Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Structural and biochemical characterization of the cytosolic wheat cyclophilin TaCypA-1

Simranjeet Singh Sekhon,[a,b]‡ Harsimran Kaur,[c,d]‡ Tanima Dutta,[c,d]‡ Khushwant Singh,[d]‡ Sumita Kumari,[d]‡ Sunghyun Kang,[a,b]* Sung Goo Park,[a] Byoung Chul Park,[a] Dae Gwin Jeong,[a,b] Ashwani Pareek,[d]* Eui-Jeon Woo,[a,b]* Prabhjeet Singh[c]* and Tae-Sung Yoon[a,b]*

[a]Medical Proteomics Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea, [b]Bio-Analytical Science Major, University of Science and Technology, 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea, [c]Department of Biotechnology, Guru Nanak Dev University, Amritsar 143 005, India, and [d]Stress Physiology and Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India

‡ The first three authors made equal contributions. S. S. Sekhon contributed to the crystallographic studies, H. Kaur and T. Dutta contributed to the cloning and biochemical studies, K. Singh contributed to the bioinformatics analysis and S. Kumari contributed to the cloning and expression of proteins in the heterologous system.

Correspondence e-mail: skang@kribb.re.kr, ashwanip@mail.jnu.ac.in, ejwoo@kribb.re.kr, singhprabhjeet62@gmail.com, yoonts@ust.ac.kr

Received 5 September 2012
Accepted 20 December 2012

PDB References: TaCypA-1, 4e1q; complex with cyclosporin A, 4hy7

Cyclophilins belong to a family of proteins that bind to the immunosuppressive drug cyclosporin A (CsA). Several members of this protein family catalyze the *cis–trans* isomerization of peptide bonds preceding prolyl residues. The present study describes the biochemical and structural characteristics of a cytosolic cyclophilin (TaCypA-1) cloned from wheat (*Triticum aestivum* L.). Purified TaCypA-1 expressed in *Escherichia coli* showed peptidyl-prolyl *cis–trans* isomerase activity, which was inhibited by CsA with an inhibition constant of 78.3 n*M*. The specific activity and catalytic efficiency (k_{cat}/K_m) of the purified TaCypA-1 were 99.06 $\pm$ 0.13 nmol s^{-1} mg^{-1} and 2.32 $\times$ 10^5 M^{-1} s^{-1}, respectively. The structures of apo TaCypA-1 and the TaCypA-1–CsA complex were determined at 1.25 and 1.20 Å resolution, respectively, using X-ray diffraction. Binding of CsA to the active site of TaCypA-1 did not result in any significant conformational change in the apo TaCypA-1 structure. This is consistent with the crystal structure of the human cyclophilin D–CsA complex reported at 0.96 Å resolution. The TaCypA-1 structure revealed the presence of a divergent loop of seven amino acids ^{48}KSGKPLH^{54} which is a characteristic feature of plant cyclophilins. This study is the first to elucidate the structure of an enzymatically active plant cyclophilin which shows peptidyl-prolyl *cis–trans* isomerase activity and the presence of a divergent loop.

1. Introduction

Cyclophilins, which bind to the immunosuppressive drug cyclosporin A (CsA), are members of the peptidyl-prolyl *cis–trans* isomerase family and catalyze the *cis–trans* isomerization of peptide bonds preceding prolyl residues (Fischer *et al.*, 1989). This isomerization enables the nascent proteins to attain their final folded state, which has been recognized as the rate-limiting step (Brandts *et al.*, 1975). Cyclophilins are ubiquitous proteins and are found in all subcellular compartments (Galat, 1999). 29 cyclophilin genes with different subcellular localizations were predicted in *Arabidopsis* (Chou & Gasser, 1997; Romano *et al.*, 2004), which is the largest number reported to date in any organism. The rice genome showed the presence of 27 putative cyclophilin genes, all of which, with the exception of OsCyp17, show putative orthology to *Arabidopsis* cyclophilins (Ahn *et al.*, 2010). The rice cyclophilins were predicted to be catalytically active, with the exception of OsCyp19-4. The lack of peptidyl-prolyl *cis–trans* isomerase activity of OsCyp19-4 was attributed to the substitution of Trp by Arg at residue 121. Genes for cytosolic cyclophilins (CypA; Johnson *et al.*, 2001; Johnson & Bhave, 2004) and endoplasmic

reticulum (ER)-localized cyclophilins (CypB; Wu *et al.*, 2009) have also been characterized in wheat. A chloroplast-localized cyclophilin (CypB) in fava bean which had peptidyl-prolyl *cis–trans* isomerase activity was inhibited by CsA with an inhibition constant (K_i) of 3.9 n*M* (Luan *et al.*, 1994). The cyclophilins TLP40 and TLP20 in the thylakoid lumen of spinach chloroplast showed high levels of peptidyl-prolyl *cis–trans* isomerase activity and have been implicated in intra-organelle signalling and dephosphorylation of photosynthetic proteins (Fulgosi *et al.*, 1998). However, the *Arabidopsis* homologue of TLP40 (AtCyp38) did not show any peptidyl-prolyl *cis–trans* isomerase activity, whereas the TLP20 homologue, AtCyp20-2, possessed strong enzymatic activity (Shapiguzov *et al.*, 2006). Cyp20-3 (ROC-4), which is localized in the stroma of the *Arabidopsis* chloroplast (Lippuner *et al.*, 1994), has been reported to play a vital role in cysteine biosynthesis by assisting the assembly of the enzyme complex (Dominguez-Solis *et al.*, 2008). Two maize cyclophilins of 17.5 and 17.7 kDa (Sheldon & Venis, 1996) and a nuclear-localized cyclophilin (CcCyp) in *Cajanus cajan* (Sekar *et al.*, 2010) exhibited CsA-inhibitable peptidyl-prolyl *cis–trans* isomerase activity. It is apparent from these observations that biochemical characterization has been carried out for only a few cyclophilins in plants and hence further studies are required to understand the role of these proteins.

There has been growing interest in the structural analysis of cyclophilins since the structure of human CypA at 2.5 Å resolution was determined by X-ray crystallographic analysis (Ke *et al.*, 1991). An in-house survey of the Protein Data Bank (PDB; http://www.rcsb.org/pdb/home/home.do) revealed 82 human and six nematode cyclophilin structures among over 150 available cyclophilin structures which have been determined using X-ray diffraction. Although cyclophilins are ubiquitous and present in all subcellular fractions, information on the structural aspects of these proteins in plants is scanty. Only *A. thaliana* cyclophilin (AtCyp38) has been reported in the PDB (Vasudevan *et al.*, 2012). However, AtCyp38 does not possess peptidyl-prolyl *cis–trans* isomerase activity. It is evident that despite having high overall sequence homology the plant cyclophilins show different degrees of peptidyl-prolyl *cis–trans* isomerase activity, which has been attributed to structural differences (Vasudevan *et al.*, 2012). These observations therefore highlight the need to elucidate the crystal structures of cyclophilins in plants. In the present study, we cloned a gene encoding a wheat cytosolic cyclophilin (TaCypA-1), studied its enzymatic activity and determined its structure at high resolution. Furthermore, the structure of TaCypA-1 was also elucidated as a complex with its specific inhibitor CsA.

2. Materials and methods

2.1. Plant material, RNA extraction and cDNA synthesis

Seeds of Indian wheat (*Triticum aestivum* L.) cultivar PBW-343 were procured from Punjab Agricultural University, Ludhiana, Punjab, India. The plants were raised in pots in a net house at Guru Nanak Dev University, Amritsar as described previously (Singh *et al.*, 2008). The ears were tagged on the day of anthesis and were harvested 16 d post-anthesis. Grain samples were harvested in triplicate and stored in liquid nitrogen. RNA from the grains was extracted in RAFLEX solution (Merck, USA) as per the manufacturer's instructions. cDNA was prepared from mRNA using a single-strand cDNA-synthesis kit (Fermentas, India).

2.2. Cloning and purification of a cytosolic cyclophilin (TaCypA-1) from *T. aestivum* L.

Tblastx analysis of the TIGR Plant Transcript Assemblies Database (TADB; http://plantta.jcvi.org/) was performed using the sequence of a cytosolic cyclophilin as a query (GenBank accession No. AF262982.1; Johnson *et al.*, 2001). The resultant retrieved sequence (TA58444_4565) showed 92% identity to the query. The polymerase chain reaction (PCR) primers for amplification of cDNA were designed using the *Primer*3 software (http://frodo.wi.mit.edu/). The forward (5′-CG**GAATTC**ATGGCCAACCCGAGGGTG-3′) and the reverse (5′-CCG**CTCGAG**GAGCTGGCCGCAGTCGGC-GAT-3′) primer sequences contained *Eco*RI and *Xho*I sites (shown in bold), respectively, for cloning. PCR amplification was carried out under the following conditions: initial denaturation (367 K for 4 min) using 200 ng cDNA followed by 35 cycles of denaturation (367 K for 1 min), primer annealing (328 K for 1 min) and extension (345 K for 1 min), with a final extension of 5 min at 345 K. The PCR reaction mixture contained primer (10 m*M*), dNTPs (1 m*M*) and *Taq* polymerase (2 U). The amplified products were gel-extracted using a commercial gel-extraction kit (Merck Specialities Pvt. Ltd, India) and cloned as a 6×His-tag fusion in the expression vector pET-28a(+) (Novagen, India). The presence of the insert was validated by restriction digestion of the recombinant plasmids and sequencing of the cloned fragment (516 bp; (Macrogen Inc., Republic of Korea). The cloned gene was designated TaCypA-1.

Induction of the recombinant fusion protein was carried out in *Escherichia coli* strain BL21 (DE3) by adding 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside after 3 h of incubation at 310 K (A_{600} = 0.6). The cultures were transferred to 291 K with 180 rev min^{-1} shaking overnight for cell growth before harvesting. Purification was performed using HisTrap HP, RESOURCE Q and RESOURCE S columns (GE Healthcare). The cells were lysed in 1× PBS buffer containing an EDTA-free protease-inhibitor tablet followed by five rounds of sonication (time, 30 s; pulse on/off, 1 s). The sample was centrifuged for 50 min at 16 000*g*. The supernatant was filtered using a 0.45 µm filter and loaded onto a 5 ml HisTrap HP column equilibrated with buffer *A* [1× PBS, 5 m*M* β-mercaptoethanol (2-MCE)]. The bound protein was eluted with a gradient of 0–250 m*M* imidazole. The pure fractions were collected, dialysed overnight against dialysis buffer (20 m*M* Tris–HCl pH 7.0, 30 m*M* NaCl, 5 m*M* 2-MCE) and applied onto a RESOURCE Q column (6 ml) which was equilibrated with the same dialysis buffer. Since the protein

Table 1
Data-processing and refinement statistics for the structure determination of wheat cyclophilin (TaCyPA-1).

Values in parentheses are for the highest resolution shell.

Protein	Native TaCypA-1	TaCypA-1–CsA
PDB code	4e1q	4hy7
Unit-cell parameters (Å, °)	a = 37.13, b = 52.94, c = 77.76, $\alpha = \beta = \gamma$ = 90	a = 37.91, b = 49.84, c = 78.97, $\alpha = \beta = \gamma$ = 90
Resolution range (Å)	26.86–1.25 (1.27–1.25)	27.00–1.20 (1.22–1.20)
Temperature (K)	100	100
X-ray source	BL17A, Photon Factory	7A-SB1, PAL
Wavelength (Å)	1.000	0.9773
$R_{\rm merge}$† (%)	5.2 (27.60)	6.7 (24.40)
Completeness (%)	99.50 (99.90)	99.0 (99.10)
Final R factor (%)	17.78	18.65
Free R factor (%)	18.50	20.43
Ramachandran plot statistics, residues in (%)		
Favoured regions	98.2	93.8
Allowed regions	1.8	5.7
Outlier regions	0.0	0.6

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity of the i observations.

did not bind to the RESOURCE Q column, the flowthrough fractions were further purified using a RESOURCE S column. The column was equilibrated with 20 m*M* Tris–HCl pH 7.0, 30 m*M* NaCl, 5 m*M* 2-MCE and the protein was eluted with a 30–500 m*M* NaCl gradient. 80 mg pure protein was harvested from 6 l culture.

2.3. Peptidyl-prolyl *cis–trans* isomerase activity assay

The concentration of the purified TaCypA-1 protein was estimated by the method of Bradford (1976) using BSA as a standard. The peptidyl-prolyl *cis–trans* isomerase activity was assayed at 288 K for 360 s in a coupled reaction with chymotrypsin, as described previously (Fischer *et al.*, 1984). The 1 ml assay mixture contained 40 m*M* *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilidine as the substrate peptide, assay buffer (50 m*M* HEPES pH 8.0, 150 m*M* NaCl, 0.05% Triton X-100) and varying amounts of the purified protein. The reaction was initiated by the addition of chymotrypsin (300 µg ml^{-1}) and the changes in absorbance at 390 nm were monitored using a spectrophotometer (PerkinElmer Lambda Bio20) equipped with a Peltier temperature-control system. The effect of FK506 and CsA, which are specific inhibitors of the peptidyl-prolyl *cis–trans* isomerase activity of FK506-binding proteins and cyclophilins, respectively, was determined by studying the inhibition of the reaction. The inhibitors were added to the assay mixture 30 min before the start of the reaction and were incubated at 277 K. The peptidyl-prolyl *cis–trans* isomerase activity was calculated as the product of the difference between the catalysed and uncatalysed first-order rate constants (derived from the kinetics of change in absorbance at 390 nm) and the amount of substrate in each reaction (Breiman *et al.*, 1992). The data were analysed using *GraFit* 4.0 (http://www.erithacus.com/grafit). The inhibition constant for CsA was determined as the gradient of the line of best fit from a plot of [CsA]/(1 − k/k_0) against k/k_0, where k is the rate constant at a given CsA concentration and k_0 is the rate constant in the absence of CsA (Sheldon & Venis, 1996).

2.4. Crystallization and diffraction data collection

The protein was concentrated to 20 mg ml^{-1} for crystallization studies. Crystals were grown by vapour diffusion at 291 K using the sitting-drop method. The reservoir solution consisted of 1.0 *M* $(NH_4)_2HPO_4$, 0.1 *M* imidazole pH 8.0, 0.2 *M* NaCl. To obtain crystals of the TaCypA-1–CsA complex, cocrystallization of TaCypA-1 with CsA was set up by mixing 20 mg ml^{-1} concentrated protein with 0.01 *M* CsA (dissolved in DMSO) followed by overnight incubation at 282 K. Crystals were obtained by vapour diffusion at 291 K using the sitting-drop method. The reservoir solution consisted of 0.1 *M* HEPES pH 7.5, 20% PEG 8000. A diffraction data set was collected from a native crystal at a wavelength of 1.000 Å at the Photon Factory. The diffraction data set for the TaCypA-1–CsA complex was collected at a wavelength of 0.9773 Å on beamline 7A-SBI at the Pohang Accelerator Laboratory. The diffraction data were processed with the *HKL* program suite (Otwinowski & Minor, 1997) to resolutions of 1.25 and 1.20 Å for the native TaCypA-1 and TaCypA-1–CsA complex crystals, respectively (see Table 1 for statistics). The structures were solved by molecular replacement and solutions were obtained with the program *Phaser* in *PHENIX* (Adams *et al.*, 2010) using the CeCyp-3 structure (PDB entry 2igv; Wear *et al.*, 2007) as the search model for the native TaCypA-1 structure and the native TaCypA-1 structure (PDB entry 4e1q) as the search model for the TaCypA-1–CsA complex structure.

2.5. *In silico* analysis

A multiple alignment of the amino-acid sequences of cyclophilins from *T. aestivum* (TaCypA-1), *A. thaliana* (AtCyp19-2), *Populus trichocarpa* (PtCyp), *Oryza sativa* (OsCyp19-2), *Zea mays* (ZmCyp), *Homo sapiens* (hCypA), *Caenorhabditis elegans* (CeCyp-3), *Brugia malayi* (BmCyp), *Bos taurus* (Cyp40), *Mycobacterium tuberculosis* (MtPpiA), *Plasmodium falciparum* (PfCyp) and *Macaca mulatta* (TRIMCyp) was performed using *ClustalW* (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The results were also cross-checked with *Clustal Omega* (http://www.ebi.ac.uk/Tools/msa/clustalo/). The results of multiple sequence alignment were edited using the *Jalview* editor (http://www.jalview.org/). Phylogenetic analysis was performed using *TreeView* (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The domain architecture of TaCypA-1 was predicted using the Pfam database (http://pfam.sanger.ac.uk/). The results of domain analysis were also cross-checked with the SMART database (http://smart.embl-heidelberg.de/). The subcellular localization studies of TaCypA-1 were performed using *WOLF-PSORT* (http://wolfpsort.org/). The molecular weight and pI were calculated using the *ExPASy* tool (http://web.expasy.org/compute_pi/). Protein secondary structure was predicted using *PSIPRED* (http://bioinf.cs.ucl.ac.uk/psipred/).

3. Results and discussion

3.1. Characterization of TaCypA-1

By virtue of their peptidyl-prolyl *cis–trans* isomerase activity, the cyclophilins, together with parvulins and FK506-binding proteins, are the only proteins that can catalyse the conversion of the peptidyl-prolyl bond from the *cis* to the *trans* conformation (Fischer *et al.*, 1989). Recent studies carried out in our laboratory demonstrated that the peptidyl-prolyl *cis–trans* isomerase activity in grains of wheat was primarily owing to cyclophilins and was regulated in a developmental and cultivar-dependent manner (Dutta *et al.*, 2011). Although genes for several cyclophilins have been cloned from wheat (Johnson & Bhave, 2004; Wu *et al.*, 2009), biochemical and structural characterization of these proteins had not been carried out. In this study, the gene for a wheat cytosolic cyclophilin (TaCypA-1) was isolated from elite Indian wheat cv. PBW-343 and biochemically characterized. *BLASTN* analysis of TaCypA-1 revealed that the open reading frame (ORF) of the cloned gene was 99% identical to that of *T. aestivum* CypA-3 (GenBank accession No. AF262984.1; Johnson *et al.*, 2001). *BLASTX* results for TaCypA-1 showed 100% identity to CypA-1 of *T. aestivum* (GenBank accession No. AAK49426.1). The ORF was predicted to encode a protein of 171 amino-acid residues with a molecular mass of 18.3 kDa and a pI of 8.52, as determined by the *ExPASy* tool, whereas the observed molecular mass of the cloned cyclophilin protein was 22 kDa (Fig. 1). The difference between the observed and theoretical molecular weights is attributed to the presence of additional amino acids in the 6×His tag. Conserved domain architecture analysis of TaCypA-1 by Pfam suggests that it is a single-domain protein and possesses a cyclophilin-like domain (Pfam ID PF00160). The TaCypA-1 protein has a unique 11 amino-acid stretch (43–54) which is a characteristic feature of single-domain cyclophilins (Galat, 1999) and a highly conserved peptidyl-prolyl *cis–trans* isomerase domain (residues 7–169; Fig. 2). The residues Arg62, Phe67 and His133 in cytosolic cyclophilin have been reported to be essential for peptidyl-prolyl *cis–trans* isomerase activity, whereas Trp128 has been implicated in binding CsA (Romano *et al.*, 2004). However, recent studies suggest that other structural features are also important for the enzymatic activity of cyclophilins, as demonstrated for AtCyp38 (Vasudevan *et al.*, 2012). Despite showing 82% sequence identity to its enzymatically active spinach homologue TLP40 and possessing three of the critical residues in the active site, AtCyp38 does not show peptidyl-prolyl *cis–trans* isomerase activity. In contrast, TLP40 was demonstrated to be an active PPIase (Fulgosi *et al.*, 1998).

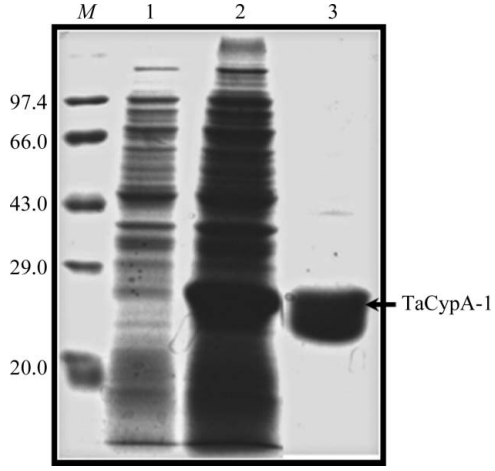


Figure 1
SDS–PAGE analysis of the TaCypA-1 protein. Total protein isolated from *E. coli* strain BL21 (DE3) before (lane 1) and after (lane 2) induction with 0.5 m*M* IPTG. Purified TaCypA-1 is indicated by an arrow (lane 3). Protein size markers are shown in lane *M* and their molecular masses are given in kDa.

Table 2
Comparison of the catalytic efficiency and inhibition constant of TaCypA-1 with those of other cyclophilins.

(*a*) Catalytic efficiency.

Cyclophilin	Catalytic efficiency (k_{cat}/K_m) (M^{-1} s^{-1})	Reference
TaCypA-1 (wheat)	2.32×10^5	This study
ZmCyp18 (maize)	11.0×10^6	Sheldon & Venis (1996)
AtCyp22 (*Arabidopsis*)	5.7×10^6	Grebe *et al.* (2000)
OsCyp2 (rice)	4.5×10^6	Kumari *et al.* (2009)
Cyp40 (bovine)	1.9×10^6	Kieffer *et al.* (1992)

(*b*) Inhibition constants.

Cyclophilin	Inhibition constant (n*M*)	Reference
TaCypA-1 (wheat)	78.3	This study
Cyp (fava bean)	3.9	Luan *et al.* (1994)
ZmCyp18 (maize)	6.0	Sheldon & Venis (1996)
hCyp (human)	2–200	Kofron *et al.* (1991)

3.2. Characterization of peptidyl-prolyl *cis–trans* isomerase activity

To demonstrate that the purified recombinant TaCypA-1 was enzymatically active, its peptidyl-prolyl *cis–trans* isomerase activity was assayed using a spectrophotometric assay method (Fischer *et al.*, 1989). The purified TaCypA-1 showed peptidyl-prolyl *cis–trans* activity since the first-order rate constant (0.039 s^{-1}) in the presence of 1 µg of this protein was almost threefold higher than the first-order rate constant (0.013 s^{-1}) observed in an uncatalysed control (Fig. 3*a*). The first-order rate constant increased with protein concentration (Fig. 3*b*). Addition of the negative control BSA had no significant effect on the first-order rate constant. These results imply that the observed peptidyl-prolyl *cis–trans* isomerase activity was specific to TaCypA-1. The purified TaCypA-1 showed a specific activity of 99.06 ± 0.13 nmol s^{-1} mg^{-1}. The catalytic efficiency (k_{cat}/K_m) of the recombinant TaCypA-1 protein was 2.32×10^5 M^{-1} s^{-1}, which was lower compared with the K_{cat}/k_m values of other cyclophilins, *i.e.* ZmCyp18 from maize, AtCyp22 from *Arabidopsis*, OsCyp2 from rice and bovine Cyp40 (Table 2*a*). The peptidyl-prolyl *cis–trans* isomerase activity of TaCypA-1 was only inhibited by CsA, with an inhibition constant of 78.3 n*M* (Figs. 3*c* and 3*d*). FK506, even at 50 µ*M*, had no apparent effect on the enzy-

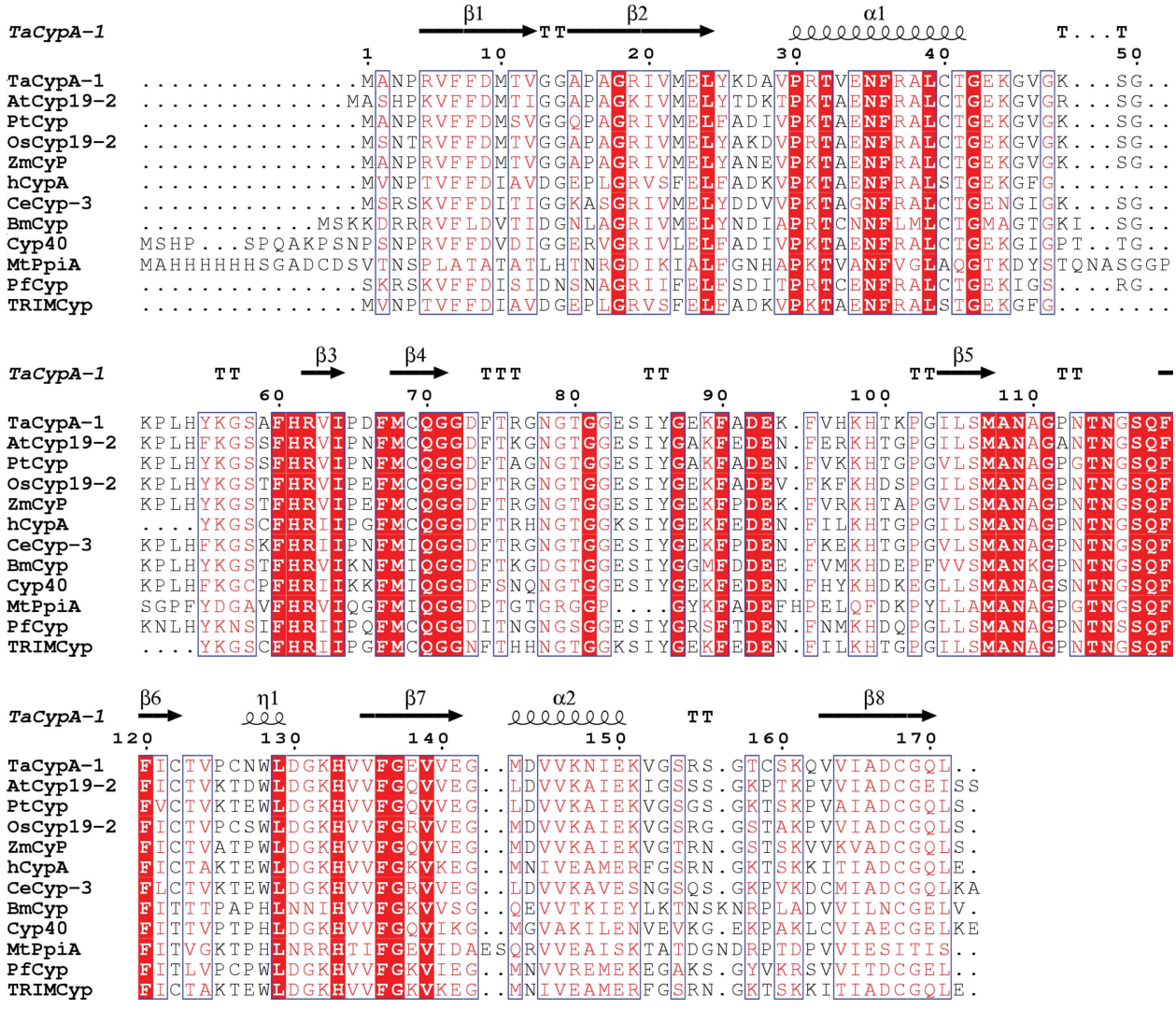


Figure 2
Multiple sequence alignment of cyclophilin proteins from *T. aestivum* (TaCypA-1; GenBank accession No. JQ678695), *A. thaliana* (AtCyp19-2; GenBank accession No. NM_127683), *P. trichocarpa* (PtCyp; GenBank accession No. XM_002313700), *O. sativa* (OsCyp19-2; GenBank accession No. NM_001052252), *Z. mays* (ZmCyp; GenBank accession No. BT042680), *H. sapiens* (hCypA; GenBank accession No. NM_021130), *C. elegans* (CeCyp-3; UniProtKB P52011), *B. malayi* (BmCyp; UniprotKB Q27450), *B. taurus* (Cyp40; UniProtKB P26882), *M. tuberculosis* (MtPpiA; UniProtKB P65762), *P. falciparum* (PfCyp; UniProtKB Q25756) and *M. mulatta* (TRIMCyp; UniProtKB P62940) was performed using *ClustalX* v.2.1. The *ESPript* 2.0 multiple-alignment editor was used for the final presentation. The positions of the various α-helices and β-sheets are indicated at the top of the figure.

matic activity of this protein (Fig. 3*c*). Since no cross-inhibition is observed between FK506-binding proteins and cyclophilins (Harding *et al.*, 1989), the cloned protein (TaCypA-1) is a true cyclophilin. The observed inhibition constant of CsA (78.3 n*M*) for TaCypA-1 is higher than those of other cyclophilins from fava bean and maize, but is comparable to human cyclophilins (Table 2*b*). The difference in the sensitivity of TaCypA-1 to CsA is in accordance with previous reports (Edvardsson *et al.*, 2003), which demonstrated that the peptidyl-prolyl *cis–trans* isomerase activity of two maize cyclophilins, TLP40 and TLP20, showed differential sensitivity to CsA.

3.3. Sequence alignment and conserved domain analysis

Multiple sequence alignment of cyclophilins from 12 different organisms revealed that TaCypA-1 shares a high degree of similarity with other reported members. It shares 71% similarity with hCypA, 87% with OsCyp19-2, 86% with ZmCyp, 83% with PtCyp, 78% with AtCyp19-2, 74% with both BmCyp and CeCyp-3, 78% with both bovine Cyp40 and PfCyp, 52% with MtCyp and 81% with TRIMCyp (Fig. 2). To study the phylogenetic relationship among these members, a tree was constructed based on the neighbour-joining (NJ) method from alignment of the full-length protein sequences using the BLOSUM62 matrix (Fig. 4). This study revealed that TaCypA-1, ZmCyp and OsCyp19-2 are closely related, implying a high degree of similarity among them. In contrast, the other two cyclophilins reported from plants, *i.e.* AtCyp19-2 and PtCyp, were grouped together in a different clade. The results from the phylogenetic tree are in accordance with the sequence identity of these cyclophilins. This analysis also revealed that cyclophilins reported from monocot species may

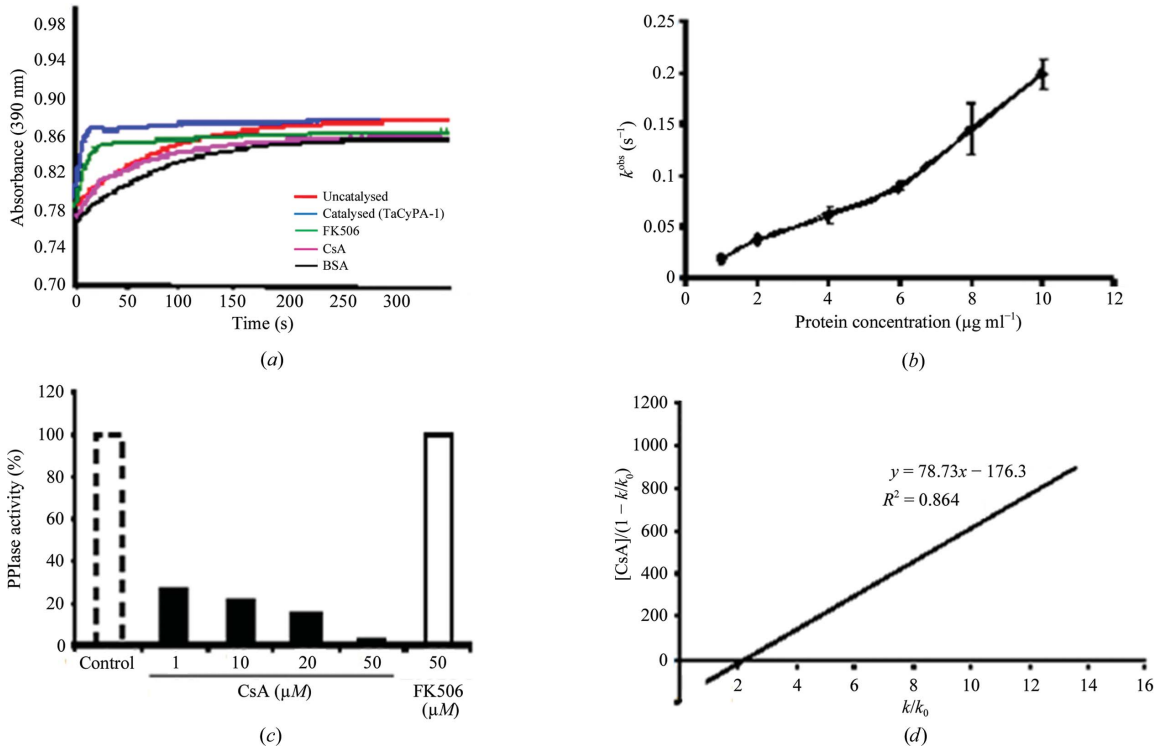


Figure 3
(*a*) Kinetics of TaCypA-1-catalysed hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilidine (showing the peptidyl-prolyl *cis–trans* isomerase activity of TaCypA-1). (*b*) Concentration dependence of the rate constant of the TaCypA-1 activity. (*c*) Effect of the cyclophilin inhibitor cyclosporin A (CsA) and the FK506-binding protein inhibitor FK506 on the peptidyl-prolyl *cis–trans* isomerase activity of TaCypA-1. The data represent the peptidyl-prolyl *cis–trans* isomerase activity as a percentage of the uninhibited control activity. (*d*) Determination of the inhibition constant of TaCypA-1 for CsA. The first-order rate constant was analysed using the *GraFit* 4.0 software. The inhibition constant (k_i) for CsA was determined as the gradient of the line of best fit from a plot of [CsA]/(1 − k/k_0) against k/k_0, where k is the rate constant at any given CsA concentration and k_0 is the rate constant in the absence of CsA. The slope of the line represents k_i. All experiments were conducted as a minimum of three replicates.

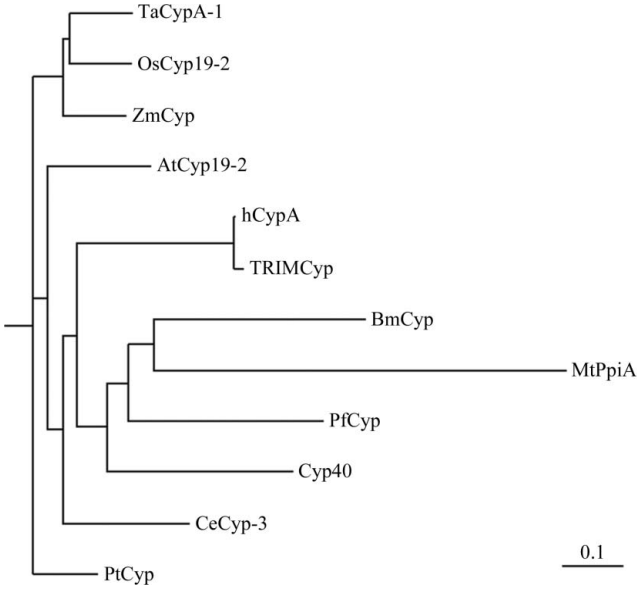


Figure 4
Unrooted phylogenetic tree of cyclophilin proteins from *T. aestivum* (TaCypA-1), *A. thaliana* (AtCyp19-2), *P. trichocarpa* (PtCyp), *O. sativa* (OsCyp19-2), *Z. mays* (ZmCyp), *H. sapiens* (hCypA), *C. elegans* (CeCyp-3), *B. malayi* (BmCyp), *P. falciparum* (PfCyp), *B. taurus* (Cyp40), *M. tuberculosis* (MtPpiA) and *M. mulatta* (TRIMCyp). The phylogenetic tree was based on sequence alignment by *Clustal Omega* using the NJ method with default options and was edited using the *TreeView* software. The scale bar indicates the number of amino-acid changes per site.

have evolved from a common ancestor different from that of cyclophilins reported from dicots. At the same time, it can also be seen that the plant cyclophilins (OsCyp-19, ZmCyp, PtCyp and AtCyp-19) have diverged from the nonplant members of the family (BmCyp, Cyp40, PfCyp, MtCyp, TRIMCyp and hCypA). Conserved domain analysis was carried out by co-aligning the amino-acid sequences of TaCypA-1 with hCypA, AtCyp19-2, OsCyp19-2, ZmCyp and PtCyp (Kallen *et al.*, 1991). In hCypA, His54, Arg55, Phe60, Gln111, Phe113, Trp121 and His126 have been implicated in CsA binding (Ahn *et al.*, 2010). Sequence alignment of hCypA with TaCypA-1 also shows the presence of many conserved motifs, such as VFFD (amino acids 6–10) and PKTAENFRAL (amino acids 30–39), which are highly conserved in eukaryotes (Trandinh *et al.*, 1992), FMCQGGDFTR (amino acids 67–76) and PGILSMANAGPNTNGSQFFICT (amino acids 102–123). However, despite the presence of conserved motifs, comparison of the hCypA and TaCypA-1 amino-acid sequences also revealed several significant differences (Fig. 2)

3.4. Overall structure

To determine whether the differences in the amino-acid sequences of TaCypA-1 and hCypA result in the structural changes, we analysed the crystal structure of the apo form at 1.25 Å resolution. The TaCypA-1 crystal belonged to the

orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 37.13, b = 52.94, c = 77.76 Å. One TaCypA-1 molecule was observed in the asymmetric unit of the crystal; the Matthews coefficient (V_M) and solvent content were calculated to be 2.08 Å^3 Da^{-1} and 40.88%, respectively (Table 1). The structure showed the presence of eight β-sheets, two α-helices and one 3_{10}-helix (Fig. 6), similar to hCypA structures (Ke *et al.*, 1991; Kallen *et al.*, 1991). The active site is also similar to that found in hCypA and all of the residues that bind to the immunosuppressant drug CsA are conserved.

The structure of the TaCypA-1–CsA complex was refined at 1.20 Å resolution (Fig. 5). The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 37.91, b = 49.84, c = 78.97 Å. An $F_o - F_c$ map which was phased using the TaCypA-1 structure only showed additional clear electron density corresponding to the CsA molecule, confirming that the crystal was composed of the TaCypA-1–CsA complex. This result implies that the CsA molecule has a strong binding affinity for TaCypA-1, which further validates our biochemical studies that showed inhibition of TaCypA-1-associated peptidyl-prolyl *cis–trans* isomerase activity only by CsA, with an inhibition constant of 78.3 n*M*. The root-mean-square deviation (r.m.s.d.) of all residues between apo TaCypA-1 and the TaCypA-1–CsA complex is 0.24 Å, whereas the r.m.s.d. of the active-site residues is 0.13 Å. These observations indicate that the structures of apo TaCypA-1 and the TaCypA-1–CsA complex are similar in an overall comparison and that the binding of CsA to the active site has no significant effect on the overall structure of this protein. This is also consistent with the crystal structure of the human cyclophilin D–CsA complex reported at 0.96 Å resolution (Kajitani *et al.*, 2007).

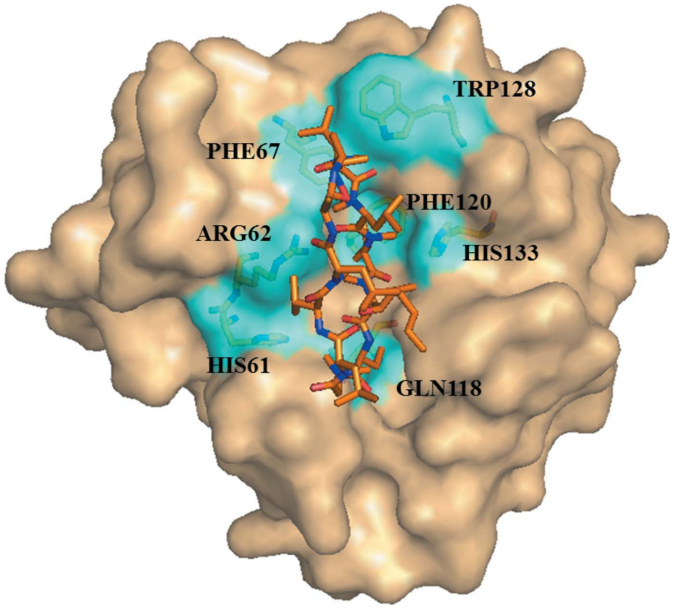


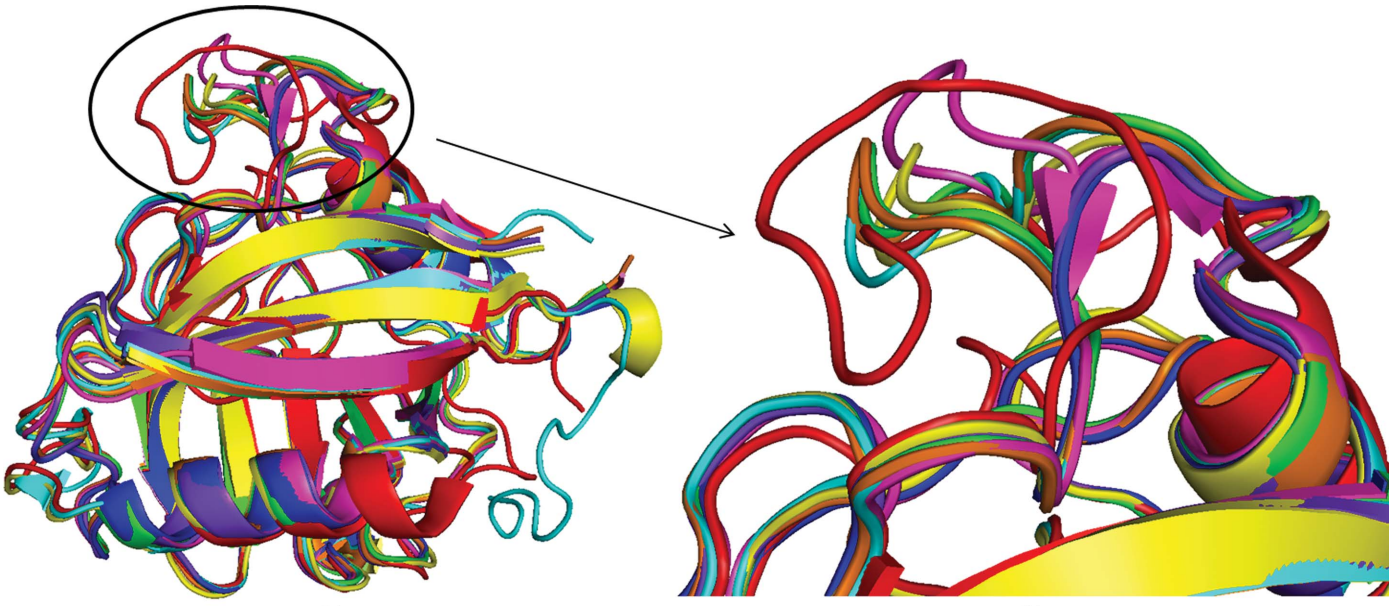
Figure 5
The TaCypA-1–CsA complex structure is represented as a surface (brown) with the active-site residues highlighted in cyan and cyclosporin A in stick representation (orange).

A comparative analysis of the structure of TaCypA-1 with the known structures of other cyclophilins was carried out. The structure of TaCypA-1 was aligned with those of cyclophilins from human (hCypD and hCypA), *C. elegans* (CeCyp-3), *B. malayi* (BmCyp), *B. taurus* (Cyp40; residues 1–172), *P. falciparum* (PfCyp) and *M. tuberculosis* (MtPpiA). The divergent loop (residues 48–54) which is observed in TaCypA-1 (Fig. 6*a*) is also present in bovine Cyp40 (Taylor *et al.*, 2001), CeCyp-3 (Dornan *et al.*, 1999), BmCyp1 (Taylor *et al.*, 1998), MtPpiA (Henriksson *et al.*, 2004) and PfCyp (Peterson *et al.*, 2000). However, it is absent in hCypD (Kajitani *et al.*, 2007) and hCypA (Fraser *et al.*, 2009). The r.m.s.d.s of TaCypA-1 from hCypD and hCypA are 0.32 and 0.36 Å, respectively, with the divergent loop being the major structural difference (Fig. 6*b*). The overall geometry of TaCypA-1 and CeCyp-3 is conserved, as reflected by the r.m.s.d. of 0.23 Å. The r.m.s.d.s on aligning TaCypA-1 with PfCyp and Cyp40 are 0.35 Å, implying structural differences. Although the divergent loop is also present in PfCyp and Cyp40, the residues forming the loop are different. TaCypA-1 also shows divergence from MtPpiA and BmCyp, with r.m.s.d.s of 1.16 and 0.51 Å, respectively. This can be attributed to differences in the two loops comprised of residues 48–54 and 152–157 (Fig. 6).

Glu83 and two highly conserved Cys residues (Cys40 and Cys168; 5.49 Å apart) that are observed in the TaCypA-1 structure and are well defined in the electron-density map constitute a conserved feature of divergent-loop cyclophilins. The reduced form of the Cys residues may play a role in the redox mechanism (Dornan *et al.*, 2003). Glu83 plays a role in locking the divergent loop into a particular conformation (Taylor *et al.*, 1998). The divergent loop provides a distinctive recognition feature which may be important during protein–protein interactions (Dornan *et al.*, 1999). The residues Ser49, Gly50 and Leu53 in the divergent loop are known to frequently be present in protein-binding sites (Betts & Russell, 2003), further suggesting that this region may play a role in protein–protein interactions. His54, which is part of the divergent loop (residues 48–54), is located 3.86 Å from the sulfhydryl group of Cys168. This arrangement of two Cys residues and a His residue may provide a suitable coordination for zinc, as observed previously for a zinc–alcohol dehydrogenase complex (Cho *et al.*, 1997).

Although the structure of another plant cyclophilin from *Arabidopsis* (AtCyp38) has recently been elucidated (Vasudevan *et al.*, 2012), our studies show significant differences between the structures of TaCypA-1 and AtCyp38. The conserved cysteine residues are absent in AtCyp38. Also, the cyclophilin domain of TaCypA-1 is an active peptidyl-prolyl *cis–trans* isomerase but AtCyp38 does not show any enzymatic activity. Alignment of the cyclophilin domains of TaCypA-1 and AtCyp38 (residues 238–423; PDB entry 3rfy) reveals an r.m.s.d. of 1.40 Å over 87 residues, implying that the structures of these two plant proteins are not conserved.

To conclude, this study is the first to elucidate the crystal structures of TaCypA-1 and its complex with CsA. On binding to the active site, CsA does not cause any significant confor-



(*a*) (*b*)

Figure 6
(*a*) The structure of native TaCypA-1 (green) aligned with those of cyclophilins from *H. sapiens* (hCypD, PDB entry 2z6w, blue; hCypA, PDB entry 3k0m, purple; Kajitani *et al.*, 2007; Fraser *et al.*, 2009), *C. elegans* (CeCyp-3, PDB entry 2igv, orange; Wear *et al.*, 2007), *P. falciparum* (PfCyp, PDB entry 1qng, pink; Peterson *et al.*, 2000), *B. taurus* (residues 1–172 of Cyp40, PDB entry 1ihg, cyan; Taylor *et al.*, 2001), *M. tuberculosis* (MtPpiA, PDB entry 1w74, red; Henriksson *et al.*, 2004) and *B. malayi* (BmCyp, PDB entry 1a58, yellow; Taylor *et al.*, 1998). (*b*) Close-up view of the divergent-loop region (residues 48–54).

mational change to the TaCypA-1 structure. This study also demonstrates that this protein is enzymatically active and possesses peptidyl-prolyl *cis–trans* isomerase activity. The divergent loop, comprising seven residues, is absent in human cyclophilins (hCypA and hCypD) but is present in TaCypA-1, bovine Cyp40 and nematode CeCyp-3. Although the role of the divergent loop needs to be validated, it may play an important role in the interaction of TaCypA-1 with other proteins. The presence of the divergent loop in TaCypA-1, bovine Cyp40 and nematode Cyps (CeCyp-3 and BmCyp) and its absence in human cyclophilins suggests that despite substantial sequence conservation among different cyclophilins these proteins have evolved to play unique and specific roles in the cell. Further studies using site-directed mutagenesis are required to dissect the significance of the divergent loop in maintaining the structural and functional organization of TaCypA-1.

This work was supported by Nuclear R&D Programs (Grant No. 2011-0006283) awarded to Korea Research Institute of Bioscience and Biotechnology (KRIBB) through the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology (MEST). This work has been also supported by the 'SEED' Program of the Korea Research Council of Fundamental Science and Technology, the 'National Space Lab' Program through the NRF of Korea funded by the MEST (Grant No. 2012-0009096), the KIST Institutional Program (Grant No. 2Z03530) and the KRIBB Research Initiative Program. Financial assistance from the Department of Biotechnology (Grant No. BT/PR-10150/FNS/20/364/2007), Ministry of Science and Technology, Government of India is gratefully acknowledged. Financial support received from JNU through 'Capacity Build-up' and 'PURSE' is also acknowledged.

References

Adams, P. D. *et al.* (2010). *Acta Cryst.* D**66**, 213–221.
Ahn, J. C., Kim, D.-W., You, Y. N., Seok, M. S., Park, J. M., Hwang, H., Kim, B.-G., Luan, S., Park, H.-S. & Cho, H. S. (2010). *BMC Plant Biol.* **10**, 253.
Betts, M. J. & Russell, R. B. (2003). *Bioinformatics for Geneticists*, edited by M. R. Barnes & I. C. Gray, ch. 14. Chichester: John Wiley. doi:10.1002/0470867302.ch14.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Brandts, J. F., Halvorson, H. R. & Brennan, M. (1975). *Biochemistry*, **14**, 4953–4963.
Breiman, A., Fawcett, T. W., Ghirardi, M. L. & Mattoo, A. K. (1992). *J. Biol. Chem.* **267**, 21293–21296.
Cho, H., Ramaswamy, S. & Plapp, B. V. (1997). *Biochemistry*, **36**, 382–389.
Chou, I. T. & Gasser, C. S. (1997). *Plant Mol. Biol.* **35**, 873–892.
Dominguez-Solis, J. R., He, Z., Lima, A., Ting, J., Buchanan, B. B. & Luan, S. (2008). *Proc. Natl Acad. Sci. USA*, **105**, 16386–16391.
Dornan, J., Page, A. P., Taylor, P., Wu, S., Winter, A. D., Husi, H. & Walkinshaw, M. D. (1999). *J. Biol. Chem.* **274**, 34877–34883.
Dornan, J., Taylor, P. & Walkinshaw, M. D. (2003). *Curr. Top. Med. Chem.* **3**, 1392–1409.
Dutta, T., Kaur, H., Singh, S., Mishra, A., Tripathi, J. K., Singh, N., Pareek, A. & Singh, P. (2011). *Food Chem.* **128**, 450–457.
Edvardsson, A., Eshaghi, S., Vener, A. V. & Andersson, B. (2003). *FEBS Lett.* **542**, 137–141.
Fischer, G., Bang, H. & Mech, C. (1984). *Biomed. Biochim. Acta*, **43**, 1101–1111.
Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. & Schmid, F. X. (1989). *Nature (London)*, **337**, 476–478.

Fraser, J. S., Clarkson, M. W., Degnan, S. C., Erion, R., Kern, D. & Alber, T. (2009). *Nature (London)*, **462**, 669–673.
Fulgosi, H., Vener, A. V., Altschmied, L., Herrmann, R. G. & Andersson, B. (1998). *EMBO J.* **17**, 1577–1587.
Galat, A. (1999). *Arch. Biochem. Biophys.* **371**, 149–162.
Grebe, M., Gadea, J., Steinmann, T., Kientz, M., Rahfeld, J. U., Salchert, K., Koncz, C. & Jürgens, G. (2000). *Plant Cell*, **12**, 343–356.
Harding, M. W., Galat, A., Uehling, D. E. & Schreiber, S. L. (1989). *Nature (London)*, **341**, 758–760.
Henriksson, L. M., Johansson, P., Unge, T. & Mowbray, S. L. (2004). *Eur. J. Biochem.* **271**, 4107–4113.
Johnson, J. & Bhave, M. (2004). *J. Cereal Sci.* **40**, 137–150.
Johnson, J., Clarke, B. & Bhave, M. (2001). *J. Cereal Sci.* **34**, 159–171.
Kajitani, K., Fujihashi, M., Kobayashi, Y., Shimizu, S., Tsujimoto, Y. & Miki, K. (2007). *Proteins*, **70**, 1635–1639.
Kallen, J., Spitzfaden, C., Zurini, M. G., Wider, G., Widmer, H., Wüthrich, K. & Walkinshaw, M. D. (1991). *Nature (London)*, **353**, 276–279.
Ke, H., Zydowsky, L. D., Liu, J. & Walsh, C. T. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 9483–9487.
Kieffer, L. J., Thalhammer, T. & Handschumacher, R. E. (1992). *J. Biol. Chem.* **267**, 5503–5507.
Kofron, J. L., Kuzmic, P., Kishore, V., Colon-Bonilla, E. & Rich, D. H. (1991). *Biochemistry*, **30**, 6127–6134.
Kumari, S., Singh, P., Singla-Pareek, S. L. & Pareek, A. (2009). *Mol. Biotechnol.* **43**, 195–204.
Lippuner, V., Chou, I. T., Scott, S. V., Ettinger, W. F., Theg, S. M. & Gasser, C. S. (1994). *J. Biol. Chem.* **269**, 7863–7868.
Luan, S., Lane, W. S. & Schreiber, S. L. (1994). *Plant Cell*, **6**, 885–892.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Peterson, M. R., Hall, D. R., Berriman, M., Nunes, J. A., Leonard, G. A., Fairlamb, A. H. & Hunter, W. N. (2000). *J. Mol. Biol.* **298**, 123–133.
Romano, P. G., Horton, P. & Gray, J. E. (2004). *Plant Physiol.* **134**, 1268–1282.
Sekhar, K., Priyanka, B., Reddy, V. D. & Rao, K. V. (2010). *Plant Cell Environ.* **33**, 1324–1338.
Shapiguzov, A., Edvardsson, A. & Vener, A. V. (2006). *FEBS Lett.* **580**, 3671–3676.
Sheldon, P. S. & Venis, M. A. (1996). *Biochem. J.* **315**, 965–970.
Singh, S., Singh, G., Singh, P. & Singh, N. (2008). *Food Chem.* **108**, 130–139.
Taylor, P., Dornan, J., Carrello, A., Minchin, R. F., Ratajczak, T. & Walkinshaw, M. D. (2001). *Structure*, **9**, 431–438.
Taylor, P., Page, A. P., Kontopidis, G., Husi, H. & Walkinshaw, M. D. (1998). *FEBS Lett.* **425**, 361–366.
Trandinh, C. C., Pao, G. M. & Saier, M. H. Jr (1992). *FASEB J.* **6**, 3410–3420.
Vasudevan, D., Fu, A., Luan, S. & Swaminathan, K. (2012). *Plant Cell*, **24**, 2666–2674.
Wear, M. A., Kan, D., Rabu, A. & Walkinshaw, M. D. (2007). *Angew. Chem. Int. Ed. Engl.* **46**, 6453–6456.
Wu, H., Wensley, E. & Bhave, M. (2009). *Plant Sci.* **176**, 420–432.