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A family portrait: structural comparison of the Whirly proteins from *Arabidopsis thaliana* and *Solanum tuberosum*

DNA double-strand breaks are highly detrimental genomic lesions that routinely arise in genomes. To protect the integrity of their genetic information, all organisms have evolved specialized DNA-repair mechanisms. Whirly proteins modulate DNA repair in plant chloroplasts and mitochondria by binding single-stranded DNA in a non-sequence-specific manner. Although most of the results showing the involvement of the Whirly proteins in DNA repair have been obtained in *Arabidopsis thaliana*, only the crystal structures of the potato Whirly proteins WHY1 and WHY2 have been reported to date. The present report of the crystal structures of the three Whirly proteins from *A. thaliana* (WHY1, WHY2 and WHY3) reveals that these structurally similar proteins assemble into tetramers. Furthermore, structural alignment with a potato WHY2–DNA complex reveals that the residues in these proteins are properly oriented to bind single-stranded DNA in a non-sequence-specific manner.

1. Introduction

In plant mitochondria and plastids, DNA lesions such as DNA double-strand breaks (DSBs) can be repaired conservatively through homologous recombination (reviewed in Maréchal & Brisson, 2010). Under some conditions, however, the DNA-repair machinery is unable to cope with all DNA damage and non-conservative repairs occur (Abdelnoor *et al.*, 2003; Cappadocia *et al.*, 2010; Kwon *et al.*, 2010; Maréchal *et al.*, 2009; Shedge *et al.*, 2007). Whirly proteins are negative regulators of a non-conservative repair pathway named microhomology-mediated break-induced replication (MMBIR; Maréchal *et al.*, 2009; Cappadocia *et al.*, 2010). Whirly proteins are mainly found in the plant kingdom, where they localize to either chloroplasts (specialized mature plastids responsible for photosynthesis) or mitochondria (reviewed in Desveaux *et al.*, 2005). In solution, the recombinant proteins form tetramers (Desveaux *et al.*, 2002; Cappadocia *et al.*, 2010) that bind single-stranded DNA with low sequence specificity (Cappadocia *et al.*, 2010). In the absence of the chloroplast-directed Whirly proteins, the plastid genome of both *Arabidopsis thaliana* and *Zea mays* (maize) becomes unstable and accumulates DNA rearrangements that contain microhomologies at their endpoint junction (Maréchal *et al.*, 2009). Also, the treatment of *Arabidopsis* plants with ciprofloxacin, an inhibitor of DNA gyrases that induces DSBs in both plastids and mitochondria (Rowan *et al.*, 2010; Parent *et al.*, 2011), results in an increase in microhomology-mediated DNA rearrangements in the plastids of plants lacking WHY1 and WHY3 (Cappadocia *et al.*, 2010). WHY2, the Whirly protein directed to mitochondria in *Arabidopsis*, also appears to be involved in repressing the MMBIR pathway in the mitochondria, although to a lesser extent (Cappadocia *et al.*, 2010).

The crystal structures of the *Solanum tuberosum* (potato) WHY1 and WHY2 proteins were solved in the free form (Desveaux *et al.*, 2002; Cappadocia *et al.*, 2008, 2010). These structures revealed that both chloroplast-directed and mitochondria-directed Whirly proteins assemble into tetramers with *C*₄ symmetry. Each Whirly domain consists of two four-stranded β -sheets that stack at a 90° angle and two α -helices. The α -helices constitute the core of the proteins, against which the β -sheets stack in a whirligig-like manner. The

Table 1
Diffraction data statistics.

Data set	WHY1	WHY2	WHY3
Data collection			
Beamline	X29, NSLS	X29, NSLS	X29, NSLS
Wavelength (Å)	1.080	1.075	1.075
Space group	<i>C</i> 222 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 42 ₁ 2
Unit-cell parameters (Å)	<i>a</i> = 81.59, <i>b</i> = 180.69, <i>c</i> = 116.07	<i>a</i> = 63.47, <i>b</i> = 72.66, <i>c</i> = 136.83	<i>a</i> = <i>b</i> = 80.60, <i>c</i> = 63.62
Resolution (Å)	50–1.88 (1.95–1.88)	50–1.75 (1.81–1.75)	20–1.85 (1.92–1.85)
No. of unique reflections	69721	64884	18616
Multiplicity	7.1 (6.5)	7.0 (5.3)	13.4 (10.2)
Completeness (%)	99.8 (99.2)	99.4 (99.3)	100.0 (99.7)
<i>R</i> _{merge}	0.048 (0.308)	0.066 (0.746)	0.093 (0.926)
<i>I</i> /σ(<i>I</i>)	19.5 (5.9)	12.8 (1.8)	8.8 (2.1)
Refinement statistics			
Resolution (Å)	50–1.88	50–1.75	50–1.85
Reflections (total/test)	69689/2000	64805/2000	18588/1858
<i>R</i> _{work} / <i>R</i> _{free} (%)	17.6/21.5	18.4/22.0	18.9/22.6
No. of atoms (excluding H atoms)			
Protein	5374	4714	1323
Ligands	73	78	10
Water	661	496	98
<i>B</i> factors (Å ²)			
Protein	31.8	37.4	38.5
Ligands	50.4	56.4	44.4
Water	36.1	40.2	44.2
R.m.s.d.s			
Bond lengths (Å)	0.012	0.012	0.012
Bond angles (°)	1.363	1.350	1.355
Ramachandran plot			
Residues in favoured regions (%)	96.9	97.6	97.0
Residues in allowed regions (%)	3.0	2.1	3.0
Outliers (%)	0.1	0.3	0

crystal structures of WHY2 in complex with different DNA molecules revealed how this protein binds ssDNA with low sequence specificity (Cappadocia *et al.*, 2010). Specifically, the DNA is sandwiched in between the β -sheets of adjacent subunits, thereby preventing spurious contacts between the nucleobases and the protein surface. This type of binding is coherent with the DNA-repair role assigned to the Whirly proteins.

To date, no structure of an *Arabidopsis* Whirly protein has been elucidated. This is unfortunate, as a large proportion of the results concerning Whirly proteins were obtained using *Arabidopsis* as a model organism. In this manuscript, we report the crystallization and crystal structure determination of the *Arabidopsis* proteins WHY1, WHY2 and WHY3.

2. Material and methods

Unless specified otherwise, the Whirly proteins mentioned hereafter refer to *Arabidopsis*.

2.1. Cloning and expression of WHY1, WHY2 and WHY3

The DNA fragments encoding the Whirly domains of WHY1, WHY2 and WHY3 (UniProt accession Nos. Q9M9S3, Q8VYF7 and Q66GR6-2, respectively) were amplified by PCR and cloned into the pET-21a vector (Novagen). WHY1_{74–241} and WHY3_{78–245} were cloned in between the *Nde*I and *Not*I restriction sites, thus adding a methionine at the N-termini of the proteins and an AAALHHH-HHHH sequence at their C-termini. WHY2_{45–212} was cloned in between the *Nde*I and *Xho*I restriction sites, thus adding a methionine at the N-terminus of the protein and an LEHHHHHH sequence at its C-terminus. The sequences were confirmed by DNA sequencing.

The expression plasmids were transformed into *Escherichia coli* strain BL21(DE3). The cells were grown at 310 K in Luria–Bertani broth. When the cells reached an OD₆₀₀ of 0.6–1.0, isopropyl

β -D-1-thiogalactopyranoside was added to a final concentration of 1 mM. After cell growth overnight at 303 K, the cells were harvested and lysed by alumina grinding. The lysate was resuspended in 20 mM sodium phosphate pH 7.5, 500 mM NaCl, 25 mM imidazole. The recombinant proteins were purified by applying the supernatant from the cell lysate onto a HisTrap Chelating nickel-affinity column (GE Healthcare). The proteins were further purified using a Superdex 200 16/60 size-exclusion column (GE Healthcare) pre-equilibrated in a buffer consisting of 10 mM Tris–HCl pH 8.0, 100 mM NaCl. The proteins were concentrated using Millipore 10K concentrators and the protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce). The proteins were diluted to a final concentration of 20 mg ml^{−1}.

2.2. Crystallization of WHY1, WHY2 and WHY3

Crystals were typically grown from a hanging drop at 296 K by mixing 3 μ l purified protein with 3 μ l reservoir solution and allowing the system to equilibrate by vapour diffusion. Initial conditions for growing WHY1 crystals were obtained using the PEG Screen (NeXtal). These conditions were refined to 5%(v/v) PEG 3350, 0.2 M potassium acetate, 0.1 M MES pH 5.5. Crystals of WHY2 were obtained using conditions similar to those used to obtain potato WHY2 crystals (Cappadocia *et al.*, 2008). The conditions for WHY2 were refined to 15%(v/v) PEG 3350, 0.1 M MOPS pH 7.0. Initial conditions for growing WHY3 crystals were obtained from the WHY1 conditions and were refined to 14%(v/v) PEG 1000, 0.2 M potassium acetate, 0.1 M sodium citrate pH 4.2.

2.3. X-ray data collection, structure determination and refinement

Crystal cryoprotection was achieved by using a modified mother-liquor solution in which the PEG concentration was raised to 25%(v/v). The crystals were mounted in CryoLoops (Hampton Research) and flash-cooled in a stream of nitrogen gas at 100 K. 360 frames were recorded using an oscillation range of 0.5° and crystal-to-

detector distances of 240, 227 and 227 mm for WHY1, WHY2 and WHY3, respectively. Diffraction data were collected using an ADSC Quantum 315 CCD detector on beamline X29 of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (BNL, USA). The data were processed, indexed and scaled using either *HKL-2000* (Otwinowski & Minor, 1997) or *XDS* (Kabsch, 2010) and *SCALA* (Evans, 1993).

The structures of WHY1 and WHY2 were solved by molecular replacement using *PHENIX* (Adams *et al.*, 2010) with the crystal structures of the potato WHY1 (PDB entry 1l3a; Desveaux *et al.*,

2002) and WHY2 (PDB entry 3n1h; Cappadocia *et al.*, 2010) as search models, respectively. The structure of WHY3 was also solved by molecular replacement using the refined structure of WHY1 as a search model. The models were improved by iterative model building in *Coot* (Emsley *et al.*, 2010) and refinement in *PHENIX*.

3. Results and discussion

3.1. Overall structures

Whirly proteins are typically composed of a transit peptide that target them to chloroplasts or mitochondria, a Whirly domain that has ssDNA-binding capacity and an acidic aromatic C-terminal tail. As both the transit peptide and the acidic aromatic C-terminal tail are predicted to be flexible in solution and could interfere with the crystallization process, they were excluded from the constructs.

WHY1 crystallized in space group $C222_1$ with four protein molecules in the asymmetric unit. The crystals of WHY1 had a Matthews coefficient value V_M of $2.67 \text{ \AA}^3 \text{ Da}^{-1}$ (considering a molecular mass of 19 978.9 Da) and diffracted to 1.88 Å resolution (Table 1). The WHY2 crystals belonged to space group $P2_12_12_1$, had a Matthews coefficient value V_M of $2.00 \text{ \AA}^3 \text{ Da}^{-1}$ (considering a molecular mass of 19 711.6 Da), diffracted to 1.75 Å resolution and contained four proteins in the asymmetric unit (Table 1). For WHY3, a splicing variant of the protein that does not possess a serine residue at position 175 was chosen for structure determination as crystallization of the protein containing the serine residue led to perfectly twinned crystals that hampered structure determination. WHY3 crystallized in space group $P42_12$ with one molecule in the asymmetric unit. These crystals had a Matthews coefficient value V_M of $2.58 \text{ \AA}^3 \text{ Da}^{-1}$ (considering a molecular mass of 19 990.9 Da) and diffracted to 1.85 Å resolution (Table 1).

Figure 1

Tetramers of (a) WHY2, (b) WHY1 and (c) WHY3 in cartoon representation. WHY2 tetramers are present in the asymmetric unit. Tetramers of WHY1 and WHY3 were generated by applying the appropriate crystallographic symmetries.

WHY1, WHY2 and WHY3 all exhibit the canonical Whirly fold. In the WHY2 structure, the four proteins present in the asymmetric unit form a Whirly tetramer with fourfold symmetry (Fig. 1a). For WHY1 and WHY3, this same quaternary arrangement can also be generated by applying the appropriate crystallographic symmetry (Figs. 1b and 1c). This, together with previous reports of Whirly proteins forming tetramers (Desveaux *et al.*, 2002; Cappadocia *et al.*, 2010), supports the idea that plant Whirly proteins minimally fold as tetramers.

3.2. Comparison of *Arabidopsis* and potato chloroplast-directed Whirly proteins

The Whirly domains of chloroplast-directed Whirly proteins from *Arabidopsis* and potato reveal good conservation at the sequence level (Fig. 2a). Structurally, WHY1 and WHY3 are also similar, with an r.m.s.d. for equivalent C α positions of 1.1–1.6 Å between the four WHY1 subunits and WHY3 (Fig. 2b). The determination of the crystal structures of WHY1 and WHY3 offers the possibility of comparing them with the structure of potato WHY1 (PDB entry 1l3a;

AtWhy1	74	AEGLPA	RFYVGH	SIYKGKAALT	VDPRAP	PEF
AtWhy3	78	AEVSSP	RFYVGH	SIYKGKAALT	TIEPRAP	PEF
StWhy1	85	QGASTP	KVEVGY	SIYKGKAALT	VEPRSP	PEF
AtWhy1	104	VAL	LD SGAFKLSKDG	FLLIQFAP	SAGVRQYD	
AtWhy3	108	VALES	GA FKLTKEGF	LLIQFAP	AAGVRQYD	
StWhy1	115	SP	LD SGAFKLSREGM	VMLIQFAP	AAGVRQYD	
AtWhy1	134	WSKKQV	FLSVTEIG	TLVSLG	PRESCFEFFH	
AtWhy3	138	WSRKQV	FLSVTEIGNL	VLVSLG	PRESCFEFFH	
StWhy1	145	WSRKQV	FLSVTEIGS	IISLG	AKDSCEFFH	
AtWhy1	164	DPFKGK	SDEGKVRKVL	KVEPLPDGSG	HFFN	
AtWhy3	168	DPFKGK	GDEGKVRKVL	KVEPLPDGSG	RFFN	
StWhy1	175	DPNKGK	RSDEGRVRKVL	KVEPLPDGSG	HFFN	
AtWhy1	194	LSVQNK	LNVDES	IYIPITRAE	FAVLISAF	
AtWhy3	198	LSVQNK	LNVDES	SVYIPITKAE	FAVLISAF	
StWhy1	205	LSVQNK	LINDEN	IYIPVTKAE	FAVLISAF	
AtWhy1	224	NFVL	PLYLIGW	HAFANS	IK	
AtWhy3	228	NFVL	PLYLIGW	SAFANS	IK	
StWhy1	235	NFVMP	YLLGW	HTAVNS	FK	

(a)

(b)

Figure 2

(a) Sequence alignment of the Whirly domains of chloroplast-directed Whirlies. AtWhy1, *Arabidopsis* WHY1; AtWhy2, *Arabidopsis* WHY2; StWhy1, *S. tuberosum* WHY1. (b) Structural alignment of chloroplast-directed Whirly proteins in cartoon representation with WHY1 in green, WHY3 in cyan and potato WHY1 (PDB entry 1l3a) in orange.

Fig. 2b). The two WHY1 models display closely related folds with an r.m.s.d. for equivalent C α positions of 1.1–1.5 Å when superposing *Arabidopsis* and potato subunits. For comparison, the r.m.s.d. between individual subunits in potato WHY1 alone varies between 0.8 and 1.0 Å and it varies between 0.4 and 0.8 Å for *Arabidopsis* WHY1 subunits. The main difference between the structures is a shift of 4–7 Å (depending on the subunit) in the position of the 174–185 loop of WHY1. Together with our previous report (Cappadocia *et al.*, 2010), the present results suggest strong similarity of *Arabidopsis* WHY1, WHY3 and potato WHY1 both at the sequence and at the structure levels.

3.3. Comparison of *Arabidopsis* and potato mitochondria-directed Whirly proteins

The Whirly domains of mitochondria-directed Whirly proteins from *Arabidopsis* and potato also reveals good conservation at the sequence level (Fig. 3a). The four subunits of WHY2 display a high degree of structural variation, with an r.m.s.d. ranging from 1.4 to 4.8 Å. However, most of this variation is limited to a β -hairpin encompassing residues 74–90 which exhibits significant flexibility (Fig. 3b). Indeed, in the absence of this region WHY2 display a lower degree of structural variation, with an r.m.s.d. ranging from 0.7 to 1.9 Å. Residues 74 and 90 indeed appear to act as a hinge enabling a near-70° rotation of the β -hairpin relative to the core of the protein. It is the first time that such a large movement has been reported for a protein with a Whirly fold. Except for this loop, the *Arabidopsis* WHY2 displays great structural similarity to its potato homologue (PDB entry 3n1h), with an r.m.s.d. varying between 0.9 and 1.9 for

AtWhy2	45	KDA	AKPS	GRL	FAPYS	IFK	GKAALS	VEP	VLP
StWhy2	48	ADA	GKRE	GRV	FAPYS	SVF	GKAALS	SAEP	RLP
AtWhy2	75	SFTE	IDSGN	LRI	DRRS	LM	MTF	MPA	IGERK
StWhy2	78	TFNR	LD SG	GVKL	NRRG	VIM	LTF	WPS	VGERRK
AtWhy2	105	YDWEK	KQK	FALS	PTEV	GSLISM	GSKDS	SEF	
StWhy2	108	YDWEK	RQL	FALSA	TEV	GSLISM	GTRDS	SEF	
AtWhy2	135	FHDPS	MKSS	NAGQVR	KSLSV	VKPH	ADGSG	GYF	
StWhy2	138	FHDPS	MLSS	NAGQVR	KSLSI	KPN	ADGSG	GYF	
AtWhy2	165	ISLSV	NNNS	I	LKTND	YFV	VPVT	KAEFA	VMKT
StWhy2	168	ISLSV	VNNN	N	LKTND	RFT	VPVT	TAEFA	VMRT
AtWhy2	195	AFS	FALPHIM	GWNR	L	TGH			
StWhy2	198	AFS	FALPHIM	GWDR	F	TNR			

(a)

(b)

Figure 3

(a) Sequence alignment of the Whirly domains of mitochondria-directed Whirlies. AtWhy2, *Arabidopsis* WHY2; StWhy2, *S. tuberosum* WHY2. (b) Structural alignment of mitochondria-directed Whirly proteins in cartoon representation with WHY2 in yellow and potato WHY2 (PDB entry 3n1h) in light blue. The black arrows point to the β -hairpin encompassing residues 74–90.

Figure 4

(a) Alignment of *Arabidopsis* Whirly ssDNA-binding sites. Proteins are shown in cartoon representation and residues equivalent to those of potato WHY2 (PDB entry 3n1i) that contact ssDNA are shown in stick representation with C atoms in grey. Those of WHY1, WHY2 and WHY3 are shown in cyan, magenta and green, respectively. The potato WHY2 nomenclature was used for clarity. The ssDNA is shown in stick representation with its C atoms in yellow. (b) Close-up of residues Trp100 and Trp110. The representation is similar to that in (a).

individual subunits (Fig. 3b). This suggests that the mitochondria-directed Whirly proteins adopt similar structures.

3.4. Conservation of the ssDNA-binding interface

Following the elucidation of the crystal structure of potato WHY2 bound to ssDNA, we proposed a general model for the binding of ssDNA by Whirly proteins (Cappadocia *et al.*, 2010). Our present report of the crystal structures of the three *Arabidopsis* Whirly proteins offers a unique opportunity to verify the actual scope of this model. With this aim, we generated the Whirly tetramers by applying the crystallographic symmetry when necessary and aligned the three *Arabidopsis* Whirly structures with that of the potato complex (Fig. 4a). Generating the complete tetramer is important as each ssDNA-binding site encompasses two adjacent subunits. Only three clashes were observed between the *Arabidopsis* Whirly structures and the ssDNA. Importantly, these clashes were only observed for the side-chain moieties and could be prevented in all cases if the side chain adopted a different rotameric configuration. We also observed that the residues involved in ssDNA binding were either conserved or replaced by residues with similar biochemical propensities at equivalent positions in WHY1, WHY2 or WHY3, notably residues equivalent to Phe64, His139 and Lys153 of potato WHY2. The case of residue Trp100 (potato WHY2 nomenclature), however, merits further consideration. This residue interacts with the ssDNA nucleobases through hydrophobic interactions (Fig. 4b). In *Arabidopsis* WHY2 this residue is replaced by a methionine that can fulfil a similar role. In WHY1 and WHY3 this residue is replaced by an alanine. This enables a rotation of the tryptophan at position 110 that would place this residue in a good conformation to interact with the ssDNA nucleobases through hydrophobic interactions. Globally, our results suggest that all *Arabidopsis* Whirly proteins can interact with ssDNA through similar binding interfaces.

4. Conclusion

We have elucidated the structure of the three *Arabidopsis* Whirly proteins. These structures demonstrate a high degree of structure

similarity between plant Whirly proteins but also capture previously unforeseen movements of certain structural elements. The high structure similarity of plant Whirly proteins suggests that the capacity of Whirly to interact with ssDNA is dependent on a preformed DNA-binding platform.

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