

PROTEIN STRUCTURE REPORT

Solution structure of the phytotoxic protein PcF: The first characterized member of the *Phytophthora* PcF toxin family

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Abstract: The PcF protein from *Phytophthora cactorum* is the first member of the “PcF toxin family” from the plant pathogens *Phytophthora* spp. It is able to induce withering in tomato and strawberry leaves. The lack of sequence similarity with other proteins hampers the identification of the molecular mechanisms responsible for its toxicity. Here, we show that the six cysteines form a disulphide pattern that is exclusive for PcF and essential for the protein withering activity. The NMR solution structure identifies a novel fold among protein effectors: a helix-loop-helix motif. The presence of a negatively charged surface suggests that it might act as a site of electrostatic interaction. Interestingly, a good fold match with Ole e 6, a plant protein with allergenic activity, highlighted the spatial superimposition of a stretch of identical residues. This finding suggests a possible biological activity based on molecular mimicry.

Keywords: NMR solution structure; backbone dynamics; PcF toxin family; oomycete plant pathogen; extracellular effector

Additional Supporting Information may be found in the online version of this article.

Abbreviations: HSQC, heteronuclear single-quantum coherence; NOE, nuclear Overhauser effect; PcF, *Phytophthora cactorum-Fragaria* protein; PDB, Protein Data Bank; RMSD, root mean square deviation; SCR, secreted cysteine-rich protein.

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Introduction

Phytophthora plant pathogens are fungus-like oomycetes causing destructive diseases in many cultivated crops, thus with relevant impact on staple food production. These pathogens manipulate the host plant metabolism using an array of secreted protein effectors.¹ As part of a long-term project, while studying secreted effector proteins from *P. cactorum*, we isolated a novel phytotoxic protein, that we named PcF (*Phytophthora cactorum-Fragaria* protein).² In its mature form, PcF is a 52-residues acidic protein

presenting three disulphide bonds.³ Purified PcF triggers withering symptoms on strawberry and tomato leaves, that is, the localized cell-death phenotype known as the “hypersensitive response.”^{4,5} The only known PcF homologues are predicted proteins: SCR74 and SCR91 encoded by polymorphic genes from *P. infestans*,^{6,7} and the products of 19 and 4 genes from *P. sojae* and *P. ramorum*, respectively.⁸ These candidate apoplastic effectors have been grouped into the “PcF toxin family” (Pfam PF09461); none of them has been characterized yet.

This work reports the NMR solution structure of the lead member of the PcF family.

Results

Assignment of disulphide bonds

Combining chemical derivatization with HPLC separation,⁹ the three disulphide bonds of PcF were unambiguously identified as Cys⁶-Cys⁴⁰, Cys¹¹-Cys⁴⁴, and Cys²⁶-Cys³⁹ (Supporting Information). Although the cysteines pattern of PcF is also found in the SCR74 and SCR91 putative proteins, the disulphide bonds pattern is an exclusive prerogative of PcF (Fig. 1 bottom). Integrity of the disulphide connectivities is essential for the withering activity (Supporting Information).

Solution structure

Using NOE-derived distance restraints, 20 models were selected on the basis of their lower energy, of systematic violations less than 0.5 Å for residual NOE and less than 5° for dihedral angles. PcF is an all- α -protein presenting a helix-loop-helix motif, Figure 1(A,C). The structural statistics indicate good stereochemical and nonbonded interaction properties, Table I. The two helices α_1 (Ala¹⁸-Asp²⁸) and α_2 (Asp³⁵-Gln⁴³) are connected by a six-residue loop, Gln²⁹-Asp³⁴, and their axes make an interhelical angle of $\sim 140^\circ$ [Fig. 1(A)]. The N- and C-terminus stretches Tyr⁴-Pro³ and Gly⁴⁵-Ala⁵², respectively, are unstructured and spatially disordered. Conversely, the region Leu⁴-Glu¹⁷, though lacking secondary structure elements, appears spatially well defined [Fig. 1(A) and Table I]. This feature is due to the Cys⁶-Cys⁴⁰ and Cys¹¹-Cys⁴⁴ bonds that anchor that region to the α_2 helix and to C-terminus, respectively. The Cys²⁶-Cys³⁹ bond, instead, connects the two helices. The RMSDs in the region Leu⁴-Cys⁴⁴ (Table I) indicate only a satisfactory structures convergence primarily because of a reduced definition of the two termini and of the interhelical loop. The protein presents a largely negative surface opposing a mainly neutral one that is crossed by a positively charged strip [Fig. 1(B)].

Backbone dynamics

The PcF rotational diffusion tensor indicates an average rotational correlation time τ_c^{eff} of 4.07 ± 0.18 ns,

and a ratio of the rotational diffusion coefficients $D_{\parallel}/D_{\perp}$ of 1.99, consistent with a monomeric cylindrical status. The helices have an average S^2 of 0.92, indicative of restricted backbone motion. The interhelical loop shows S^2 averaging at ~ 0.80 indicating a higher flexibility than the helices. Thus, the not ideal structural definition of this region [Fig. 1(A) and Table I] likely results from a combination of its flexibility and solvent exposure that leads to a reduced number of measurable NOEs. The Leu⁴-Glu¹⁷ region presents S^2 from 0.6 to 0.9, suggesting that it undergoes restricted coordinated fluctuations. R_2 values above average, for residues clustered before helix α_1 , and located in the loop- α_2 helix junction, suggest slow (μ s-ms timescale) conformational exchange. Finally, both N-terminus (Glu¹-Leu⁴) and C-terminus (Ser⁴⁶-Ala⁵²) exhibit a pronounced structural flexibility (low or even negative ^1H - ^{15}N -NOE and small R_2 values). A reduced spectral density mapping analysis confirms this description (Supporting Information).

Structural homology evaluation and K⁺ channel inhibitory test

The PcF fold has been used to query the whole PDB in search for functionally characterized structural homologues. Based on fold superposition, charge similarity, extracellular location, and cysteines relative abundance, a few small all- α -proteins were retained. They included three scorpion toxins acting as K⁺-channel inhibitors (OmTx1, OmTx3,¹⁰ and κ -hefutoxin¹¹) and Ole e 6 from *Olea europaea*, a pollen protein of unknown biochemical function in plant but acting as allergen in humans.¹² The proteins, with respect to PcF, display very similar orientation of the two α -helices, in all cases kept in place by multiple disulphide bonds, whereas their interhelical loop appears shorter and poorly superimposed (see Fig. 2). Fold alignments also revealed the spatial superposition of the stretch CEExC, at the end of helix α_2 (OmTxs), and KECxD, at the end of helix α_1 (Ole e 6).

As K⁺ channels have been recently characterized in plant cells and it has been shown that they are influenced by protein effectors,¹⁴ we tested the possibility that the basis of PcF activity might reside in its ability to inhibit plant K⁺-channels. The results indicated that up to 1 mM PcF does not affect any of the selected plant channels (the inward-rectifying KAT1, the weakly rectifying AKT2/3, and the outward-rectifying GORK). In addition, no other effects on oocyte viability were observed.

Discussion

There is a widespread uncertainty on the molecular mechanisms of plant-pathogen interactions. In fact, also for PcF, though its selectivity for tomato and strawberry strongly suggests a receptor-based mechanism of action, no gene-for-gene model has been described, yet. Nonetheless, the PcF negatively charged

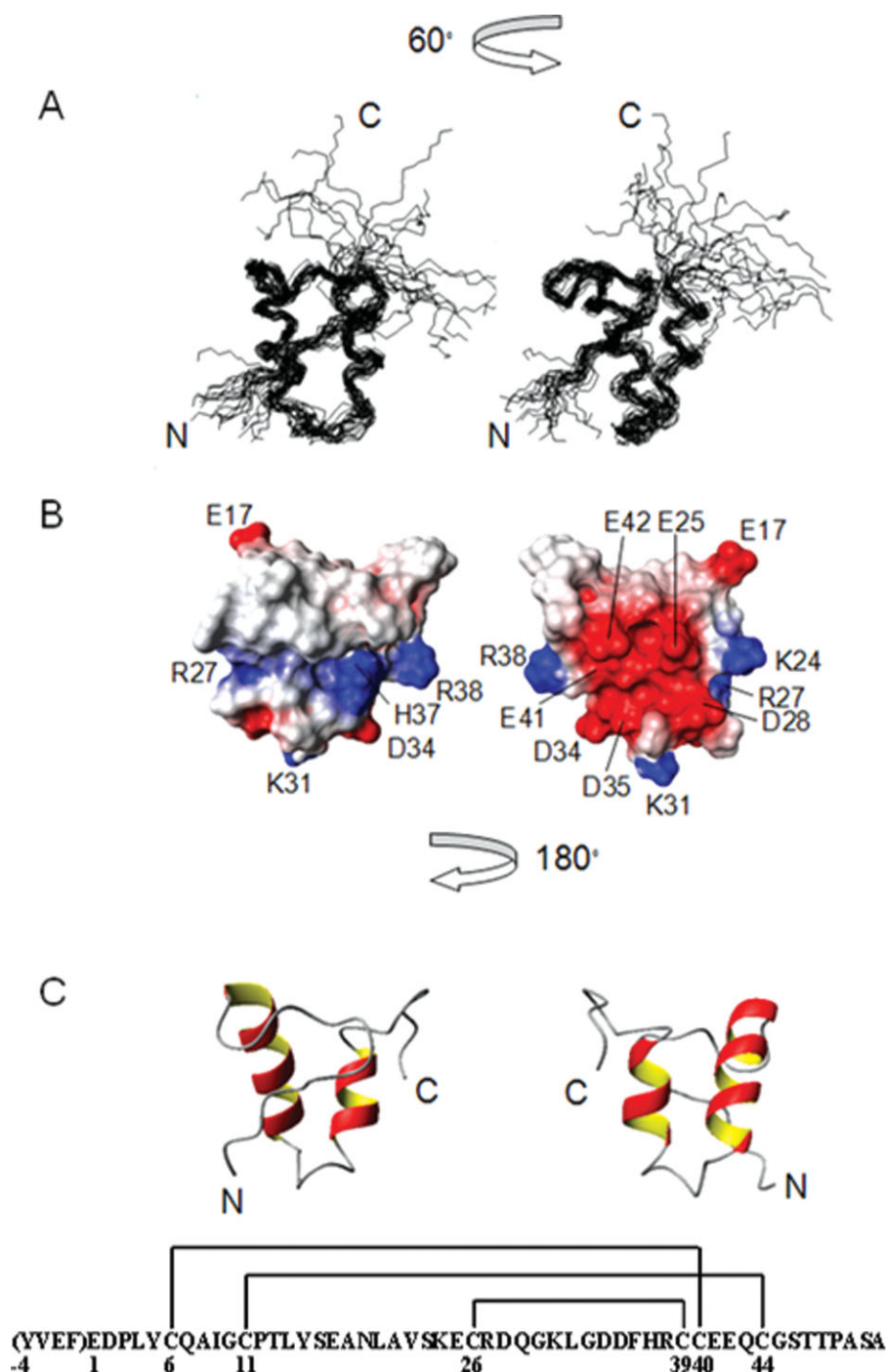


Figure 1. Solution structure of PcF. (A) Backbone superposition of the trace of the 20 lower energy NMR-derived models, selected from 100 calculated structures. (B) Color-coded electrostatic protein surface: (red) negative electrostatic potential; (blue) positive electrostatic potential; (white) neutral electrostatic potential. The right-hand view is rotated by 180° around the long axis of the molecule. (C) Ribbon representation of the protein in the same orientation used for the surface map. (Bottom): PcF disulphide bonds pattern.

face seems fit for the interaction with a positively charged ligand, an event that might contribute to receptor-mediated recognition and therefore could justify the observed host-plant selectivity.² Moreover, the protein tightly bound structure appears appropriate to tolerate the rather harsh plant apoplast where it is delivered during plant infection.

PcF's all- α organization is atypical among known fungal effectors, usually exhibiting a β -structured fold,^{15,16} and not even resembles the mainly α -structured elicitors from *Phytophthora* spp.¹⁷ However, fold alignment with Ole e 6 highlighted a possible structural determinant, that is, the KECxD homologous stretch that, noteworthy, turns out to be present

Table I. Structural Restraints and Statistics for the 20 NMR-Derived Structures Ensemble of PcF

Restraints for structure calculation		
NOE restraints	Total	481
	Intraresidue	253
	Sequential	143
	Medium range	47
	Long range	63
Dihedral angles	ϕ	35
	χ^1	9
Statistics for structure calculation		
RMSD from ideal geometry	Bond lengths (Å)	0.0025 ± 0.0005
	Bond angles (°)	1.02 ± 0.02
RMSD pairwise	Backbone (N, C α , C) (Å) (aa 4–44)	1.30 ± 0.22
	Heavy atoms (Å) (aa 4–44)	2.33 ± 0.31
	Backbone (N, C α , C) (Å) (aa 18–43)	1.28 ± 0.25
	Heavy atoms (Å) (aa 18–43)	2.43 ± 0.38
	Backbone (N, C α , C) (Å) (aa 4–17)	0.94 ± 0.22
	Heavy atoms (Å) (aa 4–17)	1.86 ± 0.36
	Backbone (N, C α , C) (Å) (aa 18–28)	0.55 ± 0.16
	Heavy atoms (Å) (aa 18–28)	1.56 ± 0.31
	Backbone (N, C α , C) (Å) (aa 35–43)	0.81 ± 0.33
	Heavy atoms (Å) (aa 35–43)	1.96 ± 0.45
PROCHECK-NMR parameters	Most favoured region (%)	69.8
	Additional allowed (%)	27.9
	Generously allowed (%)	2.3
	Disallowed (%)	0.0
	Number of bad contacts (%)	2.7

both in plants, as in the predicted protein NtP-CysR, the tobacco homologue of Ole e 6,¹² and in plant pathogens, as in the PcF toxin subfamily SCR91.⁶ Indeed, in the absence of other sequence homologies, this finding suggests that PcF might mimic the structural signature of a plant signaling protein. A similar structural mimicry in pathogenesis is novel for oomycete effectors but has been already described: the anthrax protein BclA¹⁸ and a number of effectors from both plant and human pathogens.^{19,20}

Materials and Methods

NMR studies and structure calculation

¹⁵N-labeled PcF was expressed by yeast culturing as reported in Ref. 3. ¹⁵N-PcF was dissolved at 0.7 mM in 10 mM sodium phosphate, pH 5.0, 10% D₂O. Data were recorded at 25°C, using a Varian Inova spectrometer operating at 600 MHz for the ¹H. Backbone and aliphatic side-chain signals were assigned by analysis of 2D ¹H-¹⁵N HSQC, 3D-TOCSY-HSQC, 3D-NOESY-HSQC, 2D-TOCSY, and 2D-NOESY spectra. A total of 35 ϕ backbone torsion angles were obtained using the ³J_{NH α} coupling constants, derived from analysis of a 3D HNHA spectrum.²¹ Stereospecific assignments of β -methylene resonances and χ^1 torsion angles were determined from the relative intensities of intraresidue H^N-H ^{β} NOEs in a 50 ms ¹⁵N-edited 3D NOESY-HSQC spectrum in conjunction with the relative size of ³J _{$\alpha\beta$} coupling constants estimated from a 30 ms ¹⁵N-edited

3D TOCSY-HSQC spectrum and the ³J_{NH β} coupling from 3D HNHB.²²

Structures were computed using the simulated annealing method.²³ The final energy minimization was performed with full consistent valence force field to a maximum derivative of 1 cal/Å. A total of 100 simulated annealing structures were calculated, and the 20 lower-energy structures were selected to represent the protein solution structure. The structures were analyzed with PROCHECK-NMR and MOLMOL.^{24,25} Atomic coordinates and chemical shifts have been deposited in the PDB under accession number 2BIC and in the BMRB (www.bmrb.wisc.edu) under accession number 6520, respectively.

¹⁵N relaxation measurements and analysis

T₁, T₂, and ¹H-¹⁵N-NOE values were acquired using pulse sequences adapted from standard schemes.²⁶ The T₁ and T₂ intensities were extracted using nonlinear spectral lineshape modeling and fitted to single exponential using routines within NMRPipe.²⁷ The ¹⁵N heteronuclear relaxation parameters were analyzed using the TENSOR2 program.²⁸

Structure comparisons

Cysteine and S-S pairing pattern similarities were evaluated by MOTIF Search (<http://motif.genome.jp>) and by CysView,²⁹ respectively. Fold comparison was carried out by secondary-structure matching algorithm (SSM),¹³ using the PcF structured core (residues Leu⁴-Cys⁴⁴) as the query.

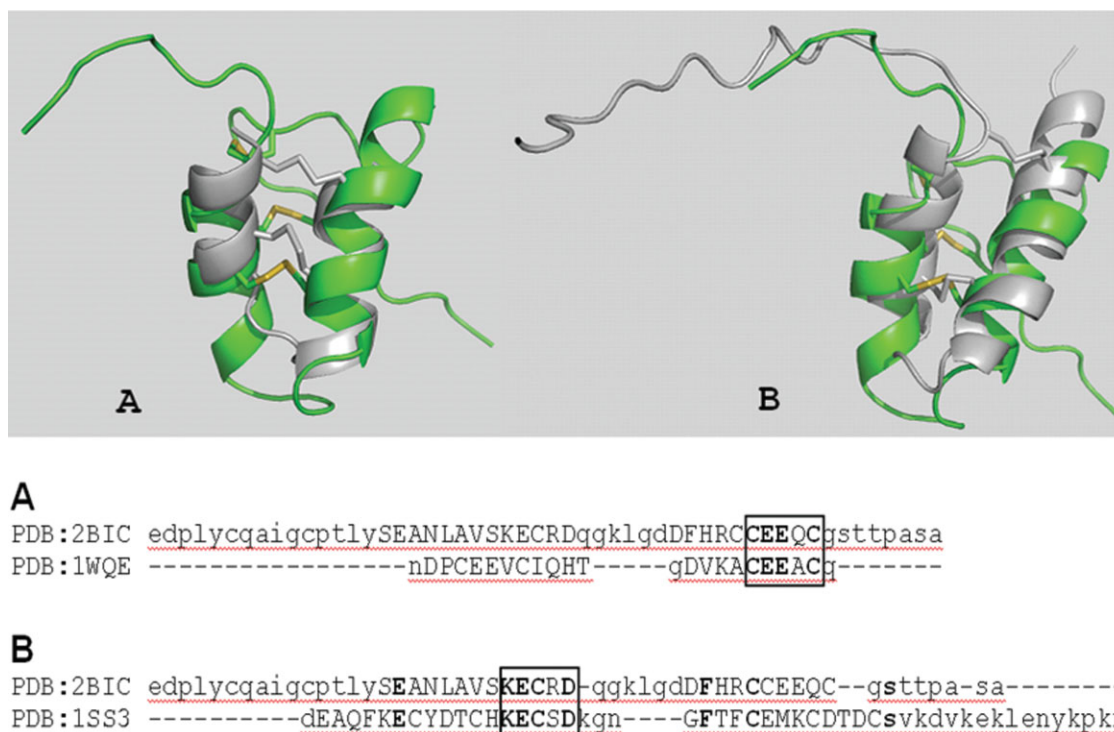


Figure 2. PcF structural alignments. (Top) Cartoon representation of the fold alignment of PcF (PDB 2BIC) with: panel A, a representative K⁺-channel blocker scorpion toxin (OmTx3, PDB 1WQE) and panel B, the pollen allergen Ole e 6 from *Olea europea* (PDB 1SS3). Molecular rendering was performed with PyMol program. All structures are in right-left orientation starting from N-terminus. PcF is highlighted in green, whereas the other proteins are in gray. Disulphide bonds are in yellow. (Bottom) Structure-based sequence alignment was performed using SSM.¹³ α -helical regions are in capital letters and identical residues are in bold. Boxed: the structurally superimposed conserved residues.

K⁺-channels inhibition assay

Assays were performed in *Xenopus laevis* oocytes expressing three Shaker-like K⁺ channels from *Arabidopsis thaliana*, that is, the inward-rectifying KAT1, the weakly rectifying AKT2/3, and the outward-rectifying GORK³⁰ For KAT1 and AKT2/3, experiments were carried out at pH 6.0 to mimic apoplastic conditions,³¹ whereas pH 7.4 was used for GORK to obtain measurable and stable currents.

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