Abstract

Effector proteins secreted by plant-pathogenic fungi play central roles in host colonization by modulating cellular processes and suppressing plant immunity. Despite their importance, the molecular mechanisms underlying the functions of most fungal effectors remain poorly understood, largely due to a lack of structural and biophysical information. This knowledge gap has limited our ability to rationally interpret effector evolution and to develop structure-guided strategies for disease control. EPC3 (Effector Protein for Cucurbit Infection 3), identified from *Colletotrichum orbiculare*, is one such virulence factor required for full pathogenicity during cucurbit anthracnose. Although EPC3 has been shown to contribute to infection in planta, the structural basis of its function and stability has remained unexplored.

In this study, I focused on the N-terminal half of EPC3, a region previously demonstrated to be essential for virulence-associated activity. I successfully established a recombinant expression and purification system for this domain and determined its three-dimensional solution structure by nuclear magnetic resonance (NMR) spectroscopy. This work provides the first experimentally determined structure of EPC3 and represents one of the few high-resolution NMR structures reported for small cysteine-rich fungal effector proteins.

The EPC3-ND (EPC3-N domain) adopts a compact β-rich fold stabilized by three intramolecular disulfide bonds. These covalent constraints generate a rigid structural scaffold composed of five β-strands arranged in a β-sandwich topology, a fold frequently observed among secreted fungal effectors. Backbone dynamics analyses based on ^{15}N relaxation measurements revealed a clear segregation of dynamic properties: the β-sheet core exhibits uniformly high rigidity on the ps-ns timescale, whereas several surface-exposed loop regions display pronounced flexibility, including motions on slower timescales. This structural organization suggests a functional division in which a stable core supports adaptable surface elements that may participate in molecular recognition.

Structural homology searches and direct comparisons with known effector structures, including members of the SIX (SIX; Secreted in xylem) family, revealed significant similarity at the fold level despite low amino acid sequence identity. Notably, conserved cysteine residues and disulfide connectivity patterns underpin this structural conservation, while sequence variability is concentrated in solvent-exposed loop regions. These observations indicate that EPC3 belongs to a conserved structural family of fungal effectors in which functional diversification is achieved primarily through variation in surface residues rather than changes in the overall fold.

To explore the potential involvement of EPC3-ND in carbohydrate recognition, I conducted ^{1}H-^{15}N HSQC-based titration experiments using representative soluble disaccharides (maltose, lactose, and D-(+)-cellobiose). No detectable chemical shift perturbations, peak broadening, or intensity changes were observed even at high sugar-to-protein molar ratios. Consistently, structural inspection revealed the absence of a canonical aromatic-rich carbohydrate-binding pocket. These results indicate that EPC3-ND does not directly bind these soluble disaccharides under the experimental conditions tested, suggesting that carbohydrate recognition is unlikely to represent its primary mode of molecular interaction.

Importantly, disruption of individual disulfide bonds by site-directed mutagenesis caused severe structural destabilization and loss of biological activity, demonstrating that Correct disulfide bond formation is essential for maintaining the native, functional conformation of EPC3-ND. Together, these findings highlight the critical role of disulfide-stabilized topology in preserving both the structural integrity and functional competence of this effector domain.

Overall, this work provides the first structural and biophysical characterization of EPC3, integrating high-resolution structure determination, backbone dynamics, and exploratory carbohydrate-binding analysis. These findings establish a foundation for future studies aimed at identifying the physiological host targets of EPC3 and elucidating its role in fungal infection and immune modulation.

Keywords: *Colletotrichum orbiculare*, effector, EPC3, NMR, functional domain