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PROTEOMIC ANALYSIS OF THE APOPLAST OF *CLADOSPORIUM FULVUM*-INFECTED TOMATO REVEALS NOVEL VIRULENCE FACTORS

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During growth on its host tomato, the biotrophic fungal pathogen *Cladosporium fulvum* secretes several effector proteins into the apoplast (Thomma *et al*., 2005). Eight of these *C. fulvum* effectors have previously been characterized. To discover novel *C. fulvum* proteins that play a role in virulence, we utilized a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) approach to visualize proteins secreted during compatible and incompatible *C. fulvum*-tomato interactions. Several proteins that accumulated during infection were identified by mass spectrometry (MS) and were shown to be previously described. For proteins that were not identified by MS, N-terminal sequencing and peptide fragment spectra obtained with liquid chromatography (LC) MS/MS provided peptide sequence information. PCR with degenerate primers based on peptide sequences yielded coding sequence for three novel *C. fulvum* proteins; PhiC, Ecp6, and Ecp7. PhiC shows homology to fungal phialides, proteins that occur in sporogenous cells that release conidia. Ecp6 contains LysM domains that may be involved in chitin binding, similar to the function of *C. fulvum* Avr4 (van Esse *et al*., 2007). Ecp7 codes for a small, cysteine-rich protein with no homology to any known proteins. We demonstrate that Ecp6 and Ecp7 are crucial for *C. fulvum* virulence by exploiting RNAi-mediated gene silencing.

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van Esse, H. P., Bolton, M. D., Stergiopoulos, I., de Wit, P. J. G. M. and Thomma, B. P. H. J. 2007. The chitin-binding *Cladosporium fulvum* effector protein Avr4 is a genuine virulence factor. (submitted).

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THE CHITIN-BINDING *CLADOSPORIUM FULVUM* EFFECTOR PROTEIN AVR4 IS A GENUINE VIRULENCE FACTOR

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The biotrophic fungal pathogen *Cladosporium fulvum* (syn. *Passalora fulva*) is the causal agent of tomato leaf mold. The Avr4 protein belongs to a set of effectors that is secreted by *C. fulvum* during infection, and is thought to play a role in pathogen virulence. Previous studies have shown that Avr4 is a chitin-binding lectin that binds to chitin present in fungal cell walls. *In vitro* assays have shown that this affinity of Avr4 for chitin is the basis of protection of fungal hyphae against hydrolysis by plant chitinases. In this study, we demonstrate that *Avr4*, when heterologously expressed in *Arabidopsis*, increases the virulence of several fungal pathogens with exposed chitin in their cell walls, whereas the virulence of a bacterium and an oomycete remained unaltered. Heterologous expression of *Avr4* in tomato also increased the virulence of *Fusarium oxysporum* f. sp. *lycopersici*. Through tomato GeneChip analyses we could show that *Avr4*-expression in tomato results in the induced expression of only few genes. Finally, we demonstrated that silencing of the *Avr4* gene in *C. fulvum* decreases its virulence on tomato. This is the first report on the intrinsic function of a fungal avirulence protein that has a counter-defensive activity required for full virulence of the pathogen.

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THE *CLADOSPORIUM FULVUM* EFFECTOR PROTEIN AVR2 IS A NOVEL CYSTEINE PROTEASE INHIBITOR.

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Cladosporium fulvum (syn. *Passalora fulva*) is an extracellularly growing biotrophic fungal pathogen that causes tomato leaf mold. The Cf2-dependent resistance towards *C. fulvum* is based on the indirect recognition of the fungal elicitor protein Avr2. Our previous studies have demonstrated that the interaction of Avr2 with the tomato cysteine protease Rcr3 triggers a *Cf2*-mediated hypersensitive response (HR), which complies to the so-called guard hypothesis.

Although the Avr2 protein acts as a cysteine protease inhibitor, its amino acid sequence does not show significant homology with other sequences present in public databases. Since disulphide bridge formation is important for the 3-dimensional structure of proteins and may support the identification of functional homologues, we elucidated the disulphide bridging pattern of the Avr2 protein. Subsequent bio-informatic analysis has led to the identification of a number of amino acids that could be important for the interaction of Avr2 with Rcr-3.

PCR-based site-directed mutagenesis was applied to obtain mutant Avr2 proteins upon heterologous expression in the yeast *Pichia pastoris*. All proteins were tested for HR-inducing activity in *Cf-2* tomato plants. A selection of the Avr2 mutant proteins were used in Rcr3-inhibition assays to determine their binding affinity when compared to the native Avr2 protein. This study indicates that the C-terminal part of Avr2 is crucial for the HR inducing activity as well for the inhibition of Rcr3. Since the Avr2 protein only shows very limited homology to known protease inhibitors, it can be concluded that Avr2 is a novel type of cysteine protease inhibitor.