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Expression, purification and preliminary X-ray diffraction studies of VERNALIZATION1$_{208–341}$ from *Arabidopsis thaliana*

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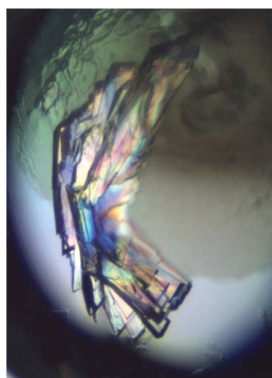
VERNALIZATION1 (VRN1) is required in the model plant *Arabidopsis thaliana* for the epigenetic suppression of the floral repressor *FLC* by prolonged cold treatment. Stable suppression of *FLC* accelerates flowering, a physiological process known as vernalization. VRN1 is a 341-residue DNA-binding protein that contains two plant-specific B3 domains (B3a and B3b), a putative nuclear localization sequence (NLS) and two putative PEST domains. VRN1$_{208–341}$ includes the second B3 domain and a region upstream that is highly conserved in the VRN1 orthologues of other dicotyledonous plants. VRN1$_{208–341}$ was crystallized by the hanging-drop method in 0.05 *M* sodium acetate pH 6.0 containing 1.0 *M* NaCl and 18%(*w*/*v*) PEG 3350. Preliminary X-ray diffraction data analysis revealed that the VRN1$_{208–341}$ crystal diffracted to 2.1 Å and belonged to space group *C*2, with unit-cell parameters a = 105.2, b = 47.9, c = 61.2 Å, α = 90.0, β = 115.4, γ = 90.0°. Assuming that two molecules occupy the asymmetric unit, a Matthews coefficient of 2.05 Å^3 Da^{-1} and a solvent content of 40.1% were calculated.

1. Introduction

Vernalization is a physiological process in which prolonged cold (namely winter) accelerates flowering. In *Arabidopsis*, vernalization is predominantly achieved by repressing the MADS-box floral repressor *FLOWERING LOCUS C* (*FLC*; Michaels & Amasino, 1999; Sheldon *et al.*, 1999). Using molecular-genetic approaches, *Arabidopsis* vernalization has been shown to require a number of proteins including VIN3, a plant homeodomain (PHD) finger protein whose expression is induced by prolonged cold (Sung & Amasino, 2004), VRN5, a VIN3-related protein that is constitutively expressed (Greb *et al.*, 2007; Sung, Schmitz *et al.*, 2006), VRN2, a homologue of the Polycomb protein Su(z)12, a well characterized chromatin regulator in *Drosophila* and humans (Gendall *et al.*, 2001), and VERNALIZATION1 (VRN1), a DNA-binding protein (Levy *et al.*, 2002). Maintenance of *FLC* silencing also requires the *Arabidopsis* homologue of HETEROCHROMATIN PROTEIN1 (Mylne *et al.*, 2006; Sung, He *et al.*, 2006). Recently, TAP-tagged VRN5 was found to be able to pull down a complex of proteins including the aforementioned VRN2 and VIN3 with Polycomb core proteins FIE (an ESC homologue), MSI1 (a p55 homologue) and a PHD-finger protein VEL1 (De Lucia *et al.*, 2008). Vernalization stably suppresses *FLC* *via* changes to the histones associated with the *FLC* locus, which result in the mitotic stability of vernalization (Bastow *et al.*, 2004; Sung & Amasino, 2004).

VRN1 is a plant-specific protein and so far appears to only have dicotyledonous homologues. VRN1 possesses two B3 DNA-binding domains, putative PEST protein-turnover domains and nuclear localization sequences (Levy *et al.*, 2002). Overexpression of VRN1 protein does not affect the requirements for vernalization but caused very deformed and early flowering plants, revealing that VRN1 needs other factors to suppress *FLC* and that VRN1 has additional roles in plant development (Levy *et al.*, 2002). *In vitro*, recombinant full-length VRN1 protein was shown to bind naked dsDNA in a non-sequence-specific manner (Levy *et al.*, 2002). A fully functional



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genomic VRN1-GFP fusion associated throughout the nucleus *in vivo* and remained associated with mitotic chromosomes (Mylne *et al.*, 2006). Immunolocalization showed VRN1 to be excluded from heterochromatic chromocentres. VRN1 protein dissociated from nuclei undergoing meiosis and this was supported by a dramatic drop in mRNA expression in pollen (Mylne *et al.*, 2006).

Although full-length VRN1 protein may be produced in *Escherichia coli*, it is unstable and even rapidly prepared protein extracts exhibit degradation. Following purification from the degraded forms, full-length VRN1 continues to degrade *in vitro*. We produced a stable 134-residue fragment $VRN1_{208-341}$ which includes the second B3 domain (B3b) and a region upstream that is both highly conserved in the VRN1 orthologues of other dicotyledonous plants and predicted to possess helical secondary structure. Here, we report the crystallization and preliminary X-ray analysis of *A. thaliana* $VRN1_{208-341}$.

2. Materials and methods

2.1. Expression and purification

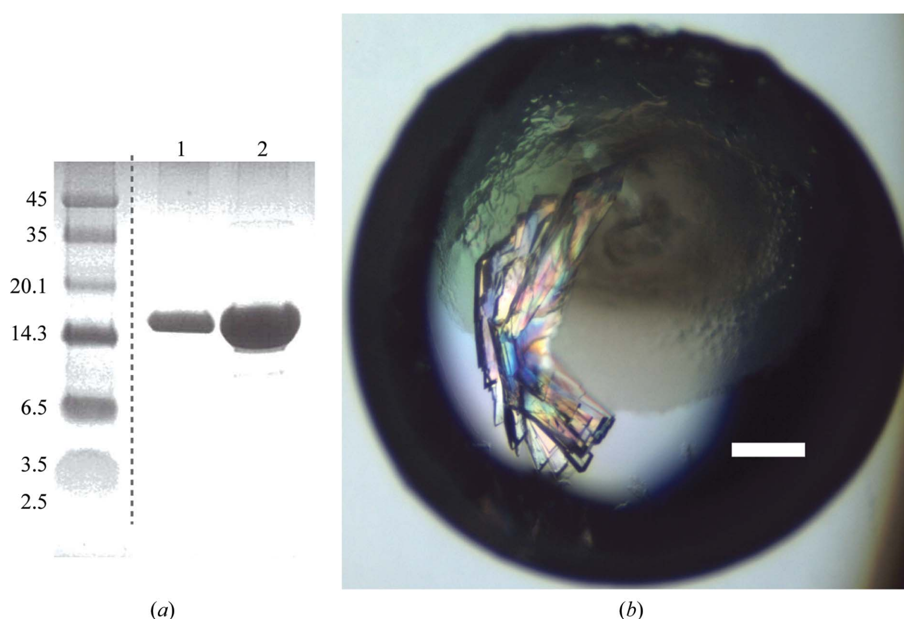
A plasmid pET19b-V1 (Levy *et al.*, 2002) containing a full-length *A. thaliana VRN1* (At3g18990) open reading frame was PCR amplified using primers JM91 (5′-GAA TTC GGT ACC **GGA TCC** CGT TCA AAG TTC TAC GAG AGT GCT-3′) and JM93 (5′-ACG CAC GCA CGC **GTC GAC** TCA GAC GTA CTC GTT GAC TCG AAA-3′). The product was digested with *Bam*HI and *Sal*I (sites shown in bold in the primer sequences above) and ligated into the *Bam*HI/*Sal*I sites of pQE30 (Qiagen). This construct introduces an N-terminal sequence MRGSHHHHHHGS to the 134 residues of VRN1 from Arg208 to Val341 (total deduced molecular weight of 16 988 Da).

The pQE30-$VRN1_{208-341}$ plasmid (JMDNA27) and pREP4 (Qiagen) were co-transformed into *E. coli* strain BL21 (Novagen) and selected on 100 $\mu g\ ml^{-1}$ ampicillin and 35 $\mu g\ ml^{-1}$ kanamycin Luria–Bertani (LB) agar plates. A single colony was picked and grown overnight at 310 K in 40 ml LB broth containing 100 $\mu g\ ml^{-1}$ ampicillin and 35 $\mu g\ ml^{-1}$ kanamycin. This culture was split to start two separate 500 ml LB cultures containing 100 $\mu g\ ml^{-1}$ ampicillin and 35 $\mu g\ ml^{-1}$ kanamycin. When the cultures had reached log-phase growth ($OD_{600\ nm}$ = 0.8), they were moved to 289 K and $VRN1_{208-341}$ protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside to 1 m*M* and growth overnight.

Cells were pelleted and resuspended in 50 m*M* Tris–HCl pH 8.0, 1 *M* sodium chloride, 30 m*M* imidazole, 10 m*M* benzamidine hydrochloride and lysed by five passes through a French press at 6.9 MPa. The lysate was cleared by centrifugation and loaded onto a PolyPrep column (BioRad) containing 3 ml chelating (Ni–NTA) Sepharose (GE Heathcare) pre-loaded with 10 ml 100 m*M* nickel sulfate. The column was washed with 50 ml 50 m*M* Tris–HCl pH 8.0, 1 *M* sodium chloride, 30 m*M* imidazole and the $VRN1_{208-341}$ protein was eluted from the column with 11 ml 50 m*M* Tris–HCl pH 8.0, 1 *M* sodium chloride, 250 m*M* imidazole. The protein was further purified by size-exclusion chromatography using a Sephacryl S100 column (GE Healthcare) in buffer containing 50 m*M* sodium phosphate pH 7.0, 150 m*M* NaCl and 1 m*M* DTT. Pure $VRN1_{208-341}$ was dialysed into 10 m*M* HEPES pH 7.0, 50 m*M* sodium chloride, 5 m*M* DTT and concentrated for crystallization by centrifugation in an Amicon Ultra-4 concentrator with a 5 kDa cutoff (Millipore). A Coomassie-stained gel of the purified $VRN1_{208-341}$ protein is shown in Fig. 1(*a*).

2.2. Crystallization

Crystallization-condition screening was performed at 293 and 277 K using the following eight commercial screening kits: Systematically Controlled Crystallization Screen Set 101 (Axygen Bioscience), Index Screen and SaltRx (Hampton Research), JCSG Plus and Pact Premier (Molecular Dimensions Ltd), Precipitant Synergy (Emerald Biosystems) and Wizard I and II (Emerald Biostructures). The hanging-drop vapour-diffusion method was used and drops were set up using a Mosquito (TTP Labtech) on ViewDrop II (TTP Labtech) seals in 96-well titre plates (TPP) containing 85 µl well solution. The hanging drop was comprised of 100 nl well solution and 100 nl protein solution (34 mg ml^{-1}, A_{280} = 34) in 10 m*M* HEPES pH 7.0 containing 50 m*M* sodium chloride and 5 m*M* DTT. Crystal-



(*a*) (*b*)

Figure 1
(*a*) Coomassie-stained gel with low molecular-weight markers (GE Healthcare; labelled in kDa) and $VRN1_{208-341}$ protein at two different concentrations: 1 µl (lane 1) and 5 µl (lane 2). (*b*) Cluster of $VRN1_{208-341}$ crystals obtained in 0.05 *M* sodium acetate pH 6.0 containing 1.0 *M* NaCl and 18%(*w*/*v*) PEG 3350 using the hanging-drop vapour-diffusion method. The scale bar corresponds to 0.2 mm.

lization experiments were stored and imaged using two RockImagers (Formulatrix), with one set at 277 K and the other at 293 K.

Optimization experiments were set up in 24-well VDXm plates (Hampton Research) that contained 400 µl well solution; the drop was comprised of 1 µl protein solution and 1 µl well solution on siliconized glass cover slides (Hampton Research).

Crystals were placed in cryo-solution [sodium acetate pH 4.5 containing 1.0 *M* NaCl, 20%(*w*/*v*) PEG 3350 and 20%(*v*/*v*) ethylene glycol] for less than a minute before being placed directly in the cryo-stream.

2.3. Data collection and refinement

Diffraction data were collected on a Rigaku FR-E copper rotating-anode generator operated at 45 kV and 45 mA with Osmic Confocal Vari-Max HF optics at a wavelength of 1.5418 Å. The crystal was kept at 100 K during data collection using a CRYO Industries of America CRYOCOOL-LN2 gas-stream cooler. 360 images were collected with $\Delta\varphi = 0.5^\circ$ and a crystal-to-detector distance of 60 mm. The detector was a Rigaku Saturn 944 CCD.

Data were processed using *CrystalClear* 1.4 (Rigaku Corporation).

3. Results and discussion

In the initial screening trials, six conditions were found to produce crystalline precipitate at the two temperatures (277 and 293 K): Systematically Controlled Crystallization Screen Set 101 conditions 79 and 88 at 277 K, Index Screen conditions 9 and 10 at 277 K and Pact Premier conditions 35 and 61 at 277 and 293 K, respectively. Five of these conditions were reproduced in an optimization round that again used the hanging-drop format.

Optimization experiments for Systematically Controlled Crystallization Screen Set 101 conditions 79 and 88 were designed in a shallow-gradient format and used to generate crystals for diffraction studies.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the last resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	$a = 105.2$, $b = 47.9$, $c = 61.2$, $\alpha = 90.0$, $\beta = 115.4$, $\gamma = 90.0$
Mosaicity (°)	0.78
Resolution range (Å)	31.07–2.10 (2.18–2.10)
Total No. of reflections	47904
Unique reflections	15246
Average redundancy	3.14 (2.08)
Completeness (%)	93.6 (80.3)
$R_{\rm merge}$†	0.080 (0.176)
$\langle I/\sigma(I)\rangle$	9.5 (3.7)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

$\rm VRN1_{208-341}$ crystals grown using conditions based on the commercial condition Systematically Controlled Crystallization Screen Set 101 condition 79 [0.05 *M* sodium acetate pH 5.0 containing 15%(*v*/*v*) PEG 3350 and 1.0 *M* NaCl] appeared as clusters (Fig. 1*b*) from which single crystals could be separated for X-ray diffraction studies. A crystal (0.2 × 0.05 × 0.05 mm) separated from a cluster grown at 277 K in 0.05 *M* sodium acetate pH 6.0 containing 1.0 *M* NaCl and 18%(*w*/*v*) PEG 3350 (based on condition 79) diffracted to 2.1 Å resolution (Table 1, Fig. 2). The crystal belonged to space group *C*2 with unit-cell parameters $a = 105.2$, $b = 47.9$, $c = 61.2$ Å, $\alpha = 90.0$, $\beta = 115.4$, $\gamma = 90.0^\circ$. Assuming the presence of two protein molecules per asymmetric unit, acceptable values were obtained for the Matthews coefficient (2.05 $\rm Å^3\,Da^{-1}$) and solvent content (40.1%; calculated using http://www.scripps.edu/cgi-bin/cdputnam/protcalc3; Matthews, 1968).

$\rm VRN1_{208-341}$ is thought to comprise a B3 domain; two structures have been determined of the B3 domains of RAV1 (Yamasaki *et al.*,

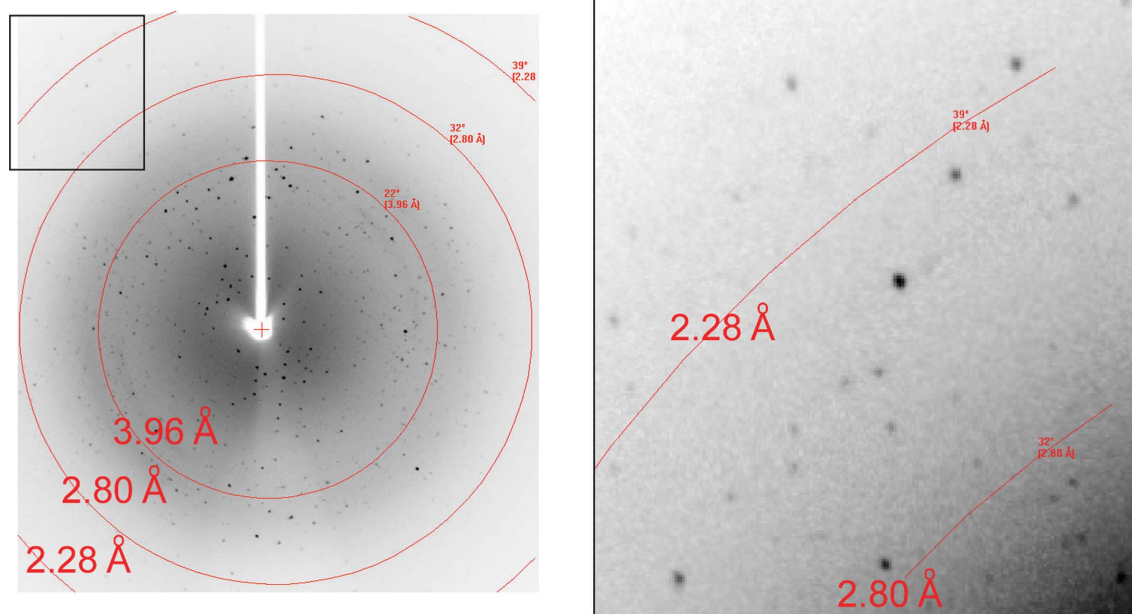


Figure 2
Diffraction image from native $\rm VRN1_{208-341}$ (Cu $K\alpha$, wavelength = 1.5418 Å). An enlargement of the boxed region is shown on the right. The crystal diffracted to 2.1 Å resolution.

2004) and At1g16640 (Waltner *et al.*, 2005). Despite only sharing 26% sequence identity, the tertiary structures of these two B3 domains are similar (backbone r.m.s.d. $\simeq$ 2 Å; Waltner *et al.*, 2005). Both structures were determined using NMR spectroscopy; this report therefore describes the first crystallization of a B3-domain protein.

The 99-amino-acid B3-domain region of $VRN1_{208–341}$ (*i.e.* VRN1 Phe243–Val341) has 25% identity to the B3 domain of RAV1 and 18% identity to At1g16640. Attempts to use these two NMR structures (RAV1, PDB code 1wid; At1g16640, PDB code 1yel) as models for a molecular-replacement solution of these $VRN1_{208–341}$ data using *Phaser* (McCoy, 2007) have so far failed. We are therefore planning to produce an SeMet derivative of this protein for structure determination by SAD or MAD phasing.

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