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Crystallization and preliminary X-ray crystallographic analysis of the plant defensin NaD1

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Plant defensins are small (~5 kDa) basic cysteine-rich proteins that are being explored in important agricultural crops for their ability to confer enhanced disease resistance against fungal pathogens. NaD1, isolated from the flowers of the ornamental tobacco (*Nicotiana alata*), is a particularly well characterized antifungal defensin. Here, the crystallization and preliminary X-ray crystallographic analysis of NaD1 is reported. Crystals of NaD1 were crystallized using the sitting-drop vapour-diffusion method at 291 K. Data were collected from two crystal forms to 1.4 and 1.6 Å resolution, respectively. The crystals of form *A* belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 32.697$, $b = 32.685$, $c = 41.977$ Å, $\alpha = 90$, $\beta = 100.828$, $\gamma = 90^\circ$, whereas crystals of form *B* belonged to the trigonal space group $P3_221$, with unit-cell parameters $a = b = 33.091$, $c = 128.77$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$.

1. Introduction

Plant defensins are a family of small proteins (~5 kDa, 45–54 amino acids) that are distributed ubiquitously throughout the plant kingdom, where they are likely to exist as multigene families (Mergaert *et al.*, 2003; Graham *et al.*, 2004; Lay & Anderson, 2005; Silverstein *et al.*, 2005, 2007; De-Paula *et al.*, 2008). They carry an overall positive net charge owing to a high number of basic amino acids and maintain eight invariant cysteine residues (the petunia defensins PhD1 and PhD2 contain an additional two cysteine residues) that form four family-defining intramolecular disulfide bonds (Lay, Brugliera *et al.*, 2003; Lay, Schirra *et al.*, 2003; Janssen *et al.*, 2003). Apart from the cysteines, there are only a very limited number of other residues that are typically conserved. They include a serine, two glycines, an aromatic residue and a glutamic acid (Broekaert *et al.*, 1995; Lay & Anderson, 2005).

Structurally, all plant defensins (as solved in solution by ^{1}H NMR spectroscopy, with the exception of one that was solved by X-ray crystallography) share a highly conserved three-dimensional fold which is centred on the cysteine-stabilized $\alpha\beta$ (CS$\alpha\beta$) motif (Lay, Brugliera *et al.*, 2003; Lay, Schirra *et al.*, 2003; Janssen *et al.*, 2003; Song *et al.*, 2011; de Paula *et al.*, 2011). This compact globular fold consists of a triple-stranded antiparallel β-sheet that is tethered to an α-helix by three disulfide bonds. A fourth disulfide bond further reinforces the protein by linking the N- and C-terminal regions. This produces a pseudocyclic protein that is resistant to both chemical and thermal denaturation (Lay, Schirra *et al.*, 2003; Lay & Anderson, 2005).

The conserved CS$\alpha\beta$ fold represents a highly versatile and stable scaffold for the presentation of amino acids of variable length and composition, primarily located in surface-exposed loops, that confers various functional specificities to plant defensins (Thomma *et al.*, 2002; Lay & Anderson, 2005; Carvalho & Gomes, 2009). These range from defence against microbial pathogens such as fungi (best characterized in this capacity) and bacteria to effects on insects. For the latter function, their varied ability to inhibit proteases, α-amylases, protein synthesis and ion channels may be a contributing mechanism (Lay, Brugliera *et al.*, 2003; Franco *et al.*, 2006; Pelegrini *et al.*, 2008; Stotz *et al.*, 2009). Other roles in development and tolerance to environmental stresses have also been implicated (Komori *et al.*, 1997;

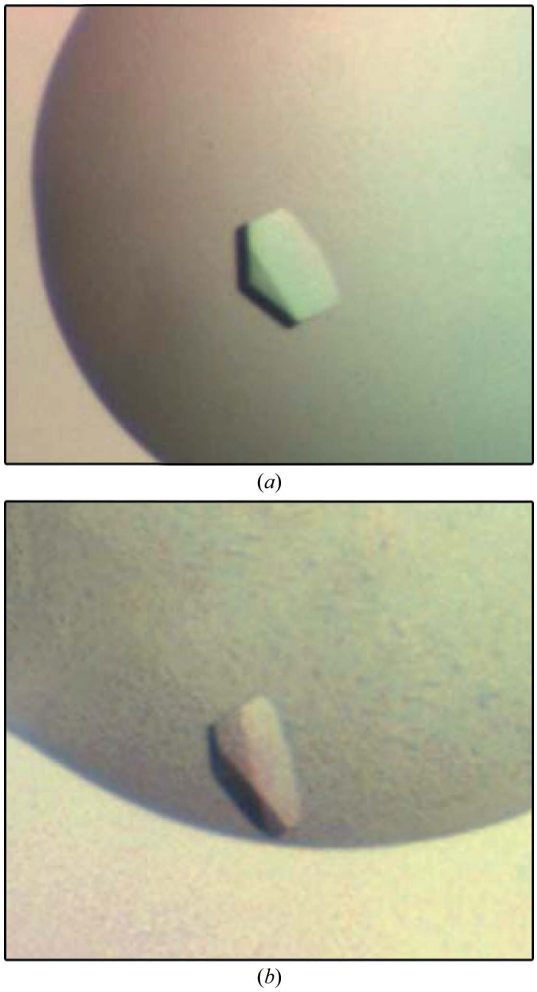
Yamada *et al.*, 1997; Koike *et al.*, 2002; Wilson *et al.*, 2005; Mirouze *et al.*, 2006; Stotz *et al.*, 2009; Spelbrink *et al.*, 2004). These activities have driven research into the potential application of defensins in the development of transgenic crops with augmented resistance to microbial pathogens and insects (reviewed in Lay & Anderson, 2005; Carvalho & Gomes, 2009).

NaD1 is a 47-amino-acid potent antifungal protein isolated from the flowers of the ornamental tobacco *Nicotiana alata* (Lay, Brugliera *et al.*, 2003; van der Weerden *et al.*, 2008, 2010). NaD1 has previously been reported to be monomeric under low-pH conditions and to lose its biological activity when reduced and alkylated (Lay, Schirra *et al.*, 2003; van der Weerden *et al.*, 2008). Here, we report the crystallization and preliminary X-ray crystallographic analysis of NaD1 in order to gain insight into the structural basis of its antifungal activity, which in turn may assist in the rational design of improved bioactive molecules.

2. Materials and methods

2.1. Purification of NaD1

NaD1 was purified from whole *N. alata* flowers up to the petal-colouration stage of flower development as described in Lay *et al.* (2003) and van der Weerden *et al.* (2008). Briefly, the flowers were ground into a fine powder and extracted in 50 m*M* sulfuric acid before neutralization with 10 *M* NaOH. Following centrifugation, the clarified supernatant was applied onto an SP Sepharose cation-exchange column (GE Healthcare Biosciences) pre-equilibrated with 100 m*M* potassium phosphate buffer pH 6.0. Bound proteins were eluted with 100 m*M* potassium phosphate buffer pH 6.0 containing 0.5 *M* NaCl. The eluted proteins were then further resolved by reverse-phase HPLC before lyophilization and reconstitution in sterile MilliQ water. The identity of NaD1 was confirmed by immunoblotting with anti-NaD1 and by mass spectrometry. The protein concentration was determined using the BCA protein assay (Pierce).



(*a*)

(*b*)

Figure 1
Crystals of NaD1 grown in (*a*) 25%(*w*/*v*) PEG 1500, 10%(*v*/*v*) succinate–phosphate–glycine buffer pH 9.1 or (*b*) 21%(*w*/*v*) PEG 1500, 10%(*v*/*v*) succinate–phosphate–glycine buffer pH 9.15.

Table 1
Data-collection statistics for NaD1 crystals of forms *A* and *B*.

Values in parentheses are for the highest resolution shell.

	Crystal form *A*	I3C derivative	Crystal form *B*
Space group	$P2_1$	$P2_1$	$P3_221$
Unit-cell parameters			
a (Å)	32.70	32.64	33.09
b (Å)	32.69	32.57	33.09
c (Å)	41.98	42.06	128.77
α (°)	90.00	90.00	90.00
β (°)	100.83	100.81	90.00
γ (°)	90.00	90.00	120.00
Wavelength (Å)	0.9537	1.5	0.9537
Resolution (Å)	41.23–1.40 (1.47–1.40)	41.32–1.58 (1.66–1.58)	64.39–1.64 (1.72–1.64)
R_{sym} or R_{merge}	0.089 (0.404)	0.079 (0.366)	0.081 (0.479)
$\langle I/\sigma(I)\rangle$	18.1 (4.6)	16.6 (5.0)	16.4 (3.1)
Completeness (%)	98.6 (90.5)	99.0 (93.3)	99.1 (93.7)
Multiplicity	6.9 (4.8)	6.7 (5.0)	9.4 (5.5)
No. of reflections	17174 (2279)		10727 (1419)
No. of observed reflections	118682 (10908)		100440 (7863)
Matthews volume (Å^3 Da^{-1})	2.08		1.93
Molecules in asymmetric unit	2		2

2.2. Crystallization

Crystallization trials were carried out with NaD1 (at a concentration of 15.7 mg ml^{-1}) at 291 K using the sitting-drop method by mixing 150 nl protein solution with an equal volume of mother liquor. The initial crystallization conditions were established using the PACT sparse-matrix protein crystallization screen (Qiagen) at the Bio21 Collaborative Crystallization Centre (Melbourne, Australia). After further optimization, crystals of good diffraction quality were obtained (forms *A* and *B*; Fig. 1).

2.3. Data collection and processing

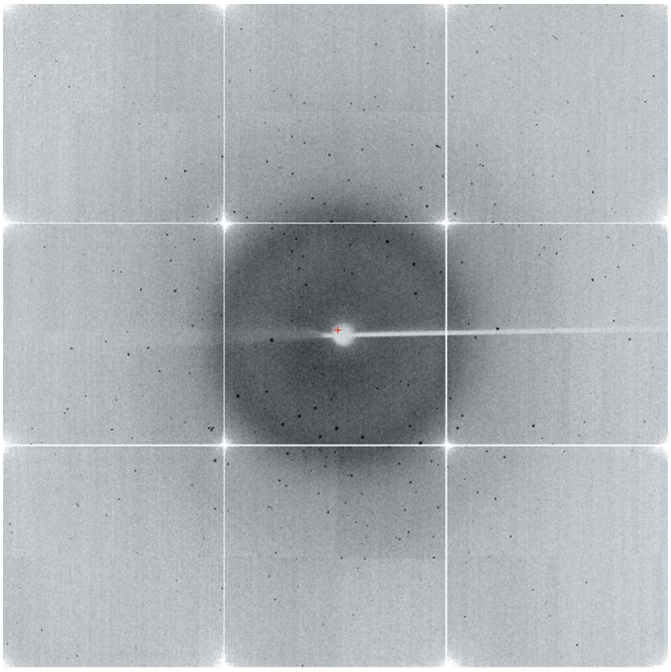
Native diffraction data were collected at 100 K from crystals flash-cooled in mother liquor supplemented with 10% ethylene glycol at a wavelength of 0.9573 Å on beamline 3ID1 at the Australian Synchrotron (Fig. 2) and were processed with *XDS* (Kabsch, 2010). For NaD1 crystal form *A*, a heavy-atom derivative was obtained by soaking crystals in mother liquor supplemented with 0.2 *M* 5-amino-2,4,6-triiodoisophthalic acid (I3C; Beck *et al.*, 2008) for 5 min. Derivative diffraction data were collected from I3C-soaked crystals at a wavelength of 1.5 Å and were processed using *XDS*.

3. Results and discussion

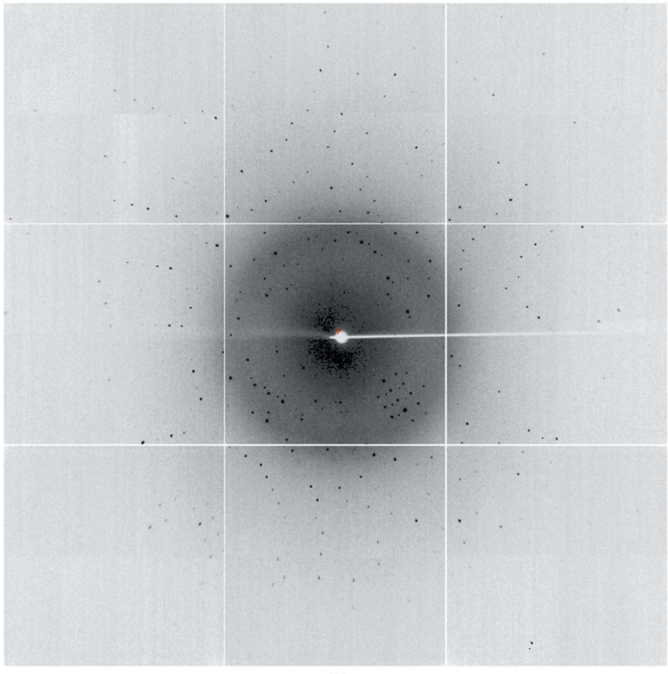
NaD1 is a member of the plant defensin family that shows promise as an antifungal protein for use in agricultural crops to confer enhanced disease resistance against fungal pathogens. In this study, we wanted to determine the crystal structure of NaD1 in order to gain some insights into its potential mechanism of action. Its purification from

N. alata flowers made use of its resilience to acid treatment and its overall positive charge. Consequently, we purified NaD1 from acidic flower extracts followed by cation-exchange chromatography and RP-HPLC.

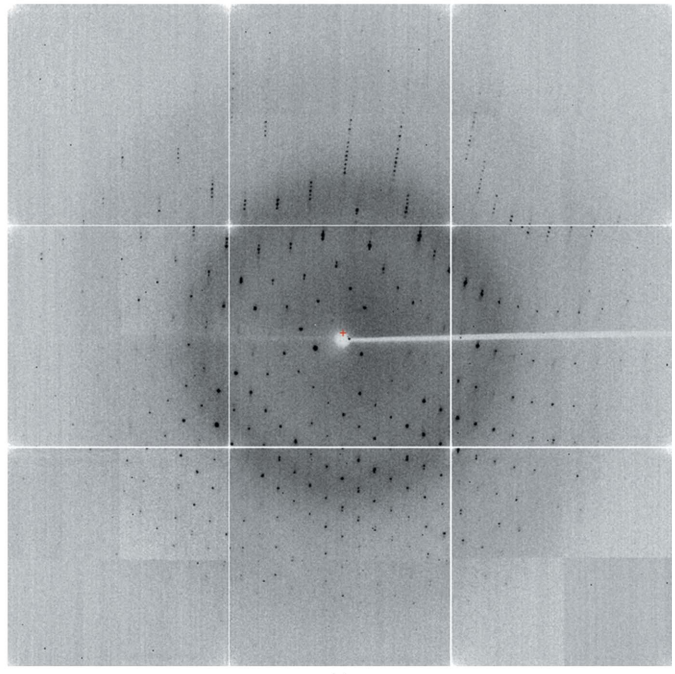
Protein crystallization was performed using the sitting-drop vapour-diffusion method at 291 K. Diffraction-quality crystals were obtained after optimization of an initial crystallization condition obtained from a sparse-matrix screen (PACT Screen, Qiagen) under two similar buffer conditions. Buffer condition *A* consisted of 25%(*w*/*v*) PEG 1500, 10%(*v*/*v*) succinate–phosphate–glycine buffer pH 9.1, while buffer condition *B* was 21%(*w*/*v*) PEG 1500, 10%(*v*/*v*) succinate–phosphate–glycine buffer pH 9.15. Native data were collected to 1.4 and 1.64 Å resolution for both crystal forms, and data from an I3C derivative of crystal form *A* were collected to 1.58 Å resolution. The crystals of form *A* (Fig. 1*a*) belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 32.697, b = 32.685, c = 41.977 Å, α = 90, β = 100.828, γ = 90°. Crystal form *B* (Fig. 1*b*) belonged to the trigonal space group $P3_221$, with unit-cell parameters $a = b$ = 33.091, c = 128.77 Å, $\alpha = \beta$ = 90, γ = 120°. Data-collection statistics for both crystal forms are shown in Table 1. Both crystals contained two molecules in the asymmetric unit, with calculated Matthews coefficients (V_M; Matthews, 1968) of 2.08 and 1.92 Å^3 Da^{-1}, respectively, and solvent contents of 41 and 36%, respectively. Experimental phasing, model fitting and refinement are in progress.



(*a*)



(*b*)



(*c*)

Figure 2
X-ray diffraction images from native form *A* (*a*), I3C-soaked form *A* (*b*) and form *B* (*c*) NaD1 crystals.

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