto nitrocellulose (Schleicher and Schuell). Western blots were blocked with 1% gelatin in Tris-buffered saline, 0.1% Tween 20, and subsequently incubated with the primary antibodies anti-ECP2 (1:1 000 dilution) and anti-ECP3 (1:500 dilution) for 2 h at room temperature. The antigen-antibody complexes were detected with the BioRad Immun-Blot goat anti-rabbit alkaline phosphatase (GAR-AP) assay kit.

Results

Disruption of ecp2 by insertion of the hph gene

In a first attempt to disrupt the *ecp2* gene, we constructed plasmid pCF172 (Fig. 1A) which contains the hygromycin B resistance (*hph*) gene cassette from pAN7-1 inserted in a *Sst*II restriction site – 450 bp downstream from the translation start – in the *ecp2* genomic clone. This insertion leaves approximately 75% of the *ecp2* gene unchanged but should prevent the formation of a full-length polyadenylated RNA transcript. In pCF172, the *hph* gene cassette is flanked on either side by 2.5 kb and 2.0 kb, respectively, of *C. fulvum* genomic DNA, this allows gene replacement by homologous recombination to occur at the *ecp2* locus (see Fig. 1A). Linearised pCF172 plasmid was used to transform *C. fulvum* wild-type race 5 to hygromycin B resistance. Ninety transformants were isolated, purified, and their genomic DNA analysed by Southern blot hybridisation. As illustrated in Fig. 1A, in *C. fulvum* wild-type race 5 the *ecp2* gene is located on a 3.1-kb *Hin*dIII fragment, whereas in the disruption mutant the replaced *ecp2* gene is located on a 6-kb *Hin*dIII fragment as a result of the insertion of the *hph* gene cassette. Two of the ninety analysed transformants contained this new 6-kb *Hin*dIII fragment (illustrated in Fig. 2A and B for mutant 4.41 with two different probes). Further analyses demonstrated the presence of the *hph* marker gene on the same *Hin*dIII fragment (Fig. 2C, the *hph* probe does not hybridise to the wild-type race-5 genomic DNA). No hybridisation to the labelled plasmid could be detected (data not shown). So, in mutant 4.41, the wild-type *ecp2* gene has been replaced by the disrupted *ecp2* gene from plasmid pCF172 through a double cross-over.

Spores from mutant 4.41 were used to inoculate seedlings of the susceptible tomato cultivar Moneymaker. Mutant 4.41 was still pathogenic on tomato and no differences in growth pattern between mutant 4.41 and the recipient wild-type race 5 could be observed. Fourteen days after inoculation, the IF was isolated from the *C. fulvum*-infected tomato leaves and analysed by SDS-PAGE and Western blotting using polyclonal antibodies raised against purified ECP2 (Wubben et al. 1994). The SDS-PAGE profile (Fig. 3A) shows the characteristic accumulation of pathogenesis-related proteins of the plant and the ECPs of *C. fulvum* (De Wit et al. 1989) which are absent in the IF of healthy tomato seedlings (lane 1). Western analysis using ECP2 antibodies (Fig. 3C) shows the presence of the ECP2 protein in the IF of wild-type-race-5-infected tomato (lane 2), but not in the IF of mutant-4.41-

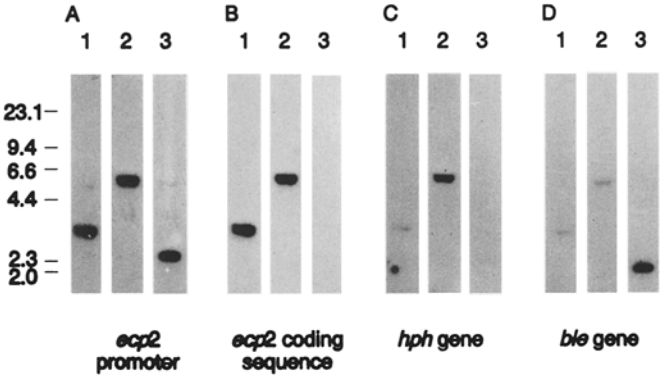

Fig. 2A–D. Southern blot analysis of *Hin*dIII-digested DNAs from wild-type race 5 (*lane 1*), the *ecp2*::*hph* insertion disruption mutant 4.41 (*lane 2*) and the Δ*ecp2*::*ble* deletion disruption mutant D39 (*lane 3*). Four different ^{32}P-labelled DNA fragments were consecutively used as probes. **A** the 1.6-kb *Xho*I-*Xho*I fragment upstream of *ecp2*. **B** the 600-bp *Sst*II-*Xho*I fragment included in the *ecp2* coding sequence. **C** the *Eco*RI-*Bam*HI fragment from pAN7-1, containing part of the *hph* gene. **D** the *ble* gene obtained as a 350-bp *Bam*HI fragment from pUT335 (Drocourt et al. 1990)

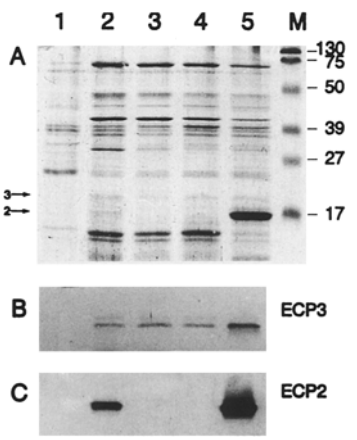

Fig. 3A–C. SDS-PAGE and Western blot analysis of the IF isolated from tomato seedlings 14 days after inoculation with (1) water, (2) *C. fulvum* wild-type race 5, (3) insertion disruption mutant 4.41, (4) deletion disruption mutant D39, (5) ECP2-overproducing transformant 4.41E. **A** proteins present in 40 µl of the original IF were separated on a 15% polyacrylamide SDS gel and stained with Coomassie brilliant blue. The molecular mass of the pre-stained SDS-PAGE standards (Bio-Rad) in *lane M* is indicated in kDa. *C. fulvum* proteins ECP2 (2→) and ECP3 (3→) are indicated by *arrows*. **B** and **C** Western blot analysis of the IF separated by SDS-PAGE (*panel A*) using ECP3 polyclonal antibodies (*panel B*) and ECP2 polyclonal antibodies (*panel C*). In addition to the 19-kDa ECP3 protein, the ECP3 antibodies recognize a fungal protein of 14-kDa (data not shown). The ECP2 antibodies weakly cross-react with a 18-kDa protein in all strains tested (not visible). Note that ECP2 is not produced by the disruption mutants 4.41 and D39 (**C**, *lanes 3 and 4*) whereas the amount of ECP3 produced is comparable to wild-type levels

infected tomato (lane 3). In the IF of mutant-4.41-infected tomato a minor 18-kDa protein was visible on the Western blot which weakly cross-reacted with the ECP2 antibodies but was masked by the native ECP2 protein in the IF of race-5-infected tomato (not visible on the blot). As a control, similar amounts of the 19-kDa ECP3 protein were detected in the IF of wild-type-race-5- and mutant-4.41-infected tomato as shown by Western analysis using ECP3 antibodies (Fig. 3B).

Although the ECP2 protein could not be detected, the *ecp*2 sequence in mutant 4.41 was still transcribed. Polyadenylated *ecp*2 transcripts were evident following Northern analysis of RNA isolated from mutant-4.41-infected seedlings. These transcripts were smaller than the *hph* transcripts (Northern blots not shown). The *hph* gene is simply inserted in the *ecp*2 gene in mutant 4.41, which could allow a translational fusion between the 3′-end of the *ecp*2 gene and the opposite strand of the *trp*C terminator sequences (Fig. 1A) and so result in the production of a hybrid protein.

Deletion of ecp2 by replacement with the ble gene

To exclude the possibility of having created a hybrid ECP2 protein, we performed a second gene disruption to completely delete the *ecp*2 coding sequences from the genome of transformant 4.41. For this purpose plasmid pCF176 was constructed (Fig. 1B) which contains the phleomycin resistance gene cassette from pAN8-1 fused to a 1.6-kb *Xho*I fragment present upstream of the wild-type *ecp*2 gene. As illustrated in Fig. 1B, a double cross-over between pCF176 and homologous sequences in transformant 4.41 would remove all remaining *ecp*2 sequences. The pCF176 transformants were first selected for resistance to phleomycin and then tested for hygromycin-B sensitivity since a perfect gene replacement would also result in loss of the *hph* gene. Of 324 phleomycin-resistant transformants selected, six were hygromycin-B sensitive and these were further analysed by Southern blot hybridisation. Two of these transformants gave hybridisation patterns which were in agreement with gene replacement as illustrated in Fig. 1B. The analysis of one of these transformants, D39, is presented in Fig. 2 (lane 3). The *ecp*2-specific (Fig. 2B) and *hph*-specific (Fig. 2C) probes no longer hybridised to genomic DNA isolated from transformant D39. The *ble* gene was shown to be present on a single *Hin*dIII fragment (Fig. 2D) and no plasmid sequences could be detected, indicating that a perfect gene replacement, as shown in Fig. 1B, had occurred.

Transformant D39, lacking the *ecp*2 coding sequence, was still fully pathogenic on tomato and no visible differences in growth pattern between mutant D39 and wild-type-race-5-infected tomato were observed (data not shown). The IF isolated from transformant-D39-infected tomato did not contain the ECP2 protein (Fig. 3, lane 4) but the minor 18-kDa protein cross-reacting with the ECP2 antibodies was still weakly detected (not visible in Fig. 3C). This result ruled out the possibility of the minor cross-reacting 18-kDa protein being a hybrid ECP2 protein.

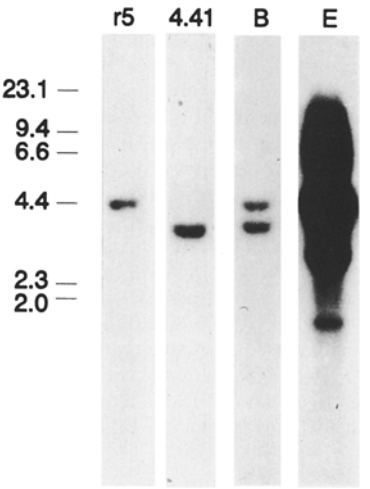

Fig. 4. Southern blot analysis of *C. fulvum* transformants containing one or multiple copies of the *ecp*2 gene. Genomic DNAs from *C. fulvum* wild-type race 5 (*r5*), *ecp*2 disruption mutant 4.41, and two *ecp*2 co-transformants (4.41 *B* and *E*), were digested with *Eco*RI and *Bam*HI and the blots hybridised to the ^{32}P-labelled 600-bp *Sst*II-*Xho*I fragment from pCF170 containing the *ecp*2 gene

Overproduction of ECP2

The effect of the level of ECP2 protein on the development of disease symptoms was studied by the construction of *C. fulvum* transformants overproducing this protein. For this purpose, transformant 4.41 was co-transformed with plasmid pCF170, which carries the wild-type *ecp*2 gene, and plasmid pAN8-1, which confers resistance towards phleomycin. The genomic DNA from six phleomycin-resistant transformants was digested with *Eco*RI and *Bam*HI and subjected to Southern blot analysis. The 600-bp *Sst*II-*Xho*I fragment containing the *ecp*2 coding sequence was used as a probe. This probe hybridised to single, but different, *Eco*RI-*Bam*HI genomic DNA fragments of *C. fulvum* race 5 and transformant 4.41 (Fig. 4). The intensity of this hybridising fragment can be used to semi-quantify the number of additional copies of the *ecp*2 gene integrated in the genome of the transformants. The analysis is only reported for transformants 4.41B and 4.41E, which both have additional copies of the *ecp*2 gene integrated in their genomes. Transformant 4.41B was characterized by a single additional *Eco*RI-*Bam*HI fragment of the same intensity as the fragment containing the disrupted *ecp*2 gene present in transformant 4.41 (Fig. 4). This suggests that transformant 4.41B has a single additional complete copy of the *ecp*2 gene in its genome. The second transformant, 4.41E, contains many copies of *ecp*2 integrated in its genome (Fig. 4). Densitometric measurements suggested that far more than ten additional copies of the *ecp*2 gene are present in the genome of this transformant.

Transformant 4.41E was chosen to inoculate tomato seedlings. Infection of tomato cultivar Moneymaker by transformant 4.41E was identical to that by *C. fulvum* wild-type race 5. The proteins present in the IF from transformant-4.41E-infected tomato leaves were characterized by SDS-PAGE and Western blot analysis (Fig. 3, lane 5). On

the Coomassie-stained gel (Fig. 3A) an intensely-stained protein band of 17 kDa is present which corresponds in size to the native ECP2 protein. The identity of this protein band was confirmed by Western blot analysis: the ECP2 antibodies reacted strongly with this protein (Fig. 3C, lane 5), which demonstrates that transformant 4.41E overproduces the ECP2 protein more than 20-fold. However, this ECP2-overproducing transformant did not cause more intense disease symptoms than the wild-type race 5 (data not shown).

Discussion

The ability of a pathogen to successfully infect and colonize a particular host plant is called basic compatibility (Ellingboe 1976; Heath 1981). Most pathogens are able to colonize only a limited number of host plants and successful infection of a host requires a range of pathogenicity factors. These factors might be involved in the attachment of fungal spores to the host plant (Hamer et al. 1988), the formation of penetration structures (Staples et al. 1986), the degradation of host cuticle and cell walls (Dickman et al. 1989; Hahn et al. 1989), toxin production (Panaccione et al. 1992), phytoalexin detoxification (Van Etten et al. 1989), or suppression of defence responses (Heath 1982).

Putative pathogenicity genes of the plant pathogenic fungi characterized so far have all been cloned by function. These include a cutinase (Dickman et al. 1989), a pisatin demethylase (Weltring et al. 1988), and a toxin synthetase gene (Panaccione et al. 1992). Pathogenicity genes with as yet unknown functions are difficult to isolate unless other experimental approaches are used. The isolation and analysis of fungal genes expressed during pathogenesis might provide a non-biased selection of putative pathogenicity genes. One such approach is the differential hybridization of a genomic library of the pathogen with cDNA synthesized from mRNA isolated from the in-vitro grown and *in planta*-grown fungus. This method was successfully used to clone genes of *Uromyces appendiculatus*, which are specifically expressed during the differentiation of infection structures (Bhairi et al. 1989; Xuei et al. 1992), and to isolate *in planta*-induced genes of *Phytophthora infestans* (Pieterse et al. 1993). In the *C. fulvum* – tomato interaction, *in planta*-induced fungal genes have been cloned and characterized based on the proteins they encode and which specifically accumulate in infected tomato leaves but not in the fungus grown in vitro (Van den Ackerveken et al. 1993). The role of these *ecp* genes during pathogenesis is unknown. As the genes encoding these ECPs are not homologous to other known genes, an obvious way to study their possible function is inactivation by gene replacement or gene disruption.

In this paper we show that transformants specifically mutated at the *ecp*2 locus are still pathogenic on seedlings of the tomato cultivar Moneymaker. The first disruption mutant isolated (mutant 4.41) has the *hph* marker gene inserted in the *ecp*2 coding sequence. In the IF from tomato cotyledons infected by this mutant the wild-type ECP2 peptide was absent. However, a protein, slightly larger than ECP2, weakly cross-reacted with ECP2 antibodies. In the IF of wild-type *C. fulvum*-infected cotyledons this protein could not be separated from ECP2 by SDS-PAGE. This cross-reacting protein was also detected in the IF from leaves infected with disruption mutant D39, in which the complete *ecp*2 coding region was deleted. This ruled out the possibility that the cross-reacting protein was the product of a truncated *ecp*2 gene present in mutant 4.41.

From the results obtained with both the *ecp*2$^-$ mutant and transformants overproducing the ECP2 protein we can conclude that the *ecp*2 gene is not essential for pathogenicity. On its own the *ecp*2 gene does not seem to have any important function, but it might be that a number of different ECPs act in concert with ECP2 and that only simultaneous disruption of several *ecp* genes would lead to a non-pathogenic phenotype. Attempts to disrupt the *ecp*1 gene (Van den Ackerveken et al. 1993) in an *ecp*2$^-$ background have so far proven unsuccessful, possibly due to the fact that the coding sequence of the *ecp*1 gene is closely linked to sequences highly repeated in the genome of *C. fulvum*, which reduces the chances of homologous recombination at the *ecp*1 locus (Marmeisse et al., unpublished data).

There are other examples in the literature where disruption of genes coding for extracellular hydrolytic enzymes did not affect the pathogenicity of fungal plant pathogens. Disruption of an endopolygalacturonase gene from the maize pathogen *Cochliobolus carbonum* (Scott-Craig et al. 1990) and of the cutinase genes of both *Magnaporthe grisea* (Sweigard et al. 1992) and *Nectria haematococca* (Stahl and Schäfer 1992), pathogens of rice and pea respectively, did not result in a decrease of pathogenicity.

The two-step gene replacement system which is described in this paper provided an efficient screening method for homologous recombination. The first step in the disruption resulted in the introduction of a marker gene (*hph*) in the *ecp*2 coding region. In the second step the complete *ecp*2 coding region was removed together with the *hph* marker gene introduced in the first step. This allowed a rapid screening of 300 transformants based on their sensitivity to hygromycin, resulting in six transformants sensitive to the antibiotic that had to be analysed at the DNA level. This system could be extended and used to target other plasmid constructs at the *ecp*2 locus such as different promoter-reporter gene fusions from *in planta*-induced avirulence and *ecp* genes. This standardized targeted insertion would rule out position effects which often hamper functional analyses of cis-acting elements.

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