Natural Disulfide Bond-disrupted Mutants of AVR4 of the Tomato Pathogen Cladosporium fulvum Are Sensitive to Proteolysis, Circumvent Cf-4-mediated Resistance, but Retain Their Chitin Binding Ability*[S]

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The extracellular AVR4 elicitor of the pathogenic fungus Cladosporium fulvum induces defense responses in the tomato genotype Cf-4. Here, the four disulfide bonds of AVR4 were identified as Cys-11-41, Cys-21-27, Cys-35-80, and Cys-57-72 by partial reduction with Tris-(2-carboxyethyl)-phosphine hydrochloride, subsequent cya-
nylation, and base-catalyzed chain cleavage. The resulting peptide fragments were analyzed by mass spectrometry. Sequence homology and the disulfide bond pattern revealed that AVR4 contains an invertebrate (inv) chitin-binding domain (ChBD). Binding of AVR4 to chitin was confirmed experimentally. The three disulfide bonds encompassing the inv ChBD motif are also required for protein stability of AVR4. Independent disruption of each of the three conserved disulfide bonds in AVR4 resulted in a protease-sensitive protein, whereas the fourth disulfide bond appeared not to be required for protein stability. Most strains of C. fulvum virulent on Cf-4 tomato contain Cys to Tyr substitutions in AVR4 involving two (Cys-11-41, Cys-35-80) of the three disulfide bonds present in the inv ChBD motif. These natural Cys to Tyr mutant AVR4 proteins did retain their chitin binding ability and when bound to chitin were less sensitive to proteases. Thus, the widely applied tomato Cf-4 resistance gene is circumvented by C. fulvum by amino acid substitutions affecting two di-
sulfide bonds in AVR4 resulting in the absence of the corre-
sponding AVR4 isoforms in apoplastic fluid. How-
ever, these natural isoforms of AVR4 appear to have retained their intrinsic function, i.e. binding to chitin present in the cell wall of C. fulvum, most likely to protect it against the deleterious effects of plant chitinases.

Gene-for-gene-based disease resistance in plants commonly requires two complementary genes, an avirulence (Avr) gene in the pathogen and a matching resistance gene in the host (1, 2). The Cf resistance genes of tomato mediate specific recogni-
tion of extracellular elicitor proteins encoded by Avr genes of the pathogenic fungus Cladosporium fulvum (3). The Avrs of C. fulvum and their matching Cf genes have become valuable instruments to investigate signal transduction pathways leading to plant disease resistance (4–7, 9). To obtain sustainable resistance, the Cf resistance genes were introgressed from wild Lycopersicon species into commercial tomato cultivars. How-
ever, because of selection pressure new strains of C. fulvum emerged that had overcome the introgressed resistance traits by modification of the Avr genes (10). Although some Avr genes in these virulent C. fulvum strains were found to be absent (11), others contained point mutations (12) or transposon in-
sertions (13). The natural strains of C. fulvum carrying these mutated Avr genes did not exhibit significantly reduced viru-
ulence under laboratory conditions (11–14), suggesting that AVR proteins are not essential for virulence or that the modi-
fied isoforms of AVRs still contribute to virulence of C. fulvum. The genetic variation is so far strictly limited to the race-
specific Avrs and is absent in genes that encode other extracel-
ular elicitor proteins of C. fulvum (15).

Although the intrinsic role of the AVR proteins of C. fulvum during infection remains obscure, they are anticipated to con-
tribute to virulence in susceptible hosts (16–18). This implies that evasion of Cf-mediated resistance by modification of Avr genes might be associated with a reduction or loss in virulence unless a functional gene remains. A candidate protein to investi-
gate the latter idea is the race-specific elicitor AVR4 because Cf-4-mediated resistance is overcome in all but one case by single amino acid substitutions in the Avr4 gene (12, 14). More-
ever, for AVR4 a virulence function is proposed in association with its ability to bind to chitin. AVR4 was found to protect fungi against degradation by plant chitinases by association with its hyphal wall.2 Mutations in the Avr4 gene, as found in natural C. fulvum isolates virulent on the tomato genotype

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1 The abbreviations used are: AVR, avirulence gene product; AF, apoplastic fluid; des-(Cys-x—Cys-y), AVR4 species lacking a specific disulfide bond (the involved half-cystines are reduced and the sulhydryl group is cyanylated); CDAP, 1-cyano-4-diethylamino-pyridinium; ChBD, chitin-binding domain; HPLC, high performance liquid chromatography; inv, invertebrate; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NIA, necrosis-inducing activity; PVX, potato virus X; TCEP, Tris-(2-carboxyethyl)-phosphine hydrochloride.

2 C. F. de Jong, A. M. Laxalt, W. Ligterink, P. J. G. M. de Wit, M. H. A. J. Joosten, and T. Munnik, submitted for publication.

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Disulfide Bond Mutants of AVR4

Cf-4, encode mostly single Cys to Tyr substitutions. In addition, two other mutations were found, i.e. Thr-66 to Ile and Tyr-67 to His. The Cys to Tyr substitutions involved the positions 64, 70, or 109 (which corresponds with Cys-35, -41, and -80 in the mature protein, respectively) (14). Some of these avr4 alleles still exhibited necrosis inducing activity when transiently expressed in Cf-4 tomato using potato virus X (PVX) (14). However, none of the AVR4 mutant isoforms could be detected in apoplastic fluid isolated from tomato leaves inoculated with *C. fulvum* (14).

Mass spectrometry revealed that all Cys residues in AVR4 are involved in disulfide bonding (20). Disulfide bond patterns and the sequential spacing between Cys residues define to a large extent the protein fold of secreted small proteins (21, 22). Here, the disulfide bond connectivities of AVR4 are elucidated. The disulfide bond pattern of AVR4 shows homologies with the disulfide bond pattern found in the recently identified invertebrate chitin-binding domain (inv ChBD) (23), i.e. three of the four disulfide bonds of AVR4 (Cys-11-41, Cys-35-80, and Cys-57-72) are represented in the inv ChBD motif. Independent disruption of each of these three disulfide bonds in AVR4 results in a protein that is sensitive to proteases present in the apoplast, which suggests that these disulfide bridges are required for conformational stability of AVR4. The Cys to Tyr mutations identified in natural strains of *C. fulvum* involve two (Cys-11-41 and Cys-35-80) of these three conserved disulfide bonds. AVR4 isoforms with a disruption in one of these two disulfide bonds are still able to bind chitin. Our data support a model where evasion of Cf-4-mediated resistance appears to be based on decreased conformational stability of the AVR4 isoform, leading to protein degradation upon release in the tomato apoplast. Noteworthy, the AVR4 isoforms were found to be more resistant to proteases when bound to chitin. These findings argue that mutant AVR4 isoforms are fully functional and can associate with chitin upon release, whereas excess of secreted and unbound protein is degraded before triggering host defense responses.

**EXPERIMENTAL PROCEDURES**

**Construction of PVX Derivatives and Transcription—avr4 mutants encoding various Cys to Ala substitutions were generated by PCR-based primer-directed mutagenesis on the plasmid pTXG3Ca3, containing the native avr4 sequence (14). PCR amplification was carried out using mutagenic primers (see Supplementary Table for primers) designated to generate two overlapping PCR fragments. PCR was used to combine the overlapping PCR fragments using the primers OX10 and N31, and the PCR product was cloned into the ClaI site of the vector pTXG3Ca3 (24) and sequenced. In vitro transcription of the plasmids and subsequent inoculation on *Nicotiana clevelandii* and tomato was performed as described (14). N. clevelandii and the tomato (*Lycopersicon esculentum*) cultivars Moneymaker (MM) and the near-isogenic line MM-Cf4 were grown as described previously (25).

**Partial Reduction and Cyanylation of the AVR4 Protein—** Expression of heterologous AVR4 was achieved in the methylophytic yeast *Pichia pastoris*, and AVR4 was purified from culture fluid (20). The disulfide bonds of AVR4 were partially reduced with TCEP (Sigma) (20, 27). A stock solution of 0.1 M TCEP was prepared in 6 M guanidine-HCl in 0.1 M citrate buffer (pH 3) and stored at −20 °C (without any deterioration for up to six months). For each reduction reaction, 100 μg of native AVR4 was dissolved in 10 μl of 6 M guanidine-HCl in 0.1 M citrate buffer (pH 3). The reaction was initiated by adding a 6-fold molar excess of TCEP to AVR4, followed by incubation at 20 °C for 15 min. Subsequently, an 80-fold molar excess of CDAP (Sigma) was added to cyanate the free thiol groups (15 min, 20 °C, in the dark). The 0.1 M CDAP stock solution in 6 M guanidine-HCl in 0.1 M citrate buffer (pH 3) was freshly prepared prior to each reaction.

**Reverse-Phase High Performance Liquid Chromatography of the Peptide Mixture—** The TCEP/CDAP reaction mixtures were separated by analytical reverse-phase high performance liquid chromatography (RP-HPLC) using a 150 × 3.9 mm Delta-Pak C18 column (300 Å, 5 μm; Waters). The separation was monitored at 215 nm, and predominant peaks were manually collected. HPLC elution solvents consisted of 0.1% (v/v) trifluoroacetic acid in water (solvent A), and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). The HPLC was operated at a flow rate of 1 ml/min. The applied gradient was 5% → 20% B (percentage B in solvent A) in 2 min, 20% → 30% in 40 min, and 30% → 60% in 3 min. AVR4 eluted at ~25% B. Integration of the HPLC profile was achieved using the supplied Waters software. Appropriate fractions (containing the AVR4 des-species) were lyophilized for storage. All solvents used were HPLC grade.

**Peptide Cleavage and Full Reduction of the Disulfide Bond/Peptide Mass Analysis—** Lyophilized HPLC fractions containing the AVR4 des-species were dissolved in two consecutive steps: first, 2 μl in 1 M NH4OH, 6 M guanidine-HCl, and second, 5 μl of 1 M NH4OH and incubated at 20 °C for 1 h. The excess of NH4OH was evaporated in a Speed-Vac system in 30 min (to almost, complete dryness). Subsequently, the remaining disulfide bonds were reduced by adding an excess of TCEP (10 μl of 0.1 M TCEP stock), and the mixture was incubated at 37 °C for 30 min. The peptide mixtures were analyzed by mass spectrometry using a MALDI-TOF MS (Perceptive Biosystems Voyager DE-RC). Small aliquots of the peptide samples were applied to a saturated matrix solution that was freshly prepared (o-cyano-4-hydroxycinnamic acid; Sigma; 10 mg/ml in acetonitrile/water/trifluoroacetic acid (50/50/1, v/v/v)); one μl was deposited on a sample plate (28, 29). Depicted spectra were averages of 100–256 consecutive laser pulses. The instrument was generally operated in the positive mode at an acceleration voltage of 23 kV combined with delayed extraction. Spectra were externally calibrated with bovine cytochrome c (12,200.9 Da), bovine insulin (5,734.6 Da) (both Sigma), and Microperoxidase 8 (MP8, 1,506.5 Da; Ref. 30).

**Incubation of the AVR4 Des-species with Apoplastic Fluid—** Apoplastic fluids (AFs) were isolated from intercellular spaces of near isogenic tomato genotypes Cf-4 and Cf-0 (21) that had been inoculated with race 4 of *C. fulvum* (strain 38, a non-AV4 producing strain) and race 5 (an AVR4-producing strain), respectively (14). Native AVR4 and AVR4 des-species (4 μg) were incubated at 30 °C for 1 h in the presence of 0.1 μl of AF (~0.1 μg of total protein). Protease inhibitors were used from a standard protein inhibitor mixture with EDTA (Roche Applied Science, 1 tablet/ml AF). Protein samples were separated on Tricine SDS-PAGE gels (32).

**Polysaccharide Substrate Binding Assay—** Native AVR4 and AVR4 des-species (4 μg) were incubated at ambient temperature for 1 h (unless stated otherwise) with an excess of 5 mg of insoluble chitin beads (New England Biolabs) or chitosan (Sigma) in 50 mM Tris-HCl (pH 8) and 150 mM NaCl (500 μl of final volume) as described (19). The insoluble material was pelleted by centrifugation (11,000 × g for 3 min). Supernatants were recovered and lyophilized. The pellet fraction was boiled in 200 μl of 1% SDS to release bound protein and centrifuged. The retrieved supernatant (containing bound AVR4) as well as the lyophilized supernatant fraction (containing unbound AVR4) were examined for protein content by Tricine SDS-PAGE.

**NMR Modeling of AVR4 with the Tachycitin NMR Structure—** The mean NMR structure of tachycin (Protein Data Bank code 1DQC) was used as template structure to model the structure of AVR4 using Modeler 6.1 (33–35). Additional loop refinement was used to model the two relatively large gaps (12 and 6 amino acids). The disulfide bridges were fixed during the calculations. One thousand models were constructed, of which the ten lowest scoring structures were further examined. The models were found reliable using standard algorithms (36, 37).

**RESULTS**

**Four Cys Residues Are Required in AVR4 to Induce Cf-4-specific Defense Responses in Tomato—** By using transient PVX-mediated expression some AVR4 alleles, i.e. C35Y, Y38H, and C80Y, were identified that exhibited reduced necrosis-inducing activity (NIA) in the tomato genotype Cf-4, whereas the other natural *Acr4* alleles induced no NIA (14). To determine whether other Cys residues, for which no mutations were found in strains of *C. fulvum*, are also required for NIA of AVR4, we independently replaced all individual Cys residues by Ala in PVX::avr4. Four-week-old tomato plants were inoculated with these PVX::avr4 derivatives, and NIA was scored (Fig. 1) as described (14). The introduction of an Ala residue at the positions 35, 41, and 80 gave similar results as previously reported for the corresponding Tyr mutations (14). Interestingly, the mutations C21A and C27A were found to result in
reduced NIA on Cf-4 tomato similar to the mutations C35A and C80A. The PVX::Avr4 constructs carrying a Cys to Ala mutation in either Cys-11, -41, -57, or -72 induced no NIA on Cf-4 tomato plants. These finding suggest that the latter four Cys residues have interrelated disulfide bonds in AVR4. For Cys-21, -27, -35, and -80, of which the single mutations showed reduced NIA, double Cys to Ala mutations were constructed. Four of the six double mutants no longer induced hypersensitive response on Cf-4 tomato, whereas mutants carrying the mutations C21A+C27A and C35A+C80A were as active as the corresponding single Cys to Ala mutants (Table I). These data suggest that disulfide bonds connect Cys-21 with Cys-27 and Cys-35 with Cys-80.

**Chemical Reduction and Cyanylation of the Disulfide Bonds in AVR4**—To determine the disulfide bond connectivities in AVR4 by a direct chemical approach, we partially reduced the disulfide bridges with TCEP at pH 3.0, thereby minimizing intramolecular rearrangements of the disulfide bridges (38, 39). The formed cysteine thiol groups were directly modified by alkylation with CDAP under acidic conditions, and the resulting peptides were separated by reverse-phase HPLC (Fig. 2). In the presence of 6 molar equivalents of TCEP/AVR4, ~50% of native AVR4 was reduced, as indicated by an increased HPLC retention time of the newly formed species (Fig. 2). Subsequent MALDI-TOF mass spectrometry identified four product peaks containing AVR4 species with one disulfide bond reduced (hereafter denoted as des-species) (Fig. 2C). The peaks eluting at 30.8 and 30.9 min could not be separated by one HPLC run, but after an additional run both species appeared more than 85% pure (Fig. 2B). The peaks containing the des-species together constituted ~70% of the reduced AVR4 species, whereas peaks that eluted at higher acetonitrile concentrations contained AVR4 species with more than one disulfide bond reduced (as detected by mass spectrometry). The increased retention time reflects the more unfolded state of these species, as the results of increased hydrophobicity of the protein species.

**Assignment of the Disulfide Bonds with Mass Mapping**—To determine which disulfide bond was reduced in each des-species, the HPLC fractions were lyophilized and redissolved in 1 M NH₄OH, which induces base-catalyzed cleavage at the peptide bond that precedes the modified half-cystines (converting them to iminothiazolidine derivatives) (27). After complete reduction, the reaction mixtures were analyzed by MALDI-TOF MS (Fig. 3). Theoretically, the reaction should yield five peptide fragments per des-species, i.e. three peptide fragments originating from the double chain cleavage reaction and two fragments originating from a β-elimination (26, 40). The latter is a side reaction that occurs at either one of the two half-cystines, thereby preventing cleavage at this half-cystine.

Assignment of the disulfide bonds was performed in a two-step approach. Mass peaks that correspond with peptide fragments from the N and C terminus up to the reduced half-cystines were first assigned. In Fig. 3A, the mass peaks m/z 2286.6 and 6673.9 Da correspond to the peptide fragments encompassing the residues 1–20 and 27–86; Cys-27 is converted to an iminothiazolidine derivative in the latter fragment (Table II). This assignment could subsequently be confirmed by other mass peaks that originate from β-eliminations, i.e. m/z 2882.3 and 7271.3 Da (the peptide fragment 1–26 with a β-elimination at Cys-21 and 21–86 with a β-elimination at Cys-27, respectively). The remaining fragment (iminothiazolidine 21–26) was too small to be detected because of the settings of the lower mass detection limit (1000 Da). Together, these data establish the disulfide bond Cys-21-27. The relative mass deviations between measured and calculated mass were less than 0.05% for the majority of the peptide fragments. Comparable analyses of the other reaction mixtures resulted in the assignment of the other disulfide bridges, i.e. Cys-11-41, Cys-57-72, and Cys-35-80 (Fig. 3, B, C, and D, respectively). The intensity of the reoccurring mass peaks at 3805.4 and 5031.8 Da in fraction 3 (Fig. 3C) would suggest more overlap between fractions 3 and 4 than shown in the HPLC elution profile in Fig. 2B. However, these two mass peaks were consistently readily observed in multiple independent replicate experiments, which suggests that these two peptides are easily ionized by MALDI.

The intensity of the mass peaks, therefore, does not correspond to the actual concentration of the purified des-species. In conclusion, we established the connectivities of the disulfide bonds. Moreover, these are consistent with the PVX data (Tables I and III).

**Three Disulfide Bonds Are Required for Conformational Stability of AVR4**—Joosten et al. (14) showed previously that AVR4 isoforms encoded by natural avr4 alleles appeared not to accumulate in tomato leaves during a compatible interaction (genotype Cf-4), whereas the mutant avr4 genes showed no altered expression levels as compared with the wild-type gene. We compared the stability of the four des-species and native AVR4 in the presence of AF isolated from genotype Cf-4 infected by a non-AVR4 producing strain of C. fulvum. In the
absence of AF, native AVR4 and the four des-species were found to be stable over the time span of 1 h (Fig. 4). However, in the presence of low concentrations of AF, rapid degradation occurred for three of the four des-species, whereas native AVR4 and des-(Cys-21-27) were not affected by the incubation. Addition of a mixture of protease inhibitors effectively blocked the degradation of the three sensitive des-species, establishing that proteases are responsible for this degradation process. When the des-species were incubated with AF obtained from tomato Cf-0 infected by a C. fulvum race 5 (an AVR4 producer), similar results were obtained (data not shown). It can be concluded that the disulfide bonds Cys-11-41, Cys-35-80, and Cys-57-72 are important for conformational stability of mature AVR4 upon secretion in the tomato apoplast (independent from the tomato genotype). In the absence of any of these three disulfide bonds, AVR4 is likely not only to be trimmed to its elicitor (active mature form of 86 residues), but degradation appears to proceed until complete degradation of the protein.

AVR4 Contains a Single Invertebrate Chitin-binding Domain—The identified disulfide pattern of AVR4 was further exploited to perform a query (motif.genome.ad.jp). This search identified a homologous sequence stretch in genes of the invertebrates Manduca sexta, Brugia malayi, and Penaeus japonicus. These genes encode for chitinases. The homology is restricted to the C-terminal domain, which contains six conserved Cys residues. This C-terminal domain was recently identified as a chitin-binding domain designated the inv ChBD (23). For one family member, tachycitin of Japanese horseshoe crab (Tachypleus tridentatus) (41), the disulfide bond pattern has been solved. Unlike AVR4, tachycitin contains five disulfide bonds, of which three reflect the conserved disulfide bonds. Sequence alignment helped to appoint the conserved disulfide bridges, i.e. Cys-11-41, Cys-57-72, and Cys-35-80. The additional disulfide bond Cys-21-27 within AVR4 does not share homology with the disulfide bonds in tachycitin. In contrast to tachycitin and AVR4, the other inv ChBD family members do not contain additional disulfide bonds (Fig. 5). A three-dimensional model of AVR4 was constructed using the three-dimensional structure of tachycitin as a template structure (42) (data not shown). The modeled structure of AVR4 contains the secondary structure elements as found in tachycitin, but the sequence insertion encompassing the disulfide bridge Cys-21-27 was too large to construct a reliable model for this part of the protein.

**Fig. 2.** Purification and analyses of the AVR4 protein mixture after partial reduction and cyanlation. A, after reduction and cyanlation, the reaction mixture was separated by reverse-phase HPLC. B, the four predominant peaks were collected and subjected to an additional round of purification. C, MALDI-TOF MS of these fractions demonstrated that the four predominant peaks contained the four singly reduced and cyanlated AVR4 species (des-species), as concluded from the increased mass (+52 ± 1 Da). The reaction involved 100 μg of AVR4 that reacted with a 6-fold excess of TCEP. The relative abundance of native AVR4 and the four des-species was: 48% (retention time 27.2 min; native AVR4), 17% (28.4 min), 6.1% (29.9 min), 4.3% (30.8 min), 8.2%, (30.9 min).

**Fig. 3.** MALDI-TOF mass spectra of the four peptide mixtures obtained after base-induced cleavage of the peptide bond and full reduction of the des-species. Mass per charge (m/z) is given for those peaks that were used to assign the disulfide bonds in AVR4. From top-to-bottom in order of elution from HPLC: (A) Cys-21-27, (B) Cys-11-41, (C) Cys-57-72, and (D) Cys-35-80. Reoccurring mass peaks (indicated by #) reflect cross-contamination of some des-species. Mass peaks corresponding to double-charged mass peaks ([M+2H]²⁺) are marked *, and mass peaks corresponding to a β-elimination are marked †.
Following incubation, each of the different AVR4 des-species denoted singly reduced and cyanylated AVR4 species. \( \beta, \beta \)-elimination (40). Itz, 2-aminothiazolino-4-carboxyl group.

Table II

| Fragment | \( m/z \) | Fragment | \( m/z \) | Fragment | \( m/z \) | Fragment | \( m/z \) |
|----------|---------|----------|---------|----------|---------|----------|---------|
| des-(11-41) | 1144.3 | des-(21-27) | 2285.6 | des-(35-80) | 3805.4 | des-(57-72) | 6180.2 |
| des-(11-40) | 3415.9 | des-(21-26) | 1766.8 | des-(35-79) | 5031.8 | des-(57-71) | 1800.0 |
| des-(11-86) | 5075.9 | des-(27-86) | 6673.7 | des-(59-86) | 798.9 | des-(72-86) | 1875.9 |
| \( \beta \)-(1-40) | 4482.2 | \( \beta \)-(1-26) | 2884.4 | \( \beta \)-(1-79) | 8759.2 | \( \beta \)-(1-71) | 7882.2 |
| \( \beta \)-(11-86) | 8413.8 | \( \beta \)-(21-86) | 7272.4 | \( \beta \)-(35-86) | 5752.7 | \( \beta \)-(57-86) | 3397.9 |

Table III

Summary of the observed phenotypes per disrupted disulfide bond in AVR4

| Disrupted disulfide bond | Cys residue pair | Necrosis inducing activity | Stability in apoplastic fluid | Binding to chitin | Stability of the AVR4 chitin complex | Number of strains of Cladosporium fulvum |
|--------------------------|-----------------|---------------------------|-----------------------------|-------------------|-------------------------------------|--------------------------------------|
| Native | - | ++ + + | + | + | + | 0 |
| Cys-21–27 | 2-3 | ++ | + | + | + | 0 |
| Cys-57–72 | 6-7 | - | - | +/− | +/− | 0 |
| Cys-11–41 | 1-5 | + | + | + | + | 2 |
| Cys-35–80 | 4-8 | ++ | + | + | + | 6 |

\( \times \) Disruption of one disulfide bond by either a cysteine substitution in PVX:Avr4 or by partial reduction.

\( \mu \) Successive numbering of the Cys residues as in Fig. 5.

\( \beta \) Affinity of AVR4 des-species for chitin; +, binds to chitin; +/−, decreased affinity for chitin (Fig. 6).

\( \gamma \) Stability of AVR4 des-species when bound to chitin in the presence of AF; +, stable; +/−, partially stable in the presence of chitin (Fig. 7).

\( \delta \) Number of strains of Cladosporium fulvum identified so far with a single Cys substitution (12,14).

![Fig. 4. Stability of disulfide mutants of AVR4 in the presence of apoplastic fluid.](image)

Protein samples were incubated for 1 h at 30 °C in the absence (−AF) or presence (+AF) of apoplastic fluid from tomato cv. moneymaker-Cf4 using PVX:Avr4 (Fig. 1). As control, AF was supplemented with protease inhibitors (+ Prot. Inh.). Following incubation, the stability of the proteins was examined by SDS-PAGE. Similar results were obtained with AF obtained from Cf-0 inoculated with C. fulvum race 4 (an AVR4 producer). Sample loading order reflects the order of elution from the HPLC (Fig. 2).

**Disulfide Bond-disrupted Mutants of AVR4 Display Affinity for Chitin**—In a concurrent paper, it is demonstrated that native AVR4 binds specifically to chitin but not to other cell-wall polysaccharides. Moreover, for human chitinase, it has been shown that the six conserved Cys residues that belong to the inv ChBD need to be intact for chitin binding (43). To examine whether AVR4 des-species still exhibited chitin binding activity, we incubated the des-species with chitin (Fig. 6). Following incubation, each of the des-species was detected together with chitin. This suggests that absence of one disulfide bridge in AVR4 does not abolish chitin binding. However, for des-(Cys-57–72), binding was repeatedly less complete than for the other three des-species (as more protein remained in solution) (Fig. 6), but after prolonged incubation (from 1 to 4 h) all of the des-(Cys-57–72) protein was found in the pellet. This finding suggests a decreased affinity of this AVR4 isoform for chitin. It is noted that des-(Cys-57–72) was potentially contaminated with des-(Cys-35–80), which could have interfered with the chitin binding assay. Because des-(Cys-35–80) has a comparable chitin binding affinity as native AVR4, the des-(Cys-57–72) might even exhibit a lower affinity than observed in our assay.

**Binding to Chitin Extends the Lifetime of the Des-species in the Presence of Apoplastic Fluid**—During growth in tomato, C. fulvum remains confined to the intercellular spaces of tomato (44). It has been proposed that after release AVR4 associates directly to regions of the hyphal walls of C. fulvum where chitin is exposed (19). When chitin is saturated, excess of AVR4 is thought to be distributed throughout the apoplast. We investigated whether the various des-species were less sensitive to proteolytic degradation after binding to chitin. Therefore, we incubated the des-species with chitin for 4 h, rather than 1 h, to ensure complete association between these proteins and chitin. After incubation with chitin, the solutions were...
supplied with AF and stability of the AVR4 isoforms was followed in time. Over the period of 4 h, native AVR4 and des-(Cys-21-27) bound to chitin remained fully stable in the presence of AF (Fig. 7). Moreover, association with chitin resulted in an increased half-life time of des-(Cys-11-41), des-(Cys-35-80), and des-(Cys-57-72) in the presence of AF. Thus, when bound to chitin, normally unstable des-species are protected against proteases present in AF.

**DISCUSSION**

Here, we have elucidated the disulfide bridge pattern of AVR4 by a method known as partial reduction/mass mapping. The method allowed us to selectively disrupt a single disulfide bond in AVR4 and to purify these so-called des-species for further research. The following disulfide bonds were found in AVR4: Cys-11-41, Cys-21-27, Cys-35-80, and Cys-57-72. A query based on this disulfide bond pattern and the spacing between the Cys residues indicated that AVR4 contains the motif of the inv ChBD (23). Except for tachycitin, no disulfide bridge pattern has been solved for the other family members (29). For AVR4, we found that three disulfide bonds, excluding Cys-21-27, are essential for protein stability and the six Cys residues involved in these bonds are conserved among the inv ChBD family (23). For human chitinase it has been demonstrated that all these six Cys residues are essential for recognition of chitin (43) because AVR4 disruption of one of the conserved disulfide bonds does not result in the loss of the ability to bind to chitin. However, disruption of the disulfide bond Cys-57-72 appeared to reduce the affinity for chitin in the case of this particular des-species.

Strains of *C. fulvum* were found to evade Cf-4-mediated resistance by producing AVR4 mutant proteins. The majority of these modifications involved substitution of Cys residues at positions 35, 41, and 80 by Tyr (14), indicating that disruption of only two of the three conserved disulfide bonds has, so far, contributed to evasion of AVR4 recognition (Table III). AVR4 mutant proteins carrying a disruption in one of these conserved disulfide bonds could not be detected in AF from tomato leaves inoculated with *C. fulvum* race 4 carrying these alleles, which suggested degradation of these isoforms. It was previously reported (14, 45) that both AVR4 and AVR9 are processed by extracellular proteases as an integral part of their maturation to elicitors. We have now established that AVR4 isoforms are indeed rapidly degraded as a result of protease activity present in AF, as suggested by Joosten et al. (14). In wild-type AVR4, the presence of the conserved disulfide bonds prevents further degradation of mature AVR4 protein. Although Cf-4-mediated resistance is evaded by the production of protease-sensitive AVR4 isoforms, we noticed that the unstable AVR4 isoforms are still able to bind to chitin.

As mentioned before, disruption of the disulfide bond Cys-57-72 appeared to reduce the affinity for chitin. Supporting data for a role of Cys-57-72 in chitin binding comes from the three-dimensional-structure of tachycitin. Part of the three-dimensional structure of tachycitin can be superimposed on the structure of hevein, a plant chitin-binding lectin (42). This part of the tachycitin was, therefore, proposed to act as the chitin-binding domain. The shared structural motif encompasses the second β-sheet in tachycitin, a short helical turn, and the third disulfide bridge conserved in the ChBD motif (Cys-57-72 in AVR4). An NMR study of AVR4 recently showed that AVR4 and tachycitin seem to adopt a similar protein fold and that the residues important for the interaction with chitin could be superimposed on the structure of hevein. Hence, our findings predict that strains of *C. fulvum* producing isoforms of AVR4 lacking this specific disulfide bond Cys-57-72 will evade Cf-4-mediated resistance. However, strains of *C. fulvum* producing such a disulfide bond-disrupted mutant have not yet been identified. A possible explanation for the absence of such a strain could be that selection pressure exists associated with maintaining the chitin binding ability of AVR4. Modification of this disulfide bond Cys-57-72 could, therefore, come with a virulence penalty for *C. fulvum*.

Different from the three conserved disulfide bonds, our data indicate that the disulfide bond Cys-21-27 does not contribute to conformational stability of AVR4. Possibly, this disulfide bond is required for local conformational stability around the sequence insertion. However, preliminary NMR data indicate that the sequence insertion surrounding Cys-21 adopts an α-helix in AVR. Because the α-helix apparently increases the stability of this part of the structure, the disulfide bond Cys-21-27 itself would not be essential for the conformational stability of AVR4. Similar observations were previously reported for the cystine-knot fold (46).

**Fig. 6.** Chitin binding affinity of the disulfide bond-disrupted isoforms of AVR4 (des-species). The des-species were incubated together with insoluble chitin, and the chitin binding affinity was estimated by SDS-PAGE. Chitosan, to which native AVR4 does not bind, was included as negative control. Note that more des-(Cys-57-72) (*) remains in solution as compared with the other des-species. Although semi-quantitative, reduced binding of des-(Cys-57-72) was consistently found in five independent replicate experiments.

**Fig. 7.** Binding to chitin stabilizes the disulfide bond-disrupted isoforms of AVR4 (des-species). AVR4 and the des-species were trapped to chitin before incubation with apoplastic fluid. Upon addition of AF, the stability of the protein species was followed over the course of 4 h and analyzed by SDS-PAGE. As control, a parallel experiment was performed without AF.
When the NIA was tested of the AVR4 isoform lacking the disulfide bond Cys-21-27 in Cf-4 tomato using PVX, the NIA was found to be similar to the NIA of AVR4 isoforms lacking Cys-35-80. NIA of both mutant proteins, however, is less than that of native AVR4. However, in contrast to disulfide bond Cys-35-80, disulfide bond Cys-21-27 does not contribute to protein stability, suggesting that the mechanisms underlying the reduced NIA of both AVR4 des-species are different. When the disulfide bond Cys-35-80 is disrupted, a certain fraction of the resulting protein will be degraded by proteases present in the intercellular space, whereas the remaining fraction of protein triggers to some degree the Cf-4-mediated defense responses. The reduced NIA of AVR4 mutant carrying a disrupted disulfide bond Cys-21-27 cannot be explained by sensitivity to proteases but is most likely because of the amino acid substitution or because of a conformational change of the protein. The remaining NIA could be a reason why, despite the fact that disruption of disulfide bond Cys-21-27 does not affect chitin binding activity of AVR4, no natural C. fulvum strains have thus far been found that carry such a mutation.

In addition to the three Cys substitutions found in natural isolates, two other natural amino acid mutations have been found in AVR4 (T37I and Y38H). These modifications are thought to affect the conformational stability of AVR4, based on a report by Zhu and Braun (47). The latter two mutations affect the strand β2 of the first anti-parallel β-sheet, which is in the core of the protein structure (42). In AVR4, Thr-37 and Tyr-38 are putatively paired with Pro-30 and Ile-29 in strand β1, respectively. These four residues are well conserved in the inv ChBD.3 The report of Zhu and Braun indicates that Pro-Ile cross-strand contact pairs are allowed (47). Similarly, aromatic residues are exclusively found at position 38 (Tyr/PhexTrp>98%) of the inv ChBD. Hydrophobic residues are found to be the favorite cross-strand contact partners, whereas His residues are disfavored. Based on these statistical analyses, we propose that the mutations T37I and Y38H both will lead to a partially destabilized first anti-parallel β-sheet, which could decrease the overall conformational stability of AVR4 and increase the sensitivity to proteases.

Overall, the disulfide bond-disrupted AVR4 isoforms as they are produced by natural strains of C. fulvum show increased sensitivity toward proteases present in the apoplast as compared with native AVR4. However, after binding to chitin in the cell wall of C. fulvum, the mutant isoforms seem to escape degradation by proteases present in the apoplast. Thus, although Cf-4-mediated recognition is evaded, the chitin binding activity of natural AVR4 mutants of C. fulvum remains, thereby contributing to the protection of C. fulvum against plant chitinases.
Natural Disulfide Bond-disrupted Mutants of AVR4 of the Tomato Pathogen *Cladosporium fulvum* Are Sensitive to Proteolysis, Circumvent *Cf*-4-mediated Resistance, but Retain Their Chitin Binding Ability

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