Affinity of Avr2 for tomato cysteine protease Rcr3 correlates with the Avr2-triggered Cf-2-mediated hypersensitive response

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SUMMARY

The *Cladosporium fulvum* Avr2 effector is a novel type of cysteine protease inhibitor with eight cysteine residues that are all involved in disulphide bonds. We have produced wild-type Avr2 protein in *Pichia pastoris* and determined its disulphide bond pattern. By site-directed mutagenesis of all eight cysteine residues, we show that three of the four disulphide bonds are required for Avr2 stability. The six C-terminal amino acid residues of Avr2 contain one disulphide bond that is not embedded in its overall structure. Avr2 is not processed by the tomato cysteine protease Rcr3 and is an uncompetitive inhibitor of Rcr3. We also produced mutant Avr2 proteins in which selected amino acid residues were individually replaced by alanine, and, in one mutant, all six C-terminal amino acid residues were deleted. We determined the inhibitory constant (K_i) of these mutants for Rcr3 and their ability to trigger a Cf-2-mediated hypersensitive response (HR) in tomato. We found that the two C-terminal cysteine residues and the six amino acid C-terminal tail of Avr2 are required for both Rcr3 inhibitory activity and the ability to trigger a Cf-2-mediated HR. Individual replacement of the lysine-17, lysine-20 or tyrosine-21 residue by alanine did not affect significantly the biological activity of Avr2. Overall, our data suggest that the affinity of the Avr2 mutants for Rcr3 correlates with their ability to trigger a Cf-2-mediated HR.

INTRODUCTION

Cladosporium fulvum (*Passalora fulva*) is an asexual fungal pathogen causing leaf mould of tomato, a disease that complies with a gene-for-gene relationship (De Wit *et al.*, 2009; Thomma

et al., 2005). The runner hyphae produced by germinating spores enter tomato leaves through stomata and colonize the apoplastic space between mesophyll cells. During infection and subsequent colonization of tomato, the fungus secretes several small cysteine (Cys)-rich effectors known as Avr (avirulence proteins) and Ecps (extracellular proteins). Genes encoding these effectors have been cloned and, for a number of them, a role in fungal virulence has been demonstrated (Bolton *et al.*, 2008; De Wit *et al.*, 2008; Stergiopoulos and De Wit, 2009; Thomma *et al.*, 2005; Van Esse *et al.*, 2007, 2008). Resistant tomato plants contain Cf proteins that mediate the recognition of cognate effectors and subsequently trigger an array of defence responses culminating in the hypersensitive response (HR) (De Wit *et al.*, 2008). The cognate Cf-2, Cf-4, Cf-4E and Cf-9 genes, whose encoded proteins mediate the recognition of Avr2, Avr4, Avr4E and Avr9, respectively, have also been cloned (Dixon *et al.*, 1996; Jones *et al.*, 1994; Takken *et al.*, 1999; Thomas *et al.*, 1997).

The Avr2 effector is a virulence factor of *C. fulvum* that inhibits tomato cysteine proteases, including Rcr3, Pip1, aleurain and TDI-65, which are important for basal host defence (Krüger *et al.*, 2002; Rooney *et al.*, 2005; Shabab *et al.*, 2008; Van Esse *et al.*, 2008). Avr2 also increases the virulence of other fungal pathogens, such as *Botrytis cinerea* and *Verticillium dahliae*, as shown in tomato and *Arabidopsis thaliana* transgenic for the Avr2 gene (Van Esse *et al.*, 2008). In the presence of Cf-2, Avr2 behaves as an avirulence factor that triggers Cf-2-mediated HR, which also requires Rcr3^{pimp} (required for *C. fulvum* resistance), a cysteine protease originating from *Lycopersicon pimpinellifolium* (Krüger *et al.*, 2002; Rooney *et al.*, 2005).

The Avr4 effector is a virulence factor of *C. fulvum* with a functional chitin-binding domain that protects chitin present in fungal cell walls against plant chitinases (Van den Burg *et al.*, 2003, 2004, 2006; Van Esse *et al.*, 2007). In the presence of Cf-4, Avr4 behaves as an avirulence factor that triggers Cf-4-mediated HR (Joosten *et al.*, 1994).

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The virulence function of the Avr4E effector is not yet known. In the presence of Cf-4E, Avr4E behaves as an avirulence factor that triggers Cf-4E-mediated HR (Westerink *et al.*, 2004).

The Avr9 effector contains a cystine knot with structural but, so far, no functional homology to carboxypeptidase inhibitor (Van den Ackerveken *et al.*, 1993; Van den Hooven *et al.*, 2001; Van Kan *et al.*, 1991; Vervoort *et al.*, 1997). Disruption of *Avr9* in *C. fulvum* does not affect virulence on tomato plants, suggesting that it is not required for full virulence (Marmeisse *et al.*, 1993).

All Avr effectors trigger Cf-mediated HR, which could suggest a direct interaction between effector and cognate Cf receptor protein. Extensive studies have been performed to find evidence for interaction between *C. fulvum* effectors and their cognate Cf proteins (Luderer *et al.*, 2001). However, using different biochemical approaches, direct interaction between the Cf-9 protein and the Avr9 effector could not be shown (Luderer *et al.*, 2001). Avr9 binds to a high-affinity binding site (HABS) that could represent a virulence target that is guarded by the Cf-9 protein (Kooman-Gersmann *et al.*, 1996, 1998). Expression of the *Avr4* gene in tomato plants without the *Cf-4* gene does not induce or suppress the expression of host genes, suggesting that Avr4 is a defensive virulence factor that protects the host colonizing fungus against deleterious effects of plant chitinases rather than attacking the host plant itself (Van Esse *et al.*, 2007, 2009). As plants containing the Cf-4 resistance protein respond with a strong HR, a direct interaction between Avr4 and Cf-4, mediating this response, is suggested. Recent studies have demonstrated that an Avr4 homologue (MfAvr4) from the *C. fulvum*-related fungus *Mycosphaerella fijiensis* is recognized by the Cf-4 protein of tomato, whereas it is only 42% homologous to *C. fulvum* Avr4 (Stergiopoulos *et al.*, 2010). Further analysis has shown that the chitin-binding domain of MfAvr4 is most homologous to *C. fulvum* Avr4, a suggestion that this domain most probably interacts with the Cf-4 protein (Stergiopoulos *et al.*, 2010).

As already mentioned above, the interaction between Avr2 and Rcr3 triggers a Cf-2-mediated HR which complies with the so-called guard hypothesis (Van der Biezen and Jones, 1998), with Rcr3 as the guardee and Cf-2 as the guard that is triggered by (modified) Rcr3 after binding to Avr2. We have shown previously that Avr2 binds to, and inhibits, the cysteine protease Rcr3, but the detailed mechanism of interaction between the Rcr3–Avr2 complex and the Cf-2 protein is not yet understood (Rooney *et al.*, 2005).

Cysteine proteases are endopeptidases that use a cysteinyl thiol group for their catalytic activity and belong to the C-clan that harbours several protease families (Rawlings *et al.*, 2004). The majority of the members of the CA-1 subfamily of cysteine proteases are of plant origin and are referred to as papain-like cysteine proteases (PLCPs) as they show similarity to papain, a well-studied cysteine protease present in the latex of papaya (*Carica papaya*) (Van der Hoorn, 2008). All PLCPs are involved

in important physiological and cellular processes, including organogenesis, the turnover of storage proteins, programmed cell death, tolerance to abiotic stress and defence against both herbivorous predators and microbial pathogens (Hatsugai *et al.*, 2004; Kiyosaki *et al.*, 2009; Van der Hoorn, 2008). PLCPs have similar folds and contain a Cys and a histidine (His) residue in their catalytic centre, which consists of seven subsites (S_1 – S_4 and S_1' – S_3'), each accommodating a side-chain of a corresponding amino acid residue (P_1 – P_4 and P_1' – P_3' , respectively) present in the peptide substrate or peptide inhibitor (Turk *et al.*, 1998).

It is difficult to classify protease inhibitors on the basis of sequence information, as the reactive site residues of inhibitor domains are often not conserved like the active site residues present in target proteases. Some protease inhibitors, such as phytocystatins, target plant proteases of the CA-1 subfamily (Rawlings *et al.*, 2004; MEROPS peptidase database, <http://merops.sanger.ac.uk>; Rawlings *et al.*, 2008). Most phytocystatins have molecular masses between 12 and 16 kDa (Martinez and Diaz, 2008) and act as pseudosubstrates that form tight, reversible complexes with target enzymes by entering the active site cleft. Not many fungal and oomycete cysteine protease inhibitors have been described so far, but most show homology with phytocystatins.

The oomycete pathogen *Phytophthora infestans* secretes a number of protease inhibitors with similarity to phytocystatins, including Epic1 and Epic2B, which also interact with, and inhibit, Pip1 and Rcr3 (Shabab *et al.*, 2008; Song *et al.*, 2009; Tian *et al.*, 2007; Van Esse *et al.*, 2008), but do not trigger a Cf-2-mediated HR like the Rcr3 inhibitor Avr2 (Rooney *et al.*, 2005).

Although we have no experimental evidence that Avr2 of *C. fulvum* shares functional homology with phytocystatins, we mention here a few motifs present in phytocystatins that have provided the basis for introducing amino acid substitutions into Avr2. They include the QXVXG motif [where Q, V and G stand for glutamine (Gln), valine (Val) and glycine (Gly), respectively; X stands for any amino acid residue], one or two Gly residues at the N-terminus, and many lysine (Lys) residues scattered over the entire inhibitor protein.

By assuming that Avr2 enters the active site cleft of Rcr3, with a preference for aromatic amino acid residues in the P_2 subsite of Avr2 to interact with the S_2 subsite of Rcr3, we individually substituted the tyrosine-21 (Tyr21) and phenylalanine-54 (Phe54) residues by alanine (Ala) (Fig. 1). As Lys residues occur frequently in phytocystatins, we also individually substituted the Lys17 and Lys20 residues in Avr2 by Ala (Fig. 1). In addition, we individually substituted all eight Cys residues in Avr2 by Ala (Fig. 1).

In this article, we report the overall structure of Avr2 and show that it is a novel type of cysteine protease inhibitor with eight Cys residues that are all involved in disulphide bonds. We pro-

Fig. 1 (A) Amino acid sequence of wild-type Avr2 protein (residues 1–58) including the histidine (His) (HHHHHH) and the Flag (DYKDDDDK) tags at the N-terminus. The enterokinase site is indicated by the filled triangle. Underlined amino acid residues were substituted by alanine in individual mutant Avr2 proteins. The constructs encoding mutant Avr2 proteins were produced in *Pichia pastoris*. One mutant Avr2 was produced lacking all six C-terminal amino acid residues (indicated in bold), leading to a 52-amino-acid mutant Avr2 protein. (B) The disulphide bond pattern of wild-type Avr2 as determined by disulphide mapping combined with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis (see Experimental procedures for further details). The disulphide bonds between the cysteine residues (Cys7–Cys33, Cys12–Cys52, Cys29–Cys43 and Cys53–Cys57) are indicated as full lines.

duced wild-type Avr2 protein in *Pichia pastoris* and determined its disulphide bond pattern by mass spectrometry. By site-directed mutagenesis, we show that three of the four disulphide bonds are required for Avr2 stability, whereas the C-terminal disulphide bond is required for inhibitory activity and Cf-2-mediated HR-inducing activity. Avr2 is not processed by Rcr3 and behaves as an uncompetitive inhibitor of Rcr3 cysteine protease. In addition, we produced mutant Avr2 proteins in which four selective amino acid residues were substituted by Ala, whereas, in another case, all six C-terminal amino acid residues were deleted. We determined the inhibitory constant (K_i) of these mutants for Rcr3 and their ability to trigger a Cf-2-mediated HR in tomato. Two C-terminal Cys residues and the six C-terminal amino acid residues of Avr2 are required for both Rcr3 inhibitory activity and the ability to trigger a Cf-2-mediated HR. Overall, our data suggest that the affinity of the Avr2 mutants for Rcr3 correlates with their ability to trigger a Cf-2-mediated HR.

RESULTS

Avr2 contains four disulphide bonds

Wild-type Avr2 tagged at the N-terminus with six His residues and Flag (His::Flag::Avr2) containing an enterokinase cleavage site behind the Flag signature was produced in a fermentor by *P. pastoris*, as described previously (Rooney *et al.*, 2005; Van den Burg *et al.*, 2001), and purified on an Ni^{2+} -nitrilotriacetate (Ni-NTA) column (Fig. 1A). The His-Flag tag was removed after treatment with enterokinase and the reaction products were separated by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) in order to obtain pure mature untagged Avr2. The molecular mass of purified wild-type Avr2, as determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis, was 6085 Da, which suggests that all Cys residues present in wild-type Avr2 are involved in intramolecular disulphide bonds. This

could be verified by showing that the molecular mass of completely reduced Avr2 increased by 8 Da (6093 Da).

Disulphide mass mapping of Avr2

In order to determine the disulphide bond pattern of Avr2, disulphide mass mapping using cyanylation and CN-induced cleavage by hydroxide or ammonia was performed as described previously (Wu and Watson, 1998). First, Avr2 was partially reduced with an 18-fold molar excess of Tris-(2-carboethyl)phosphine hydrochloride (TCEP). Subsequently, Avr2 was cyanylated with a 300-fold molar excess of 1-cyano-4-dimethylamino-pyridium tetrafluoroborate (CDAP) and then cleaved by ammonia, after which the products were separated by RP-HPLC. Two singly reduced cyanylated Avr2 species were obtained, in which the presence of disulphide bonds between Cys7–Cys33 and Cys53–Cys57 could be assigned by mass determination after CN-induced cleavage. The disulphide bonds of doubly reduced species could not be assigned in this way, but could be determined by a combination of partial reduction with TCEP, followed by alkylation with *N*-ethylmaleimide (NEM), separation on RP-HPLC and digestion with *Staphylococcus aureus* endoprotease GluC, which cleaves directly C-terminal of a glutamic acid residue, followed by mass determination of the products obtained. In this way, it was shown that the two remaining disulphide bonds are present between Cys12–Cys52 and Cys29–Cys43. The complete disulphide bond pattern of Avr2 is shown in Fig. 1B. The disulphide bonds between Cys7–Cys33, Cys12–Cys52 and Cys29–Cys43 in Avr2 provide a very compact and stable structure, which is presumably difficult to digest by extracellular plant proteases that are often present in the apoplastic space of tomato leaves. The overall disulphide bond pattern of Avr2 is different from the disulphide bond patterns reported for two other *C. fulvum* effectors. Avr9 contains a cystine knot structure consisting of three disulphide bonds (Van den Hooven *et al.*, 2001), whereas Avr4 contains four disulphide bridges including a chitin-binding domain structure (Van den Burg *et al.*, 2003).

Fig. 2 Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of wild-type His-Flag-tagged Avr2 and mutant Avr2 proteins containing various amino acid substitutions or a C-terminal deletion of six amino acids (C53X). Equal amounts of protein (as indicated) were loaded on the gel. Proteins were stained with Coomassie Brilliant Blue (CBB) and identified by Western blot analysis using anti-Flag and anti-Avr2 antibodies.

Production of mutant Avr2 proteins

We decided to generate Avr2 mutants by introducing amino acid substitutions and deletions in domains of the protein that might be involved in cysteine protease binding and cysteine protease inhibition, based on the presumed roles of Cys residues in disulphide bridges and some presumed homology with phytocystatins. We substituted all Cys residues individually by Ala and also deleted all six C-terminal amino acid residues. In addition, Tyr21 and Phe54 were selected to be replaced by Ala as they might function as a putative P₂ subsite to occupy the S₂ subsite of Rcr3. Lys17 and Lys20 were chosen to be replaced by Ala, as many phytocystatins are Lys-rich (Martinez and Diaz, 2008). Figure 1A shows an overview of the amino acid substitutions and the C-terminal deletion that were introduced in the various mutant Avr2 proteins through polymerase chain reaction (PCR)-based site-directed mutagenesis of the *Avr2* gene. Wild-type and mutant versions of the *Avr2* gene were cloned in expression vector pPIC9 and transformed into the yeast *P. pastoris*. Proteins produced in *P. pastoris* were purified, analysed and quantified by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Mutant Avr2 proteins with the following amino acid substitutions could be produced in sufficient amounts: Avr2-K17A, Avr2-K20A, Avr2-Y21A, Avr2-C53A, Avr2-F54A, Avr2-C57A and the Avr2 mutant lacking all six C-terminal amino acid residues (Avr2-C53X). Substitution of all individual Cys residues by Ala, except for residues Cys53 and Cys57, resulted in too little Avr2 protein produced in *P. pastoris*, most probably because of instability of the mutant proteins. For wild-type and all mutant Avr2 proteins that could be produced in sufficient amounts, equal amounts were used to determine their

inhibitory activity towards Rcr3 and their ability to trigger a Cf-2-mediated HR.

Inhibitory activity of wild-type and mutant Avr2 proteins towards Rcr3

Previously, it has been shown that wild-type Avr2 is not a substrate for Rcr3, as the overall mass of wild-type Avr2 does not change after prolonged incubation with Rcr3 (Rooney *et al.*, 2005). Thus, it is anticipated that Avr2 interacts with Rcr3 by an irreversible trapping or a reversible tight-binding reaction. To determine which type of interaction occurs, we studied the kinetics of the interaction between Avr2 and Rcr3 in more detail by Lineweaver–Burk plot analysis. In this analysis, we used a constant Rcr3 concentration and varying concentrations of the green fluorescent casein substrate (Bodipy FL-casein) at different concentrations of Avr2. Avr2 shows an inhibition pattern that is most similar to that of uncompetitive inhibition, as both V_{max} and K_m of Rcr3 are affected at different concentrations of Avr2 (Fig. 3). The pattern observed for 40 nM Avr2 is fairly similar to that observed for 20 nM Avr2 indicating that, at a concentration of 20 nM Avr2, nearly all binding sites are occupied and increasing the Avr2 concentration has only minor effects. Uncompetitive inhibition is consistent with Avr2 only binding to the Rcr3–substrate complex outside the active centre of Rcr3. This is also in agreement with the finding that Avr2 is not a substrate of Rcr3, and that binding of the irreversible inhibitor E-64 to Rcr3 can be achieved by a three-fold molar excess of Avr2 over E-64 (Rooney *et al.*, 2005).

We also measured the inhibitory constants (K_i values) of wild-type Avr2 and the different mutant Avr2 proteins for Rcr3 by

Fig. 3 Lineweaver–Burk plot analysis. Rcr3 activity was measured for each Avr2 concentration indicated in the inset of the figure at four different Bodipy FL-casein substrate concentrations (230, 460, 920 and 1840 nM) and constant Rcr3 concentration. The reciprocal of the substrate Bodipy FL-casein concentration is shown on the x-axis. The Rcr3 rest activity is indicated on the y-axis as the reciprocal of the relative amount of Bodipy FL released from the Bodipy FL-casein substrate over 24 h at room temperature. The relative amount of released Bodipy FL was determined by measuring its fluorescence at 530 nm. The observed inhibition pattern is consistent with uncompetitive inhibition of Rcr3 by Avr2.

measuring the remaining protease activity at constant Bodipy FL-casein substrate concentration after incubation with varying concentrations of wild-type or mutant Avr2 proteins for 24 h. The K_i value for wild-type Avr2 was 16.1 nM and the K_i values for the various mutant Avr2 proteins varied between 20 and 1444 nM (Fig. 4). Substitution of Cys53, Phe54 or Cys57 by Ala increased the K_i value of Avr2 by approximately five-fold, whereas deletion of all six C-terminal amino acid residues increased the K_i value of Avr2 by more than 70-fold. Individual substitution of the Lys17, Lys20 or Tyr21 residue in Avr2 by Ala did not change significantly the K_i values towards Rcr3 relative to wild-type Avr2, suggesting that these residues are not crucial for the affinity of Avr2 for Rcr3.

Cf-2-mediated HR triggered by wild-type and mutant Avr2 proteins

The mutant Avr2 proteins with various affinities for Rcr3 were also assayed for their ability to trigger a Cf-2-mediated HR in tomato. Equal amounts (100 μ L volume with a concentration of

2 μ M) were injected into leaflets of Cf-2 tomato plants and the Cf-2-mediated HR-inducing activity was scored 3 days post-injection (Fig. 5). The Cf-2-mediated HR triggered by wild-type Avr2 and various Avr2 mutant proteins varied significantly. For the Cys residues in Avr2 that were substituted by Ala, only results for C53A and C57A substitutions could be obtained, because, for the Avr2 mutants in which the individual substitutions C7A, C12A, C29A, C33A, C43A and C52A were introduced, insufficient amounts of Avr2 mutant proteins could be produced in *P. pastoris*, most probably because of protein stability. The Cf-2-mediated HR-inducing activities of Avr2 mutant proteins, in which one amino acid residue in the C-terminal part was substituted by Ala (Avr2-C53A, Avr2-C57A and Avr2-F54A mutants), were significantly lower when compared with the activity of wild-type Avr2. Deletion of all six C-terminal amino acid residues in Avr2 (Avr2-C53X) abolished the Cf-2-mediated HR-inducing activity nearly completely.

Substitution of the amino acid residue Lys17, Lys20 or Tyr21 by Ala (Avr2-L17A, Avr2-K20A and Avr2-Y21A) in Avr2 did not alter significantly the Cf-2-mediated HR-inducing activity. Together,

Fig. 4 The affinity of wild-type and mutant Avr2 proteins for Rcr3. Wild-type or mutant Avr2 proteins were incubated at different concentrations (x-axis) at constant Rcr3 protease and substrate Bodipy FL-casein concentrations. The rest activity of Rcr3 (y-axis) was measured as the relative fluorescence of released Bodipy FL at 530 nm, 24 h after incubation at room temperature. The K_d values indicated in the inset were determined as described in Experimental procedures.

Fig. 5 Cf-2-mediated hypersensitive response (HR)-inducing activity triggered by wild-type Avr2 and mutant Avr2 proteins produced in *Pichia pastoris*. Photograph was taken 3 days post-injection of 100 μ L of an aqueous solution containing 2 μ M of wild-type Avr2 or mutant Avr2 protein as indicated. Note that only amino acid substitutions in the six C-terminal amino acids of Avr2 significantly affected Cf-2-mediated HR-inducing activities. Deletion of all six C-terminal amino acid residues of Avr2 (Avr2-C53X) abolished Cf-2-mediated HR-inducing activity nearly completely (compare also Cf-2-mediated HR-inducing activities with the K_d values for wild-type Avr2 and mutant Avr2 proteins presented in Fig. 4).

these results (shown in Fig. 5), combined with the observed K_i values (shown in Fig. 4), suggest a positive correlation between the affinity of the Avr2 mutant proteins for Rcr3 and their ability to trigger a Cf-2-mediated HR.

DISCUSSION

MALDI-TOF MS analysis showed that all eight Cys residues present in the cysteine protease inhibitor Avr2 are involved in disulphide bonds (Fig. 1B). Disulphide bonds are formed between Cys7–Cys33, Cys12–Cys52, Cys29–Cys43 and Cys53–Cys57. Three of these bonds (Cys7–Cys33, Cys12–Cys52 and Cys29–Cys43) are required for Avr2 stability, and therefore indirectly also for its inhibitory activity against the cysteine protease Rcr3, and its ability to trigger a Cf-2-mediated HR. So far, the spacing pattern of the Cys residues in Avr2 has not been found in protein structure databases, but the overall disulphide bond pattern present in Avr2 also occurs in members of a serine protease-inhibiting peptide family isolated from arthropods (Simonet *et al.*, 2002), in WAP (whey acidic protein) protease inhibitors (Ohashi *et al.*, 2003; Ranganathan *et al.*, 1999; Sano *et al.*, 2005) and in thionine-related fabatins, small antimicrobial peptides (Zhang and Lewis, 1997).

Individual substitution of Cys residues Cys7, Cys12, Cys29, Cys33, Cys43 or Cys52 by Ala strongly affected the stability of the Avr2 protein, and this was the main reason why we could not produce these Avr2 mutant proteins in sufficient amounts in *P. pastoris* for detailed biological studies. Substitution of the Cys residues forming the disulphide bond between Cys53 and Cys57 in the C-terminus of Avr2 by Ala did not affect the stability of the corresponding mutant protein, but showed a reduction in both the Rcr3 inhibitory activity and the ability to trigger a Cf-2-mediated HR. In addition, deletion of all six C-terminal amino acid residues of Avr2 showed a decrease in both Rcr3 inhibition and Cf-2-mediated HR-inducing activity of over 100-fold. This indicates that the C-terminal part of Avr2 is crucial for biological activity and most probably interacts with Rcr3. In order to find direct evidence for this interaction, we performed MALDI-TOF MS analysis of Rcr3 in the presence of biotinylated Avr2, and attempted to detect the Rcr3–Avr2 complex in co-immunoprecipitates in order to identify the interaction site. Although we were able to identify Rcr3 fragments after trypsin digestion, we could not identify Rcr3 fragments bound to Avr2, indicating that the interaction between Rcr3 and Avr2 is reversible and not covalent. This makes it difficult or even impossible to identify the interaction site(s) between Rcr3 and Avr2 by mass spectrometry. We have also tried to fixate the Rcr3–Avr2 complex by photo-affinity labelling but, so far, pilot experiments have failed.

Amino acid substitutions outside the six-amino-acid C-terminal part of Avr2, different from Cys, did not change the

affinity of Avr2 mutant proteins for Rcr3 and did not affect Cf-2-mediated HR-inducing activity.

As the Avr2 amino acid sequence and overall structure are distinct from those of other known protease inhibitors, such as phytocystatins, we studied the mode of inhibition in more detail. We performed inhibitor assays for each Avr2 concentration at constant Rcr3 concentrations and varying Bodipy FL-casein substrate concentrations. The data presented in a Lineweaver–Burk plot (Fig. 3) show that Avr2 is clearly not a competitive cysteine protease inhibitor, but behaves as an uncompetitive inhibitor. This indicates that Avr2 most probably only binds the Rcr3–substrate complex outside the catalytic centre of Rcr3 and affects both V_m and K_m . We used Bodipy FL-casein as a substrate to measure the enzymatic activity of Rcr3, as the native substrate in tomato for Rcr3 is not yet known. The K_i values of wild-type and mutant Avr2 proteins towards Rcr3 negatively correlate with Avr2-triggered Cf-2-mediated HR. This means that the lower the affinity of Avr2 mutants for Rcr3, the lower their Cf-2-mediated HR-inducing activity. As Avr2 does not bind to the catalytic centre of Rcr3, this suggests that the conformational change(s) of Rcr3 induced by binding of Avr2 triggers the Cf-2-mediated HR. This is in agreement with the observation that the *Lycopersicon esculentum* variant of Rcr3 (Rcr3^{esc}) triggers a Cf-2-mediated HR in an Avr2-independent manner. Rcr3^{esc} differs from Rcr3^{pimp} in only a few amino acid residues, and these seem to be sufficient to cause a conformational change in the protein to trigger a Cf-2-mediated HR in an Avr2-independent manner.

A similar finding has been reported for the relationship between the affinity of Avr9 mutant proteins for HABS and Avr9-triggered Cf-9-mediated HR (Kooman-Gersmann *et al.*, 1998). Ala scanning mutants of Avr9 with the lowest affinity for HABS also showed the lowest Cf-9-mediated HR-inducing activity.

In conclusion, Avr2 is a potent cysteine protease inhibitor that is different from other known cysteine protease inhibitors such as the phytocystatins (Rawlings *et al.*, 2004). Avr2 is required by *C. fulvum* to inhibit various extracellular plant cysteine proteases, including Rcr3 and Pip1, to enable the pathogen to grow in the harsh cysteine protease-rich environment of the apoplastic space of a tomato leaf. Indeed, it has been shown that Avr2 behaves as a genuine virulence factor of *C. fulvum* in the absence of the Cf-2 protein (Van Esse *et al.*, 2008), whereas it acts as an avirulence factor in the presence of the Cf-2 protein (Rooney *et al.*, 2005).

Recently, it has been reported that *Phytophthora infestans*, a pathogen of tomato and potato, secretes two cystatin-like protease inhibitors, Epic1 and Epic2B, which both interact with, and inhibit, Rcr3 but fail to trigger a Cf-2-mediated HR (Song *et al.*, 2009). In addition, the inhibition of Rcr3 by the irreversible inhibitor E-64 does not trigger a Cf-2-mediated HR (Rooney *et al.*, 2005). This shows that inhibition of Rcr3 can be achieved

by different cysteine protease inhibitors, but that only the interaction of Rcr3 with Avr2 triggers a Cf-2-mediated HR, indicating that the conformational change in Rcr3 induced by Avr2 is different from that induced by the phytocystatins Epic1, Epic2B and E-64. These data also indicate that Avr2 is a unique cysteine protease inhibitor, and that its interaction with Rcr3 results in a unique conformational change that is recognized by Cf-2. The C-terminal tail of Avr2 most probably interacts with Rcr3, but, so far, we have not been able to identify the exact interaction site between Avr2 and Rcr3.

EXPERIMENTAL PROCEDURES

Plasmid constructs used for protein expression in *P. pastoris*

Plasmids for the expression of Rcr3, wild-type Avr2 and mutant Avr2 proteins in *P. pastoris* were generated as described previously (Rooney *et al.*, 2005). Throughout this study the Rcr3 *pimpinellifolium* (Rcr3^{pim}) isoform was used.

Mutant Avr2 peptide production in *P. pastoris*

Pichia pastoris strain GS115 (Invitrogen, Landsmeer, the Netherlands) was transformed with plasmids encoding different mutant Avr2 proteins. The tags and various mutations introduced in Avr2 are indicated in Fig. 1A, and the different primers used to introduce the mutations are shown in Table 1. The vector pPIC9 (Invitrogen) was used for secretion of the proteins in the culture medium. Transformants were grown in small cultures and the proteins secreted into the medium were analysed by Tricine SDS-PAGE, and afterwards stained with Coomassie Brilliant Blue or analysed on Western blots, using antibodies (anti-Flag or anti-Avr2). Transformants expressing significant amounts of the different Avr2 proteins were selected for bulk fermentation. Fermentation was performed as described previously (Rooney *et al.*, 2005). The tagged proteins were subsequently purified on an Ni-NTA column and fractions containing highly purified proteins were pooled and dialysed against water. For mass spectrometric analysis of Avr2, the His-Flag tag was removed by treatment with enterokinase. Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce art. No. 23225, Landsmeer, the Netherlands). Purified wild-type and Avr2 mutants were tested for their Rcr3 inhibitory activity and their potential to trigger a Cf-2-mediated HR. Plants were monitored for HR development 3–5 days post-injection of wild-type and Avr2 mutant proteins.

Disulphide mass mapping of Avr2

The disulphide mass mapping of purified Avr2 produced in *P. pastoris* was performed by the method previously used for the

Table 1 Primers used in this study.

Code	Sequence
A2K17AF	GTGAAGGAGGCGTCTGGAAG
A2K17AR	CTTCCAGACGCTCTTCAC
A2K20AF	GGAGAAGTCTGGAGCGTATAAATG
A2K20AR	CAATTATACGCTCCAGACTTCTCC
A2Y21AF	GGAGAAGTCTGGAAGGCTAAATTGAAG
A2Y21AR	CTTCAATTAGCCTTTCCAGACTTCTCC
A2C29AF	GATTGGTGCTAAAGCCTCGGCGACATGTG
A2C29AR	CACATGTCGCCGAGGCTTAGCACCAATC
A2C33AF	GGTGCTAAATGCTCGGCGACAGCTGACGGG
A2C33AR	CCCGTCAGCTGTCGCCGAGCATTTAGCACC
A2C52AR	GGGTAATCACCTTGCTGTTTGGTCTTTGCGG
A2C52AF	CCGCAAGACCAAAACAGGCAAGGTGATTACCC
A2C53AR	GGGTAATCACCTTGCTGCTTTTGGTCTTTGCGG
A2C53AF	CCGCAAGACCAAAAGCGCAAAGGTGATTACCC
A2F54AF	CACCTTGTCTGTGCTGTTTGGTCTTTGCG
A2F54AR	GCAAAGACCAGCACAGCAAAGGTG
A2C57AR	CCTTGTGCTTTTGGTCTTGCCGGTTGATGATACG
A2C57AF	CGTATCATCAACCGGCAAGACCAAAACAGCAAAGG
A2C53XF	GGGTAATCACCTTGCTGATAGTGAGAATTCCG
A2C53XR	CGGAATTCTCACTATCAGCAAAGGTGATTACCC

F and R stand for forward and reverse primer sequences, respectively.

Primer codes should be read as follows: A2 stands for Avr2; the next four characters indicate the amino acid residue and its position in the Avr2 protein sequence to be substituted by alanine, except for A2C53XF and A2C53XR which lead to a protein with a C-terminal six-amino-acid deletion.

disulphide mass mapping of Avr4 (Van den Burg *et al.*, 2003). The molecular masses of partially and fully reduced Avr2 peptides were determined by MALDI-TOF MS.

Determination of apparent equilibrium dissociation constants

For the wild-type and mutant Avr2 proteins, the apparent inhibitory constants (K_i values) for Rcr3 were determined by titration of the enzyme using curve-fitting software as described previously (Annadana *et al.*, 2003). For determination of K_i , different concentrations of each Avr2 mutant protein were added to Rcr3 and the remaining Rcr3 enzyme activity was measured 24 h after incubation with the substrate Bodipy FL-casein in the presence of 10 mM L-cysteine, 50 mM Na-acetate, pH 5.0 (Annadana *et al.*, 2003). The relative amount of released Bodipy FL was measured in a fluorescence microplate reader with standard fluorescein excitation filters at 485 ± 12.5 nm and emission at 530 ± 15 nm. All K_i measurements were performed in triplicate. Fluorescence values were fitted to the K_i model (Beekwilder *et al.*, 2000).

Lineweaver–Burk plots

The $V_{\max}$ and K_m values for Rcr3 were measured indirectly by determining the Rcr3 activity in the presence of various substrate and Avr2 concentrations. The effects of different Avr2 concentra-

tions on Rcr3 activity were visualized using Lineweaver–Burk plots, allowing conclusions to be drawn on the mode of inhibition (competitive, uncompetitive, noncompetitive or mixed), where competitive inhibitors affect K_m , noncompetitive inhibitors affect V_{max} and uncompetitive inhibitors affect both V_{max} and K_m . Mixed inhibitors show intermediate patterns. For each Avr2 concentration, a fixed Rcr3 concentration was used and the Bodipy FL-casein substrate was varied. All tests were performed in 10 mM L-cysteine, 50 mM Na-acetate, pH 5.0. All samples were measured after incubation for 24 h at room temperature.

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