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NMR structure and conformational dynamics of AtPDFL2.1, a defensin-like peptide from *Arabidopsis thaliana*

Reza Omidvar [a,c,1], Youlin Xia [a,1], Fernando Porcelli [a,d], Holger Bohlmann [c], Gianluigi Veglia [a,b,*]

[a] Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN 55455, United States
[b] Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, United States
[c] Department of Crop Sciences, Division of Plant Protection, University of Natural Resources and Life Sciences, Vienna, Austria
[d] DIBAF — University of Tuscia — Largo dell'Universita', Blocco D, 01100, Viterbo, Italy

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ABSTRACT

Plant defensins constitute the innate immune response against pathogens such as fungi and bacteria. Typical plant defensins are small, basic peptides that possess a characteristic three-dimensional fold stabilized by three or four disulfide bridges. In addition to known defensin genes, the *Arabidopsis* genome comprises >300 defensin-like genes coding for small cysteine-rich peptides. One of such genes encodes for AtPDFL2.1, a putative antifungal peptide of 55 amino acids, with six cysteine residues in its primary sequence. To understand the functional role of AtPDFL2.1, we carried out antifungal activity assays and determined its high-resolution three-dimensional structure using multidimensional solution NMR spectroscopy. We found that AtPDFL2.1 displays a strong inhibitory effect against *Fusarium graminearum* ($IC_{50} \approx 4$ μM). This peptide folds in the canonical cysteine-stabilized αβ (CSαβ) motif, consisting of one α-helix and one triple-stranded antiparallel β-sheet stabilized by three disulfide bridges and a hydrophobic cluster of residues within its core where the α-helix packs tightly against the β-sheets. Nuclear spin relaxation measurements show that the structure of AtPDFL2.1 is essentially rigid, with the L3 loop located between β-strands 2 and 3 being more flexible and displaying conformational exchange. Interestingly, the dynamic features of loop L3 are conserved among defensins and are probably correlated to the antifungal and receptor binding activities.

1. Introduction

During evolution, many organisms have developed different mechanisms of immunity against pathogens. Antimicrobial peptides (AMPs), such as cathelicidins, defensins, piscidins, cecropins, and magainins represent an ancestral mechanism of immunity found in animals against prokaryotes [1–5]. Usually AMPs exert their activity by interacting with and disrupting bacterial membranes, but sometime AMPs can be internalized and interact with intracellular targets causing cell death [6–8]. Plants also produce a large variety of AMPs, including thionins, LTPs, and defensins, which display fungicidal, bactericidal, and insecticidal activity [9–13]. Defensins are a class of AMPs with a molecular weight in the range of 5 kDa, and are widely distributed in insects, fungi, animals, and plants. Plant defensins are ubiquitously distributed across all species and are small cationic peptides of 45–54 residues [6].

All defensins contain signal peptides and some of them mainly, but not exclusively, from *Solanaceae* are expressed as pre-pro-proteins containing an acidic domain similar to thionins [8,14]. For instance, the propeptide from NaD1 protects the plant against the toxic activity of the mature plant's defensins [15]. Many plant defensins have been isolated from seeds, leaves, tubers, and flowers [6]. While some plant defensins are constitutively expressed, others are produced only after infection by pathogens, supporting their important role in plant defense [6]. Despite their poor sequence identity, defensins share a common three-dimensional structure, comprising one α-helix and a triple-stranded antiparallel β-sheet, forming the canonical cysteine-stabilized αβ (CSαβ) motif [16–18]. In addition to this highly conserved CSαβ motif, defensins share two other conserved elements: a loop between the first β-strand and the α-helix (α-core), and a loop bridging the second and the third β-strand (γ-core). The γ-core is crucial for the antimicrobial activity [17] as deduced from the structural analysis of two defensins from *R. sativus* RsAFP1 and RsAFP2 [19,20]. To date, there are thirteen three-dimensional structures determined for several plant defensins, including γ-1-hordothionin and γ-1-purothionin [21], *Raphanus sativus* defensin RsAFP1 [20], *Pisum sativum* defensin Psd1 [22], two defensins from *Vigna radiata* VrD1/VrD2 [23], *Nicotiana alata* defensin NaD1 [24], *Medicago truncatula* defensin MtDef4 [25], *Petunia*

* Corresponding author at: 6-155 Jackson Hall, 321 Church St SE, Minneapolis, MN 55455, United States.
E-mail address: vegli001@umn.edu (G. Veglia).
[1] These authors have contributed equally to the work.

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hybrida defensin PhD1 [26], *Saccharum officinarum* defensin Sd5 [27], *Pachyrrhizus erosus* defensin SPE10 [28], *Lens culinaris* defensin Lcdef [29], *Aesculus hippocastanum* defensin AhAMP1 [30], and *Arabidopsis halleri* defensin AhPDF1.1b [31].

Arabidopsis thaliana has 13 *Pdf* genes divided into two groups [32]. Three genes of group 1 (*Pdf1.2a*, *Pdf1.2b*, and *Pdf1.2c*) are closely related and encode the same defensin peptide. The *Pdf1.2 gene* is generally regarded as a marker for pathogens through the ethylene and jasmonic acid pathways [33]. Other *Arabidopsis Pdf* genes are constitutively expressed in certain plant tissues [34,35]. Group 1 *Pdf* genes are induced in the non-host response of *Arabidopsis* to the barley powdery mildew fungus [36]. Overexpression of PDF1.1 resulted in enhanced resistance of *Arabidopsis* plants against *Cercospora beticola* [37]. In addition to plant defensin genes, *Arabidopsis* contains about 300 defensin-like (DEFL) genes [38]. Most of these genes were previously not annotated and were apparently lacking from the Affymetrix GeneChip. Similar genes were also identified in other plant species and many more cysteine-rich peptide genes are probably still undiscovered [39]. Since plant defensins do not induce harmful effects on humans or plant cells, they constitute a good starting point for developing new antimicrobial agents for plant protection [28].

Our group is studying a set of 9 *Arabidopsis* DEFL genes that were numbered CRP0240 by Silverstein et al. [38]. Among those, the *At1g35537* gene encodes for AtPDFL2.1, a 55 amino acid peptide that has antifungal activity comparable to that of plant defensins. To understand the structure-function relationship, we carried out antifungal assays and determined the high-resolution structure of AtPDFL2.1 using multidimensional solution NMR spectroscopy. We found that AtPDFL2.1 adopts the canonical cysteine-stabilized CSαβ fold comprising one α-helix and a triple-stranded antiparallel β-sheet, common to other plant defensins. NMR spin relaxation studies show that, apart from the N- and the C-termini, the γ-core pivotal for antimicrobial activity displays the highest degree of motion. Also, C14, which is involved in a disulfide bond with C36 shows conformational exchange in the fast-intermediate time scale. The flexibility of this region is a signature of all plant defensins and may be a requirement for eliciting their antimicrobial function.

2. Methods

2.1. Protein expression and purification

The sequence for mature AtPDFL2.1 (*At1g35537*) was cloned as a fusion with thioredoxin into a derivative of the pETtrx_1a vector and expressed in the SHuffle strain C3030 of *E. coli* [40]. For ^{15}N and ^{13}C isotopic labeling, bacterial cells were grown in M9 medium prepared with $^{15}NH_4Cl$ and ^{13}C-glucose (Cambridge Isotope Laboratories) as the sole nitrogen and carbon sources, respectively. The fusion protein was isolated by metal chelating chromatography and cleaved with TEV protease to release the AtPDFL2.1 as previously described for thionin proproteins [41]. AtPDFL2.1 was further purified by HPLC using a reverse phase C18 column (Vydac 218TP101510, 10 × 250 mm, 10–15 μM, 300 Å) and characterized by mass spectroscopy, which was conducted with a QSTAR XL Quadrupole TOF MS instrument from AB Sciex (Framingham, MA, USA). The peptide was diluted in a 1:1 ratio in a solution of 50% acetonitrile containing 0.1% formic acid and directly injected into the mass spectrometer at a rate of 5 μl/min. The data were deconvoluted using the Analyst Software package from AB Sciex. The peptide concentration was determined using a BCA assay kit (Pierce, Rockford, IL). The measured molecular weight of AtPDFL2.1 peptide at natural abundance was 6137.8 ± amu, which is within error of the expected mass for the peptide with three disulfide bonds (6138.2 amu). The U–^{13}C, ^{15}N labeled AtPDFL2.1 peptide gave a molecular mass of 6475.7 amu, consistent with 99% isotope labeling and the formation of three disulfide bonds.

2.2. In vitro antifungal assay

A 96-well microtiter plate assay was used to test the effect of recombinant AtPDFL2.1 on the growth of *Fusarium graminearum* [42]. Briefly, the concentration of fungal spores was adjusted to 2×10^4 spores per ml in 1/4 strength potato dextrose broth. 75 μl of the spore suspension was added to a sterile 96-well flat-bottomed microtiter plate (Greiner Bio-One) pre-loaded with 25 μl of different concentrations of filter-sterilized (0.22 μm syringe filter, Roth) AtPDFL2.1 or ddH_2O. The plates were incubated at room temperature in darkness and the OD_{600} was measured after 30 min and 24 h using the FLUOstar Omega plate reader (BMG LabTech). The IC_{50} value was determined as the concentration of the peptide required for 50% growth inhibition.

2.3. NMR sample preparation

NMR samples were prepared by dissolving the lyophilized peptide in an aqueous solution (95% H_2O, 5% D_2O) containing 40 mM potassium chloride, 20 mM potassium dihydrogen phosphate and 1 mM sodium azide at pH 6.5 to a final concentration of AtPDFL2.1 of ~0.2 mM.

2.4. NMR experiments

All NMR data for structure determination were recorded at 25 °C on a Bruker Avance III 850 MHz NMR spectrometer equipped with a 5 mm TCI CryoProbe. All of the NMR experiments performed are listed in the supplementary data (Table S1). The total acquisition time for all of these experiments was about 7.5 days. Data were processed using nmrPipe [43]. Proton chemical shifts were calibrated with respect to water signal relative to DSS ($(CH_3)_3Si(CH_2)_3SO_3Na$); ^{15}N and ^{13}C chemical shifts were indirectly referenced to DSS [44]. Linear prediction was applied for both ^{15}N and ^{13}C dimensions to double the data size and improve the digital resolution. Cosine square window function and automatic zero filling were applied to all ^{1}H, ^{15}N and ^{13}C dimensions. The processed data were analyzed with Sparky [45].

2.5. Resonance assignments

Backbone assignments were performed using AutoAssign using 3D HNCACB and CBCA(CO)NH experiments [46,47]. $^{13}C'$ chemical shifts were assigned using 3D HNCO and the first 2D ^{1}H–^{13}C plane of 3D HNCACO [48]. ^{1}H and ^{13}C side-chain assignments were performed with HCCH-TOCSY, H(CCCO)NH, and C(CCO)NH experiments with mixing times of 16 ms [49,50]. Distance restraints were extracted from ^{15}N- and ^{13}C-edited 3D NOESY spectra with 250 ms mixing times [51,52]. Scalar *J* coupling $^{3}J_{HNHA}$ were obtained from 3D HNHA spectrum [53]. The $^{1}H_\delta$ and $^{1}H_\varepsilon$ resonances of aromatic residues were assigned using 2D (HB)CB(CGCD)HD and (HB)CB(CGCDCE)HE [54]. The complete chemical shift assignment table was deposited in the BioMagResBank database (*www.bmrb.wisc.edu*: accession number 25468).

2.6. Structural restraints and calculation protocol

To determine the three-dimensional structure of AtPDFL2.1, we utilized internuclear distances from NOE spectra, dihedral angles estimated from the chemical shifts using TALOS [55], scalar $^{3}J_{HNHA}$ coupling constants from HNHA spectrum, and hydrogen bonds (Table 1) [55–57]. The intensities of NOE cross peaks of two 3D ^{15}N- and ^{13}C-edited NOESY spectra were binned into three categories: strong, medium and weak, corresponding to 1.8–3.7 Å, 1.8–5.0 Å and 1.8–6.0 Å distance restraints, respectively. A total of 1431 NOE-derived distances were derived from the NMR spectra and converted into unambiguous structural restraints. On average, we obtained 26 unique distance restraints per residue. The chemical shifts for the $^{13}C_\alpha$, $^{13}C_\beta$, ^{13}CO, and $^{1}H_\alpha$ resonances were used as an input for TALOS to predict the backbone