The race-specific elicitor AVR9 of the tomato pathogen *Cladosporium fulvum*: a cystine knot protein

Sequence-specific ^{1}H NMR assignments, secondary structure and global fold of the protein

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Abstract The secondary structure and global fold of the AVR9 elicitor protein of *Cladosporium fulvum* has been determined by 2D NMR and distance-geometry protocols. The protein consists of three anti-parallel strands forming a rigid region of β-sheet. On the basis of the NMR-derived parameters and distance geometry calculations, it is evident that the AVR9 protein is structurally very homologuous to carboxy peptidase inhibitor (CPI) of which the X-ray structure is known. The AVR9 protein reveals the presence of a cystine knot, which consists of a ring formed by two disulfide bridges and the interconnecting backbone through which the third disulfide bridge penetrates. This structural motif is found in several small proteins such as proteinase inhibitors, ion channel blockers and growth factors. The implications of the structural relationship between AVR9 and other biologically active proteins are discussed.

Key words: Elicitor protein; NMR; Cystine knot; *Cladosporium fulvum*

1. Introduction

Plant pathogens produce various types of molecules which play crucial roles in the interaction with their host plants. They include pathogenicity factors such as enzymes degrading plant cuticle and plant cell walls, enzymes degrading antimicrobial plant compounds and low molecular weight toxins that kill plant cells [1]. Pathogens also secrete molecules, the so-called elicitors, that induce active defense responses in their hosts [2]. Elicitors are divided into non-specific and race-specific elicitors. Non-specific elicitors induce a defense response in plants irrespective whether these plants are resistant or susceptible to the pathogen from which the elicitor is obtained. Non-specific elicitors can be proteins or glycoproteins [3], oligosaccharins or oligochitins [4–6]. Race-specific elicitors are compounds that only induce defense responses in genotypes of host plants which are resistant to the pathogenic race that produces the elicitor, but not in susceptible genotypes. Three fungal race-specific elicitors have been isolated so far. NIP1 is a race-specific protein elicitor from the barley pathogen *Rhynchosporium secalis* [7]. It is a 82 amino acid protein that specifically induces the accumulation of PR *Hv-1*, a pathogenesis-related protein of barley. AVR4 and AVR9, are race-specific protein elicitors containing 106 and 28 amino acid residues, respectively, produced by strains of the fungal tomato pathogen *Cladosporium fulvum*, which induce a hypersensitive response (HR) in tomato genotypes carrying the complementary genes for resistance *Cf-4* and *Cf-9*, respectively [2,8]. The primary structure of these two race-specific elicitor proteins (AVR4 and AVR9) is known and their encoding avirulence genes (*Avr*4 and *Avr*9) have been cloned [9,10]. The resistance gene *Cf-9* from tomato, complimentary to AVR9, has been cloned [11].

Neither the secondary nor the tertiary structure of the gene products of fungal avirulence genes and corresponding plant resistance genes are known. Many of the cloned plant resistance genes show sequence homology, of which the most striking property is the occurrence of leucine-rich repeats in their gene products [11–19]. The only leucine-rich protein of which the crystal structure has been determined is porcine ribonuclease inhibitor [20–22]. About the physical interaction between fungal avirulence gene products (the race-specific elicitors) and resistance gene products nothing is known yet. The three characterized fungal race-specific elicitor proteins are small molecules of which the structure could be determined by ^{1}H NMR provided sufficient amounts of the proteins are available. NIP1 is a race-specific elicitor which is constitutively produced in vitro, while both AVR4 and AVR9 race-specific elicitor proteins are only produced by *C. fulvum* in planta. In addition, the *Avr*9 gene of *C. fulvum* can be induced in vitro under nitrogen-limiting growth conditions [23]. The gene encodes a pre-pro-protein of 63 amino acids, containing a signal protein of 23 amino acids, resulting in a protein of 40 amino acids which is processed by fungal and/or plant proteases into a mature protein of 28 amino acids. The mature 28 amino acid protein, found in infected plants, induces HR in *Cf-9* genotypes [24]. By replacing the promoter of the *Avr*9 gene by the constitutive glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter of the fungus *Aspergillus nidulans*, the AVR9 protein can be produced in significant amounts. *C. fulvum* transgenic for *Avr*9 secretes the AVR9 protein in vitro into the culture medium [24]. However, in vitro not the mature protein of 28 amino acids is produced, but rather a mixture of proteins containing 32, 33 or 34 amino acids. The

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biological activity of these proteins is indistinguishable from the mature 28 amino acid protein.

Here we report on the secondary structure and global fold of the in vitro produced 33 amino acid AVR9 race-specific elicitor as determined by ^{1}H NMR. The AVR9 protein has a structural motif consisting of a cystine knot and a small triple-stranded antiparallel β-sheet. These motifs are common in proteinase inhibitor proteins [25], ion channel blocker proteins [26] and various hormonal growth factors [27]. Structurally the AVR9 protein is most related to the carboxypeptidase inhibitor protein of potato.

2. Materials and methods

2.1. Production and purification of AVR9 elicitor

To produce sufficient quantities of the AVR9 elicitor in vitro the promoter of the *Avr9* gene has been replaced by the constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene of *Aspergillus nidulans* and the resulted construct was introduced into race 5 of *C. fulvum* by transformation [24]. The transgenic race of *C. fulvum* secretes a mixture of 32, 33 and 34 amino acid AVR9 proteins into the culture medium. The transgenic race was grown in B5 medium for 2 weeks and the proteins were isolated according to the methods desribed before [24]. Using this protocol 2 mg of the 33 amino acid AVR9 protein was purified. The purity of AVR9 was checked by analytical high-performance column chromatography as described before [24]. The molecular mass of the purified AVR9 protein was verified by electrospray mass spectrometry (TSQ-70 mass spectrometer with Finnigan Mat interface). The atomic mass of the protein was determined to be 3629.2 ± 1.5 Da. From the atomic mass it can be concluded that three disulfide bonds are present in the AVR9 protein. The presence of three disulfide bonds has also been confirmed by electrospray mass spectrometry measurements of the 28 amino acid AVR9 protein where an atomic mass of 3188.5 ± 1.4 Da was found.

2.2. NMR measurements

Protein concentrations used for NMR experiments were 1 mM in 90% H_2O/10% D_2O, pH 5. NMR spectra were recorded on a Bruker AMX 500 and a Bruker AMX 600 spectrometer. All 2D-NMR spectra were recorded in the pure phase absorption mode using time-proportional phase incrementation (TPPI) [28]. Nuclear Overhauser effect spetroscopy (NOESY) spectra were recorded at 298 K and at 280 K with mixing times of 175, 250 and 500 ms. Clean total correlation spectroscopy (TOCSY) spectra [29] were recorded with the MLEV spinlocking sequences in H_2O and D_2O at 298 K with spin lock times of 60 and 85 ms. The water signal was suppressed using low power presaturation on the water frequency. In most experiments the stimulated crosspeaks under bleached alphas (SCUBA) sequence [30] was incorporated using a SCUBA delay of 40 ms to recover saturated resonances under the solvent peak. All homonuclear 2D spectra were recorded with 512 t_1 increments with 4096 data points and a spectral width of 6042 Hz at 500 MHz. ^{1}H chemical shifts are referenced at the HDO resonance at 298 K and pH 5 at 4.77 ppm [31]. All ^{1}H NMR chemical shifts are collected in Table 1.

Distance restrains were collected from the NOESY spectra with 250 ms mixing time. The NOESY peaks were compared with those in the 175 and 500 ms mixitng time spectra in order to correct for spin-diffusion. The NOESY peaks were divided into three categories: weak (1.8–5.0 Å), medium (1.8–3.5 Å) and strong (1.8–2.7 Å). Slowly exchanging amides were determined from the NH resonances (both on the diagonal as from the fingerprint region crosspeaks) present in NOESY spectra recorded at 298 K in D_2O of a AVR9 sample which was previously fully protonated and lyophilized. The spectra were recorded within the first 12 h of exchange and several days after dissolving the AVR9 sample in D_2O. In this way intermediate and slowly exchanging protons could be determined. It is assumed that the slowly exchanging NH protons form strong hydrogen bonds within the structure thereby decreasing the exchange rate of these protons. All slowly exchange amide protons form part of a β-sheet as evidenced by the intra-residue, sequential and inter-strand NOEs.

NMR spectra were processed on a Bruker X32 work station using UXNMR software or on a Silicon Graphics Indigo^2 work station, using Felix 2.3. The free induction decays were zero-filled and multiplied by appropriately matched sine bell functions prior to Fourier transformation. Backbone $^3J_{HN-H\alpha}$ coupling constants were measured from DQF-COSY spectra in H_2O zero-filled to 1K × 8K data points (digital resolution in the F2-dimension is 0.8 Hz/point).

The J-couplings were combined with the NOE intensities between the β-methylene protons and the H_α or the NH protons to allow stereospecific assignments of the β and β′ protons. Using these stereospecific assignments dihedral angle constrains were deduced as either gauche$^+$, gauche$^-$ or trans.

Structure determination was performed on a Silicon Graphics Indigo^2 work station using the Biosym software package, which includes DGII [32] for distance geometry and Discover for energy minimization and molecular dynamics. A series of structures was constructed using DGII and simulated annealing using 254 distance restraints and 26 dihedral restraints (11 φ and 15 χ). The rather low concentration of protein prohibited the determination of a larger number of NOE derived distance restraints. A total of 20 structures were calculated using disulfide bonds between cysteine residues 7–21, 11–24 and 17–31. The average structure was energy-minimized using Discover.

3. Results

3.1. NMR assignments

The resonances of amide region are well dispersed, indicative of a well-defined structure. Moreover, the splitting of the HN resonance resulting from the $3J_{HN-H\alpha}$ coupling can be determined directly from the 1D ^{1}H NMR spectrum. The resonances shown could be assigned from DQF-COSY, NOESY and TOCSY spectra in H_2O and D_2O.

The protein contains 17 AMX spin systems, four glycine AX systems, two AM(PT)X glutamine spin systems, three leucines, two valines, two arginines and one lysine, threonine and alanine, respectively. All 17 AMX spin systems can be found in the TOCSY spectrum in H_2O. Of the four glycine residues the resonances of G1 and G3 could not be found in the TOCSY spectra in H_2O, indicating fast exchange of their amide protons. Of the other spin systems all amide protons could be found, except for the amide proton of L4. Remarkable is the weak contact between the H_4 protons of the two histidine residues and their Cβ-methylene protons. The presence of these two crosspeaks unambiguously establishes the spin system identification of the two histidines H27 and H33.

On exchange of H_2O for D_2O several amide protons do not, or hardly, exchange in a time frame of days. From comparison of the NOESY data sets in H_2O and in D_2O it became immediately clear that the residues, of which the amide protons do not exchange, are all located in a β-sheet with antiparallel strands as strong $H\alpha_i$–HN_{i+1} and Hα–Hα contacts were observed in the spectra.

Sequential assignments are obtained according to standard procedures [33] and were primarily based on the 2D ^{1}H-NOESY spectra in H_2O and D_2O. Most residues in the antiparallel β-sheets were readily identified by the strong sequential dαN crosspeaks in the NOESY spectra. The three strands of β-sheet are shown in Fig. 1. The three strong Hα–Hα contacts indicated in this figure are prominently present in the D_2O NOESY spectra. Some residues were more difficult to assign (not located in the β-sheet region) as they showed overlap for their amide proton resonance and having only a limited number of NOESY crosspeaks. However, by detailed comparison of spectra obtained at 280 and 297 K these ambiguities could be solved. No inter-residual NOE connectivities could be determined for the first four amino acid residues of the AVR9

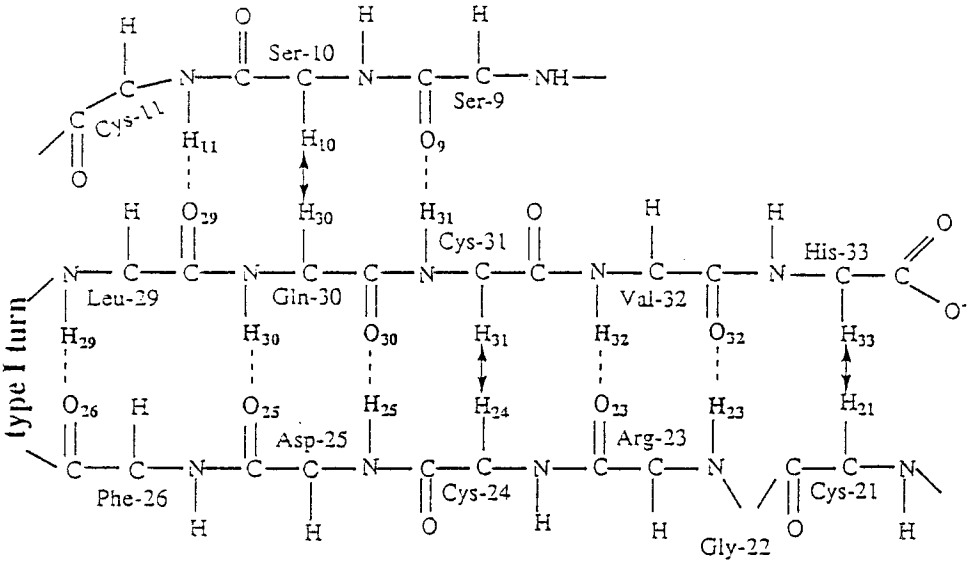

Fig. 1. β-sheet pleated structure, consisting of three anti-parallel strands, of the 33 amino acid AVR9 protein. Hydrogen bonds are indicated by dotted lines. Strong H_α–H_α NOE contacts are indicated by arrows. The loop of F26-L29 is a type-I turn.

protein, suggesting that these residues do not have a well-ordered character and are flexible.

The presence of strong sequential HN–HN contacts between Q30 and L29 suggests that the beginning of the third and last strand of the β-sheet is twisted at residue L29. The connectivity pattern of the loop from residue F26-L29 has all characteristics of a type-I turn. The strong Hα–Hα contact between H33 of the β-sheet and an AMX spin system shows that the second strand of the β-sheet has a β-bulge at residue G22. The hydrogen bond pattern is indicated in Fig. 1 and matches the slow exchange rates of the amide protons of C11, D25, L29, Q30, C31 and V32. These protons do not exchange within a time frame of days when the protein is dissolved in D_2O. The amide proton of R23 has an intermediate exchange; this proton is only present in NOESY spectra taken within 8 h after dissolving the protein in D_2O. R23 is the only residue of which the amide proton shows intermediate exchange. The amide protons of the residues S9, S10, C21, G22, C24, F26, H27, K28 and H33 do exchange fast with solvent which is consistent with the hydrogen bonding pattern as indicated in Fig. 1.

Table 1
^{1}H chemical shifts[a] of AVR9 at pH 5 and 298 K

Residue	HN	α	β	Others
Gly[1]	n.o.	n.o.		
Val[2]	8.52	4.25	2.16	γ 0.99, 0.99
Gly[3]	n.o.	n.o.		
Leu[4]	n.o.	4.52	1.37, 1.37	γ 1.15; δ 1.27, 0.76
Asp[5]	7.42	4.67	3.63, 2.19	
Tyr[6]	8.75	4.52	3.45, 3.17	δ 7.30; ε 6.87
Cys[7]	8.31	4.31	3.16, 3.06	
Asn[8]	8.89	4.56	3.01, 3.01	
Ser[9]	8.17	4.59	4.02, 3.99	
Ser[10]	8.57	4.96	4.01, 3.93	
Cys[11]	7.80	4.93	3.36, 3.03	
Thr[12]	8.64	4.50	4.39	γ 1.23
Arg[13]	7.93	4.73	1.67, 1.37	γ 1.67, 1.52; δ 3.27, 3.19; ε 7.31
Ala[14]	9.18	4.02	1.47	
Phe[15]	7.21	4.62	3.22, 3.09	δ 7.21; ε 7.37; ζ 7.27
Asp[16]	7.43	4.63	3.13, 2.97	
Cys[17]	8.47	4.77	2.97, 2.80	
Leu[18]	8.33	4.38	1.54, 1.54	γ 1.62; δ 0.93, 0.87
Gly[19]	8.46	4.02, 3.79		
Gln[20]	8.67	4.23	2.15, 2.15	γ 2.49, 2.49
Cys[21]	8.25	4.35	2.96, 2.72	
Gly[22]	7.48	3.59, 3.51		
Arg[23]	8.35	4.59	1.59, 1.59	γ 1.45, 1.27; δ 3.19, 3.12; ε 7.35
Cys[24]	8.79	4.69	2.51, 2.04	
Asp[25]	8.42	4.50	3.09, 2.62	
Phe[26]	8.32	4.25	3.29, 2.80	δ 7.16; ε 7.37; ζ 7.27
His[27]	8.63	4.67	3.45, 3.45	H_2 8.67; H_4 7.39
Lys[28]	7.81	4.25	1.89, 1.52	γ 1.42; δ 1.63; ε 2.99; ζ 7.61
Leu[29]	8.57	3.93	2.09, 1.97	γ 1.53; δ 1.02, 0.97
Gln[30]	7.12	5.19	1.79, 1.59	γ 2.28, 2.18
Cys[31]	8.58	5.27	3.31, 3.25	
Val[32]	9.63	4.77	2.44	γ 0.96, 0.83
His[33]	8.42	4.82	3.22, 3.22	H_2 8.68; H_4 7.42

[a]Proton chemical shifts are referenced to H_2O at 4.77 ppm.
n.o., not observed.

The residues D5-N8 and T12-Q20 do not show any regular secondary structure, although some HN–HN NOE contacts can be observed, possibly indicating some α-helical character. However, several long-range NOE contacts can be observed in the NOESY spectra, showing that these regions have a clear structural character. In contrast, the residues G1-L4 which have no long-range NOE-contacts are not well ordered.

The residues T12, R13, A14, F15 and D16 are strongly influenced by temperature and pH as the amide protons of these residues can shift up to 0.1 ppm depending on the conditions, indicating that these regions can undergo small structural fluctuations based on the environment.

4. Discussion

The structure of the 33 amino acid AVR9 protein consists of a compact sulfur core, three strands of antiparallel β-sheet formed by the residues S9-C11, R23-F26 and L29-H33, a very floppy region G1-L4 and a large extended loop region T12-Q20. It is evident from the electro spray mass spec. results that AVR9 contains three disulfide bonds. The structures of numerous small cysteine-rich proteins including serine proteinase inhibitors [25], ion channel blockers [26] and growth

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CgTx GVIA          C-KSZGSSCSZTSYNC-----CRSCNZYT---KRCY
Kalata B       INGLPVC----GETCV-GGT-C-NTPGC-TCSW-----PVCTR
CMTI-I             RVC-PRILMECKK-DSDCL-AE-C-VCLEH----GYCG
EETI-II             GC-PRILMRCKQ-DSDCL-AG-C-VCGPN----GFCG
CPI            EQHADPIC----NKPCKTHD-DCSGAWFCQACW---NSARTCGPYVG
AVR9           GVGLDYC----NSSCTRAF-DCLGQ--CGRCD---FHKLQCVH
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Fig. 2. Polypeptide sequences aligned on the basis of half-cystine positions and β-strand (underlined) hydrogen bonding patterns [25]. AVR9 is included in the alignment. Half-cystines are in bold.

factors [27] have been studied in recent years by ^{1}H NMR or X-ray crystallography. However, although ^{1}H NMR assignments can be obtained, defining the definitive 3D structure can still be quite tedious due to the large number of cysteine residues potentially forming disulfide bonds. These disulfide bonds provide great stability to these proteins. Structurally these proteins are very much related [25]. Fig. 2 shows the primary structure and β-strands of various cysteine-rich proteins studied by ^{1}H NMR, including ωCgTx GVIA [34], Kalata B1 [35], *Cucurbita maxima* trypsin inhibitor (CMTI-I; [36]), *Ecballium elaterium* trypsin inhibitor (EETI-II; [37]), carboxy peptidase inhibitor (CPI; [38]) and *C. fulvum* AVR9 (this article). On the basis of the secondary structure elements and the ^{1}H chemical shifts it became evident that the AVR9 protein has much in common with this class of proteins. All proteins show the same structural organization with three strands forming an antiparallel pleated β-sheet. Moreover, amide protons of residues located in the same position in the β-sheet show the same exchange behaviour. The strong resemblance in HN and Hα chemical shifts between AVR9 and CPI [38] is striking, the more so as the homology in amino acid sequence is very limited.

In the 3D structure of CPI, EETI-II and ωCgTx GVIA1 the disulfide bonds are between residues C1–C4, C2–C5 and C3–C6, where C1 is the first cysteine residue at the N-terminus. Moreover, the 3D X-ray structure of the enzyme–inhibitor complex of carboxypeptidase A and CPI is reported [39]. Also recently the X-ray structure of EETI-II has been determinded and shown to have identical disulfide bonds as inferred from the NMR study (Le Nguyen, [37] and pers. comm.). Based on these data we calculated the 3D structure of the AVR9 protein using disulfide bonds between C1–C4(=C7–C21), C2–C5 (=C11–C24) and C3–C6 (=C17–C31) residues. After DGII calculations, a set of 20 structures was obtained of which three were discarded as they showed the largest NOE violations. The RMSD over the backbone atoms of the remaining 17 structures was 1.4 Å.

A common property of the reported proteins and growth factors is the presence of the so-called cystine knot [25,40,41]. The superfamily of cystine knot-containing molecules has been divided in two classes: the inhibitor cystine knots [25] and growth factor cystine knots [40,41]. The proteins belonging to the inhibitor cystine knots show large structural similarity over motif residues forming the cystine knot. The amino acid residues making up this motif are located in a triple-stranded, anti-parallel β-sheet which consists of a minimum of 10 residues, XXC2, XC5X, XXC6X (where the numbers on the half-cystine residues refer to their positions in the disulfide pattern). The cystine knot consists of a ring formed by cystines C1–C4, C2–C5 and the intervening protein backbone, through which the third disulfide (C3–C6) passes. On alignment of an energy minimized structure of the AVR9 protein with the structurally conserved regions (underlined residues in Fig. 2) of ωCgTx GVIA, CMTI-I, EETI-II and CPI, the RMSD was found to be 0.93, 1.35, 1.01 and 0.82 Å, respectively.

The core of the β-sheets XXC2, XC5X, XXC6X, is not recognizable from sequence alone, but the spacing between the six cysteine residues is also important. The following spacing consensus of cysteine residues is found in inhibitor proteins: CX3–7CX4–6CX0–5CX1–4CX4–10C, including AVR9. The ring formed by connecting C1–C4 and C2–C5 consists of eight residues in kalata B, nine in CPI and AVR9, 11 in CMTI-I and EETI-II and 12 in ωCgTx GVIA, respectively

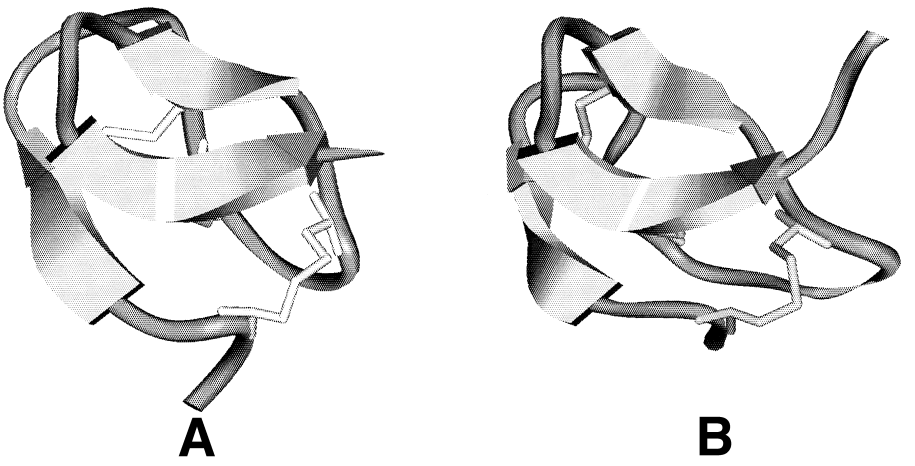

Fig. 3. 3D structures of AVR9 (A) as determined by DG-II and CPI (B) as determined by X-ray crystallography, with the three anti-parallel strands forming the β-pleated sheet. The cystine bridges are indicated.

[25]. The disulfides C2–C5 and C3–C6 superimpose very well in all inhibitor cystine knots including AVR9, and are thus most conserved. The C1–C4 bridge is only poorly superimposable in all proteins and seems to play a minor role in folding as has been determined for EETI-II [42].

Structurally AVR9 is most related to CPI. AVR9 and CPI can be aligned over a large sequence region from Y6-C11 and from C21-H33. The RMSD of alignment of the backbone atoms (19 residues representing 152 atoms) of the two proteins over this region is 1.22 Å (Fig. 3). In CPI the C-terminal residues YVG are involved in binding to carboxypeptidase. For AVR9 the residues involved in binding to a plant receptor are not known yet. Recent studies by Vogelsang et al. (unpublished results) showed that exchanging residues R13 or R23 in AVR9 by K increased biological activity in *Cf-9* tomato genotypes, exchanging residue L29 by S abolished biological activity, while exchanges of selected other amino acids had no or intermediate effects on biological activity. The loop residues PRILM of the trypsin inhibitors CMTI-I and EETI-II are involved in binding to trypsin. The basic R residue and the hydrophobic M residue are most crucial for binding. This PRILM loop is clearly not present in AVR9 nor CPI (Fig. 2). On the other hand the carboxypeptidase-binding loop of CPI (especially the C terminal residues YVG) is not present in AVR9. It would be interesting to construct a chimeric protein starting from the AVR9 backbone which has both trypsin and carboxypeptidase inhibiting activity by adding the PRILM and YVG residues. Such a bis-headed inhibitor has been created by Chiche et al. [43] by adding the amino acid residues YVG (present at the C-teminus of CPI) to the C-terminus of EETI-II. In this way they created a protein inhibitory to both trypsin and carboxypeptidase.

Whereas of most of the proteins discussed, the (intrinsic) biological activity is known, from the fungal AVR9 protein it is only known that it induces a fast HR in plant carrying the disease resistance gene *Cf-9*. The function of AVR9 for the fungus *C. fulvum* itself is yet unknown. Homologies between the promoter of the *Avr9* gene and the promoter of genes involved in nitrogen metabolism of *A. nidulans* and *Neurospora crassa* along with its induction under low nitrogen conditions [44,45] suggest that AVR9 might be involved in nitrogen metabolism [23]. For the induction of HR in Cf9 genotypes of tomato the C-terminal 28 amino acids of AVR9 are sufficient. Based on the structure presented in this paper, the five N-terminal amino acids of the 33 amino acid elicitor show no secondary structure and are therefore probably easily processed by plant and or fungal proteases. Complete reduction of the AVR9 protein abolishes HR inducing activity, indicating that secondary structure is required for biological activity. As AVR9 only induces HR in tomato plants carrying the *Cf-9* resistance gene it is tempting to speculate on the possibility that the *Cf-9* resistance gene product binds to AVR9. In tomato plants a binding site for AVR9 has been reported, but it is yet uncertain whether this binding site is the product of the *Cf-9* gene itself, one of his homologs, or a receptor that is closely associated with the product of the *Cf-9* gene [46].

Growth factors such as transforming growth factor-β2, nerve growth factor, and platelet derived growth factor-BB also belong to the growth factor cystine knots [40] They also contain three disulfides and antiparallel β-sheets, but the motifs are topologically different and cannot be superimposed. In growth factors the ring is formed by cysteine residues C2–C5 and C3–C6, through which the the disulfide bond C1–C4 penetrates. The ring varies between eight and 15 residues. The growth factors are able to form dimers either noncovalenty (nerve growth factor), by one intermolecular disulfide bond (transforming growth factor) or by two intermolecular disulfide bonds (platelet-derived growth factor). Dimerisation is required before binding to receptors can occur. Of the inhibitor cystine knot proteins described, it not is known whether they dimerize before binding to their receptor. In solution dimerization has not been observed, but this does not exclude that it could occur in vivo where redox conditions could favour disulfide formation [47]. If AVR9 would form dimers through disulfide bonds under certain conditions the C1–C4 disulfide bond (i.e. the C7–C21 disulfide bond) would probably be reduced most easily and would be available for intermolecular dimerization. This is the topic of our current research.

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