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Crystallization and preliminary crystallographic analysis of defective pollen wall (DPW) protein from *Oryza sativa*

Wei Wang,[a]‡ Yuanyuan Ma,[b]‡ Yang Suo,[c] Liming Yan,[b]* Dabing Zhang[c]* and Chen Miao[a]*

[a]State Key Laboratory of Cotton Biology, Key Laboratory of Plant Stress Biology, Henan University, Kaifeng 475004, People's Republic of China, [b]Laboratory of Structural Biology and MOE Laboratory of Protein Science, School of Medicine and Life Sciences, Tsinghua University, Beijing 100084, People's Republic of China, and [c]Institute of Plant Science, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China

‡ These authors contributed equally to this work.

Correspondence e-mail: yanlm@xtal.tsinghua.edu.cn, zhangdb@sjtu.edu.cn, miaochen928@henu.edu.cn

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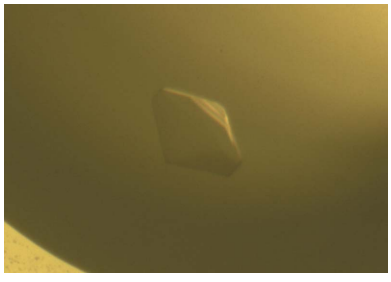

The *defective pollen wall* (*dpw*) gene of *Oryza sativa* encodes a fatty acid reductase (DPW) which plays important roles in primary fatty alcohol synthesis. DPW catalyzes the synthesis of 1-hexadecanol. The enzyme shows a higher specificity for palmitoyl-ACP than for palmitoyl-CoA as the substrate, and can only use NADPH as the cofactor. To gain an understanding of the molecular mechanism underlying the reaction catalyzed by DPW, the gene encoding DPW without the N-terminal 80 amino acids (DPWΔ80) was cloned into pET-28a vector and was overexpressed in *Escherichia coli*. DPWΔ80 was purified to homogeneity and screened for crystallization. DPWΔ80 in complex with NADPH produced crystals that diffracted X-rays to a resolution of 3.4 Å. The crystals belonged to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 222.8$, $c = 114.0$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$.

1. Introduction

Fatty alcohols and their derivatives play diverse roles in all biological systems. For example, fatty alcohols are the major components of surface lipid coatings such as the cuticle of plants, insect surface coatings and animal skin (Kunst & Samuels, 2003). In flowering plants, fatty alcohols make up the primary ingredient of the anther cuticle and pollen wall, where they play protective roles. The absence of fatty alcohols has been shown to affect the reproductive development and fertility of plants (Molina *et al.*, 2006; Chen *et al.*, 2011; Shi *et al.*, 2011). In higher plants, fatty alcohols are synthesized by fatty acyl-CoA/ACP reductases from either fatty acyl-coenzyme A (fatty acyl-CoA) or fatty acyl-acyl carrier protein (fatty acyl-ACP) (Rowland & Domergue, 2012).

In 2011, a *defective pollen wall* (*dpw*) gene was isolated and characterized from a rice (*Oryza sativa*) male-sterile mutant. Subsequently, *dpw* was shown to encode a fatty acyl reductase (FAR) that produces 1-hexadecanol. DPW is specifically expressed in the tapetal cells and microspores of rice anthers (Shi *et al.*, 2011). A striking difference between DPW and most other FARs is the ability of DPW to use fatty acyl-ACP as the substrate. DPW shows about a 270-fold higher specificity for palmitoyl-ACP than for palmitoyl-CoA as substrate, and can only use NADPH, and not NADH, as a cofactor (Shi *et al.*, 2011). Since the production of hexadecanol from C16 fatty acids may be a key step in their efficient export from the plastids (Spector & Soboroff, 1972), *dpw*-deficient mutants show a significant reduction in the content of C16 fatty alcohols. This deficiency results in structural defects of the anther and subsequent male sterility (Shi *et al.*, 2011).

Male sterility 2 (MS2) from *Arabidopsis* is an orthologue of DPW. This protein also functions as a fatty acyl-ACP reductase and is responsible for the production of fatty alcohols involved in sporopollenin biogenesis, but it can use either NAD(P)H or NADH as cofactor (Chen *et al.*, 2011). No structural information is available for either of these proteins and their mechanism of catalysis is unknown. A search of the Protein Data Bank (PDB) for homologous structures using the primary sequence of DPW retrieved no significant hits, suggesting that the structure of DPW might be different from those of known FARs. Therefore, a structural view of DPW bound with its substrates is central to understanding the catalytic mechanism and substrate specificity. In this work, we describe the expression,

purification, crystallization and preliminary crystallographic analysis of DPW.

2. Experimental procedures

2.1. Protein expression and purification

Cloning and protein expression of DPW from rice were performed following a general procedure published previously (Zhou *et al.*, 2013; Chen *et al.*, 2013; Sun *et al.*, 2012). Briefly, the *O. sativa dpw* gene with an N-terminal truncation to remove the first 80 residues was amplified from a rice cDNA library. The forward and reverse primers contained *Eco*RI and *Xho*I restriction sites, respectively. The purified PCR products were digested by restriction enzymes and cloned into the expression vector pET-28a with a 6×His tag at the N-terminus. The resulting clone was transformed into *Escherichia coli* Top 10 strain to produce the recombinant plasmid. After verifying the sequence of the cloned gene by DNA sequencing, the plasmid was transformed into *E. coli* BL21 (DE3) cells for overexpression. The transformed cells were cultured at 310 K in Luria–Bertani (LB) medium containing 100 mg l^{-1} kanamycin until the OD_{600} reached 0.5–0.8, after which the culture was induced with a final concentration of 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) at 293 K.

After overnight induction, the cells were harvested by centrifugation at 3300*g* for 10 min at 277 K and were suspended in buffer consisting of 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 4 m*M* $MgCl_2$ (buffer *A*). Cells were lysed using an ultra high pressure cell disrupter (JNBIO, Guangzhou, People's Republic of China) at 277 K and the cell debris was removed by centrifugation at 15 000*g* for 30 min at 277 K. The supernatant was loaded onto an Ni–NTA affinity chromatography column (GE Healthcare, USA) and after thorough washing with buffer *A* the bound protein was eluted using a linear gradient (20–250 m*M*) of imidazole. Fractions containing the protein were analyzed by SDS–PAGE and those showing a major band corresponding to the size of DPWΔ80 were pooled together (Fig. 1). Imidazole was removed by buffer exchange using centrifugal concentrators. At this stage DPWΔ80 was suspended in 20 m*M* Tris–HCl pH 8.0 (buffer *B*) and the protein was further purified *via* ion-exchange chromatography using a HiTrap Q HP ion-exchange column (GE Healthcare, USA) eluted with buffer *B* containing a linear gradient (0–1000 m*M*) of NaCl. Fractions containing DPWΔ80 were pooled and concentrated to 500 µl. DPWΔ80 was then loaded onto a Superdex 75 10/300 GL column (GE Healthcare) in buffer *A* and the protein eluted in a single peak. Fractions containing DPWΔ80 were pooled together, the buffer was changed to 20 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 1 m*M* DTT for storage, and the protein solution was concentrated to 20 mg ml^{-1} and stored at 193 K for crystallization.

Selenomethionyl (SeMet)-derivatized DPWΔ80 was expressed in the same strain of *E. coli* [BL21 (DE3)] applying feedback inhibition of methionine biosynthesis as described previously (Doublié, 1997). Transformed cells were incubated overnight in LB medium at 310 K and transferred into 1 l M9 medium (supplemented with 100 mg l^{-1}

Table 1
Data-collection and refinement statistics for NADPH-bound DPWΔ80.

Values in parentheses are for the highest resolution bin.

Source	BL-5, PF
Detector	ADSC Q315 CCD
Wavelength (Å)	1.000
Temperature (K)	100
No. of images	360
Oscillation range (°)	0.5
Space group	$P6_1$ or $P6_5$
Unit-cell parameters	
a (Å)	222.814
b (Å)	222.814
c (Å)	114.024
α (°)	90
β (°)	90
γ (°)	120
Resolution range (Å)	50–3.42 (3.48–3.42)
Unique reflections	48784 (2451)
Multplicity	11.1 (11.0)
Completeness (%)	97.66 (100)
Mean $I/\sigma(I)$	43.46 (8.27)
R_{merge}† (%)	13.6 (56.0)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean of the observations $I_i(hkl)$ of reflection *hkl*.

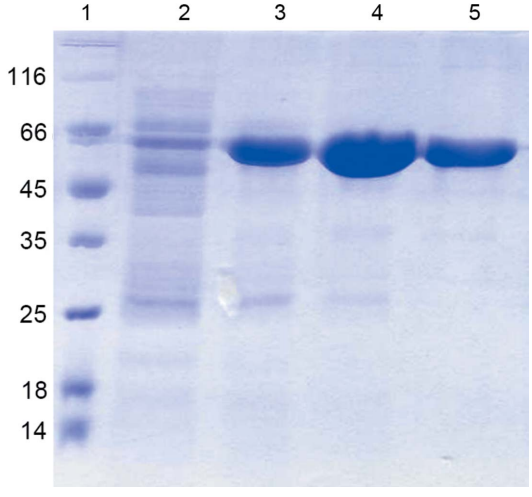

Figure 1
Purification profile of DPWΔ80 from *E. coli*. Lane 1, molecular-mass marker (labelled in kDa); lane 2, wash with 20 m*M* imidazole. Lanes 3, 4 and 5 correspond to fractions eluted with 50, 100 and 250 m*M* imidazole, respectively.

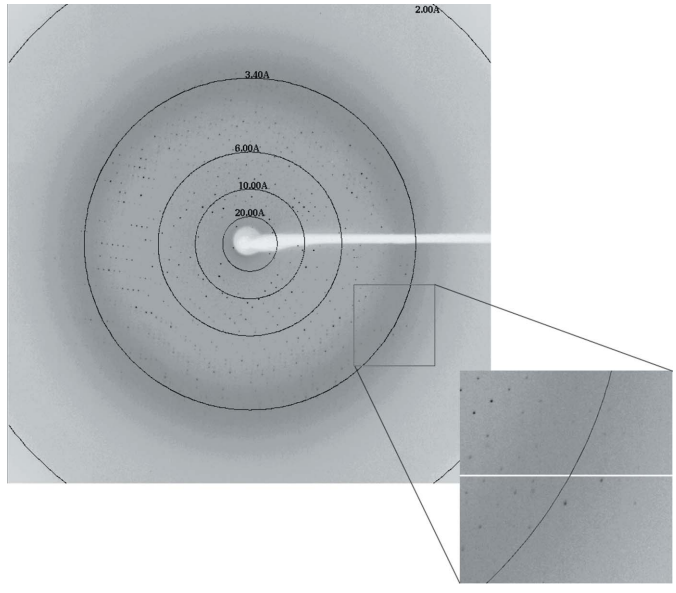

Figure 2
A typical diffraction pattern of the crystals of DPWΔ80 with NADPH. The diffraction image was collected on beamline BL-5 of the Photon Factory, Tsukuba, Japan using an ADSC Q315 CCD detector.

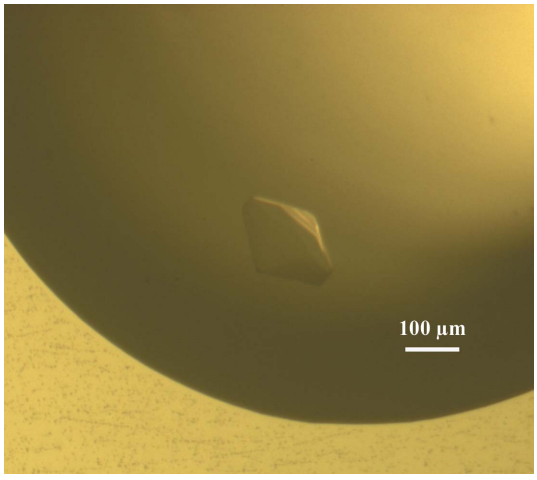

Figure 3
Single crystals of DPWΔ80 with NADPH.

kanamycin, 3% glucose, 100 mg each of Lys, Phe and Thr, and 50 mg each of Ile, Leu, Val and SeMet) at 310 K until the OD_{600} reached 0.5–0.8. After addition of the seven amino-acid supplements again and induction with 0.5 m*M* IPTG, the cells were grown at 293 K for a further 20 h. The SeMet-labelled protein was purified following the same procedure as outlined for the native protein.

2.2. Crystallization

Purified native DPWΔ80 was first incubated with NADPH (protein:NADPH ratio of 1:5) overnight at 277 K prior to crystallization. The preliminary crystallization conditions were screened by the hanging-drop vapour-diffusion method using commercially available sparse-matrix screens such as Index, Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2, SaltRx and SaltRx 2 from Hampton Research (California, USA) at 291 K. 2 µl crystallization drops containing protein solution and mother liquor mixed in a 1:1 ratio were equilibrated against 200 µl reservoir solution in 16-well plates.

Of the 384 conditions screened, crystals of the complex were only obtained in a condition consisting of 3.5 *M* sodium formate pH 7.0. However, these crystals were small and were in the form of thin plates. They diffracted poorly when tested for X-ray diffraction. In order to improve the crystal quality, optimization experiments were performed by adjusting the concentrations of precipitant, the crystallization temperature, the buffer pH and screening using the Additive and Detergents Screens from Hampton Research. Crystals suitable for data collection were obtained under the following conditions: 3.5 *M* sodium formate pH 7.0, 0.1 *M* magnesium chloride, 20%(*v*/*v*) 100 m*M* L-proline.

2.3. X-ray diffraction analysis

Initial testing of crystals for quality of X-ray diffraction suggested that the crystallization buffer was sufficient for cryoprotection. Therefore, loop-mounted crystals were directly flash-cooled in liquid nitrogen and transferred to a dry nitrogen stream (100 K) for X-ray data collection.

The data set for the DPWΔ80-NADPH crystal was collected to 3.4 Å resolution at 100 K on beamline BL-5A of the Photon Factory (PF), Japan using an ADSC Q315 CCD detector (Fig. 2). A total of 360 frames of data were collected with a 0.5° oscillation range. All intensity data were indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

The DPW protein is presumed to contain a 41-amino-acid signal peptide at the N-terminus. This signal peptide targets DPW to the chloroplast or mitochondria (Shi *et al.*, 2011). Initially, we cloned the protein without the signal peptide. However, this construct of DPW formed inclusion bodies when expressed in *E. coli*. We therefore designed N-terminal truncations. Removal of the first 80 amino acids produced soluble protein in *E. coli*. DPWΔ80 expressed in *E. coli* with a 6×His tag could be purified to homogeneity. The molecule has a molecular mass of 58.7 kDa. The protein formed crystals during screening (Fig. 3), and crystals with good diffraction quality grew to final dimensions of 200 × 200 × 100 µm within 4 d. Preliminary X-ray analysis of the crystals has been carried out. The crystals belonged to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 222.8$, $c = 114.0$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. We assumed the presence of six molecules per asymmetric unit of DPW, which gives a Matthews coefficient of 2.32 $Å^3\ Da^{-1}$ with 47% solvent content (Matthews, 1968). The final statistics of data collection and processing are summarized in Table 1. We obtained crystals of SeMet-substituted protein using the same crystallization conditions, but these crystals only diffracted to about 5 Å resolution. Optimization of the crystals of the labelled protein is currently under way.

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