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The expression of the large rice FK506 binding proteins (FKBPs) demonstrate tissue specificity and heat stress responsiveness

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Abstract

The FK506 binding proteins (FKBPs) are abundant and ubiquitous proteins belonging to the large peptidyl prolyl *cis–trans* isomerase superfamily. In this study we have identified and characterized the expression of three large FKBPs in rice: the rice rFKBP64, rFKBP65 and rFKBP75. These FKBPs contain three FKBP12-like domains and a tetratricopeptide repeat (TPR) domain. The expression of the rice FKBPs was found to be regulated by heat stress in various organs. The expression of rFKBP64 at RNA level was elevated by heat stress in roots and shoots and low in mature leaves.

The expression of rFKBP65 was detected at the RNA level only after heat stress in all cultivars whereas at the protein level there were differences in the expression between the rice cultivars. The rFKBP75 was expressed at the RNA in all tissues before and after heat stress and the rFKBP75 protein appears to be more abundant after heat stress. The only FKBP to be expressed in seeds was the rFKBP75 which was higher in the embryos and endosperm of dry seeds then in the same organs separated from imbibed seeds, indicating that the protein is important in the steps of seed maturation.

In this study we have characterized three large rice FKBPs and have demonstrated that rice FKBPs are heat stress induced and differentially expressed in various tissues indicating specific physiological functions.

Keywords: FK506 binding proteins (FKBP); Rice; Immunophilins; Heat stress; Peptidyl prolyl *cis–trans* isomerases

1. Introduction

The FK506 binding proteins (FKBPs) belong to the superfamily of peptidyl prolyl *cis–trans* isomerases (EC 5.2.1.8) which is present in all organisms and almost all subcellular compartments [1,2]. PPIases catalyze the rotation of the peptide bond preceding proline in the proteins and their enzymatic activity was extensively studied [3,4].

The FKBPs are a distinct set of cellular receptors which bind the immunosuppressive drugs FK506 and rapamycin. The complexes formed by the FKBPs and their ligands are the functional modules for immunosuppression and therefore the FKBPs are named also immunophilins. When FKBPs bind these drugs, their peptidyl prolyl *cis–trans* isomerase (PPIase) activity is inhibited [5].

During the past decade, a growing number of immunophilins from mammalian, bacterial and plants have been characterized [1,6–8]. The high level of conservation and ubiquitous distribution suggest that these proteins participate in essential cellular processes.

The FKBP family consists of multiple members that are distinguished by their molecular weights ranging from 12 kDa (hFKBP12) to over 77 kDa (wFKBP77) [9,10].

They are found in almost all subcellular locations including ER [11–13], chloroplast [14–16], cytosol [9,17], nucleus [13,18,19] and mitochondria [20].

High molecular weight FKBP members have been characterized in a few plants species such as *Arabidopsis* [21–23] wheat [10,24], maize [25] and bean [11].

The role of several FKBPs in higher plants has started to emerge. For instance, the *Arabidopsis* mutant *pas1* displays abnormal developmental pattern [22]. Disruption of the *Arabidopsis* AtFKBP42 which is the twisted dwarf (TWD1)

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mutant caused developmental defects [23,26,27]. Recently it was shown that AtFKBP42 functionally interacts with the vacuolar ABC transporters regulating their activity [23,26]. A chloroplast FKBP has been shown to interact with a photosynthetic electron carrier affecting the accumulation of the Rieske subunit of cytochrome bf complex protein, an important component of the photosynthetic electron transport chain [15].

Recent completion of the *Arabidopsis* genome revealed 23 FKBPs present in the genome, 16 being single domain FKBP and 7 members with multiple domains FKBPs [2].

The single domain FKBPs contain only one FKBP12-like domain whereas the multiple domain members contain several FKBP12-like domains in addition to the TPR and in some cases the calmodulin binding domains.

The domain structure of the multiple domain FKBPs in *Arabidopsis* and wheat resemble the mammalian FKBP59 which is a member of the steroid receptor complex [28]. However, the mammalian FKBP59 possess only two FKBP12-like domains, whereas the wheat and *Arabidopsis* possess three FKBP12-like domains [24]. Similar to the mammalian FKBP59, the wheat FKBP73 and FKBP77 possess PPIase activity which is inhibited by the drug FK506 [24] bind to HSP90 and to dynein via their TPR domain and PPIase domain respectively [29,30]. Expression analysis of the *Arabidopsis* FKBPs has shown that they are expressed in most tissues at various intensities [2], and studies of the wheat FKBPs have shown that FKBP73 is expressed under normal growth conditions whereas FKBP77 is heat stress induced [10].

This study is the first to report on molecular cloning and characterization of in vivo expression pattern of the large multi domain rice FKBP genes.

2. Materials and methods

2.1. Computer methods

In order to identify rice putative FKBPs proteins, sequence homology searches amongst the entries in the GeneBank database were performed using the BLAST server at the National Center for Biological Information (NCBI, http://ncbi.nlm.nih.gov). Sequence alignments were done with the human, hFKBP12 (NP_000792); *Arabidopsis*, AtFKBP12 (AAB57847); the wheat, wFKBP73 first FKBP12-like domain (X86903; FKBP12-like: 42–148 amino acid (a.a.)) and the full wFKBP73. Multiple alignments were done using the programs Clustal W EMBL-EBI provided by European Bioinformatics Institute and the Box Shade 3.21(http://Ch.EMBnet.org).

Rice sequences that showed over 40% identity to the above sequences were considered as putative rice FKBPs.

2.2. Sequence analysis

To search for functional domains within the immunophilin protein sequences, SMART (http://smart.embl-heidelberg.de/), PROSITE (http://us.expasy.org/tools/scanprosite/), ELM (http://elm.eu.org/), TargetP (http://www.cbs.dtu.dk/services/TargetP/) and ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) were used.

The M_r and PI of the full-length proteins were calculated by MacVector™ (accelrys).

Whenever DNA sequence was done, it was performed using the Applied Biosystems PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, following the manufacture's instructions. Samples were run in an Applied Biosystems automatic sequencer model (3100 Genetic Analyzer).

2.3. Plant material

Rice seeds (*Oryza sativa* cultivar Bengal, Lebonnet and Cypress) were dehusked and surface sterilized by shaking in a 10% solution of NaOCl for 15 min and then rinsed in 70% ethanol and then three times in sterilized water. The seeds were imbibed in water for 4 h, after which they were placed on a moist sterile filter paper in a sterilized box. They were subsequently germinated in darkness at 25 °C for 7–9 days. The etiolated shoots and roots of the seedlings were harvested and used for RNA and protein extraction. For growth of mature plants, the dehusked seeds were imbibed at 37 °C for 24 h. They were transferred to wet filter paper in Petri dishes containing the wet filter paper and germinated at 25 °C with an 8-h photoperiod. Upon germination, the plantlets were transferred to small pots containing vermiculite. Subsequently the plantlets were transferred to bigger pots containing soil and grown in net house. The plants were watered every other day and fertilizer applied whenever appropriate. Middle leaves were harvested from 1-month-old plants and used for RNA and protein extraction.

For imbibition, the seeds were soaked in water for 12 h. For heat stress treatment, the incubator was set at 42 °C with high humidity to avoid water or drought stress. All tissues that were heat stressed were subjected to this condition for 3 h. For mature leaves, the whole plant was exposed to 42 °C and subsequently the leaves were harvested.

2.4. RNA isolation

Total RNA was isolated from various tissues using SV total RNAs Isolation Kit (Promega, USA). The isolation was carried out according to the manufacturer's specification. All RNA samples were quantified spectrophotometrically at 260 nm using Nano Drop-ND-1000 Spectrophotometer (Rockland, DE). The RNA quality was also checked by 1% agarose gel electrophoresis stained with 0.5 mg/ml ethidium bromide.

2.5. Cloning and expression of rFKBP 64 in Escherichia coli

Primers were designed for one of the identified rFKBP (AK103172) to amplify the whole of the coding sequence, which is 1746 bp long. The primers were designed in such a way that they contained BamHI restriction site containing in frame start and stop codons of the DNA, from 106 to 1852 bp (5′-CGCGGGATCCATGGACGACGACTTCGAG-3′ and 5′-

CGCGGATCCTTAGGCAGCAGTAACAGG-3′). In addition, nested primers were designed to amplify the middle part of gene starting from 499 to 1231 bp to yield a fragment of 732 bp (5′-GTTCACCTCCAACTATTCCAGCC-3′ and 5′-GGTGG-AATACAGCGAGATCC-3′). A two-step RT-PCR method was used to amplify the gene from mRNA. In the first step, 5 μg of total RNA was used for the synthesis of the first strand cDNA using oligo(dT)15 primer and M-MLV Reverse Transcriptase according to the manufacturer's instructions (Promega, USA). The cDNA was diluted two times and 2 μl used for the second step (PCR). In the second step, the full gene and nested primers were used to amplify the cDNA. It should be noted that wheat cDNA was prepared along side the rice to serve as positive control and amplified with wFKBP73 primers [24].

The PCR reactions were performed on a PTC-200 PCR machine Peltier Thermal Cycler (MJ Research, USA) in a final volume of 25 μl containing 0.2 mM dNTPs, 25 pmol of each primer and 2.5 units of Taq polymerase. The cycling parameters were an initial temperature of 94 °C for 3 min, 35 cycles, each comprising of denaturation at 94 °C for 45 s, annealing temperature of 55 °C for 45 s and synthesis temperature of 72 °C for 90 s. This was followed by extension period of 5 min at 72 °C. The PCR products were analyzed by standard agarose gel electrophoresis. The amplification of the rice cDNA with the full gene primers generated the expected fragment of 1742 bp, corresponding to the coding region of rFKBP64 gene. The nested primers also amplified the expected band of 700 bp further confirming that the amplified product is actually the rFKBP64 gene. The comparison of the nucleotide sequence of the rFKBP64 cloned in PTZ57R/T with the data base sequence showed that the two sequences matched except for one silent mutation for serine 86. The fragment digested with BamHI and ligated with pGEX2TK expression vector (Pharmacia Biotech) was designated pGEX2TK-rFKBP64. The expression of pGEX2TK-rFKBP64 in BL21 cells resulted in the production of the expressed protein was affinity purified using GST system and a protein of ca.77 kDa was obtained after digesting with thrombin to remove the GST portion. The plasmid was used to transform *E. coli* BL21 cells and the conditions for the expression of the recombinant protein were optimized by varying IPTG concentration, induction period and OD. The best induction was obtained by 0.5 Mm IPTG OD_{595} 0.6 at 28 °C for 2 h. The expressed protein was extracted from transformed cells and purified using Bulk Glutathione Sepharose 4B methods (Pharmacia Biotech) according to manufacturer's manual. Protein quantification was by Bradford method [31] with bovine serum albumin as the standard.

2.6. SDS-PAGE and Western blot

The SDS-PAGE was performed as described [24]. The Western blot was done according to the standard method [32] with wFKBP73 and wFKBP77 antibodies diluted at 1:3000 dilution [24,10]. Labeling was done with anti-rabbit IgG horseradish peroxidase-linked whole antibodies (Pharmacia) diluted 1:5000. Detection was carried out using ECL Western Blotting Detection Reagents and Analysis System (Amersham Biosciences, UK). The apparent molecular weight of the plant protein bands observed in the Western blot were estimated using pre-stained standard marker as reference proteins purchased from PageRuler^{TM} Prestained Protein Ladder, Fermentas, Life Sciences.

2.7. Expression of the rice FKBP genes established by RT-PCR

Primers were designed specific to each of the three rice FKBP genes using the regions of the genes that are non-homologous (Table 1). The primers were analyzed using NTI software program. β-Actin I was chosen as an internal standard and was found amplified using actin primers designed according to *Arabidopsis* [33] and the actin sequence was fully conserved in rice found conserved in rice. The summary of the primers used are shown in Table 1. A two step semi-quantitative RT-PCR method was used to determine the expression of the three rice FKBP genes at the mRNA level. Rice cDNA was prepared from the etiolated shoots and PCR performed as described in Section 2.6. The PCR products were analyzed by standard agarose gel electrophoresis. The amplified products of each gene were purified from the gel and cloned into pTZ57R/T (Promega) and sequenced as described in Section 2.2. The tissues used were shoot and roots of etiolated seedlings and mature leaves before and after heat stress treatment. For this study, β-actin was used as an internal standard. The internal standard was calibrated by varying the number of PCR cycles ranging from 18 to 35 to determine the

Table 1
Primers designed for identification of the rice FKBPs

Gene	Primer pairs and sequences (5′–3′)	Specific location in the gene (nt)	Expected size of PCR product (bp)
rFKBP64	Fwd AGATGACCAAGCAGCCGA Rev TCAGCTAATGCTCTCTTGATCTT	1765–2094	329
rFKBP65	Fwd GGAGGAAGCTGGAACACATG Rev TTATGGCATTTTTGCTCT	1762–2083	321
rFKBP75	Fwd ATTTCGAAGCTAAGCAAGC Rev TTCCTTTGTATGTTTCAATC	1868–2322	454
β-Actin	Fwd GGTAACATTGTGCTCAGTGGTGG Rev AACGACCTTAATCTTCATGCTGC	976–1084	108

Primers were designed according to sequences from the NCBI database.

exponential phase of the RT-PCR. Twenty five cycles were used to check expression pattern of the three genes in different tissues using β-actin as the internal standard. For semi-quantitative analysis, the intensity of the bands was determined by densitometry using Image Master ID Prime Software version 4.1 (Amersham Pharmacia Biotech, USA)

2.8. Extraction of plant protein

Protein extracts were prepared from 50 to 100 mg of fresh weight of plant tissues. The plant material was ground in liquid nitrogen with mortar and pestle and suspended in 300 μl of extraction buffer (50 mM Tris–HCl pH 7.6, 300 mM sucrose, 100 mM NaCl, 10 mM EDTA pH 8.0, 1 mM DTT and 1× protein inhibitors cocktail (Roche Diagnostics GmbH Germany)). The crude extract was cleared by two successive centrifugations at 14,000 rpm for 10 min. The protein was quantified by Bradford method [31] with bovine serum albumin as the standard.

3. Results and discussion

3.1. Sequence alignment of the large rice FKBPs

In order to obtain information about the putative FKBPs present in the rice genome we have performed a combined search in the rice database with three FKBP sequences: the human FKBP12 which is used to define the FKBP family (Galat 2000, 2003), the *Arabidopsis* FKBP12 (Faure, 1998) and the wheat FKBP73 whose first FKBP12-like domain was demonstrated to possess PPIase activity and bind the drug FK506 [24]. The three large rice FKBPs described in this study, the rFKBP64 (XP_483423), rFKBP65 (CAE05842) and rFKBP75 (XP_465763) showed the highest homology to the human, *Arabidopsis* and first wheat FKBP12-like domain of wFKBP73 (Fig. 1). The screen with the hFKBP12 revealed homology only with the three large FKBPs whereas the other FKBPs were detected by homology to either the *Arabidopsis* FKBP12 and/or the first FKBP12-like domain of the large wFKBP73.

The rice genes which showed more than 40% identity to the first wheat FKBP12-like domain are represented schematically (Fig. 1). All of them contain a putative FKBP12-like domain: six FKBP12-like genes are of low molecular weight. Two of the low molecular weight FKBPs possess transmembranal or secretion signals, three large FKBPs possess tetratricopeptide (TPR) repeats and two of them also possess calmodulin binding motifs. Sequence comparison showed that the three large FKBPs characterized in this study show the highest identity (between 81 and 91%) to the first FKBP12-like domain of the wFKBP73 (Fig. 1) and between 74 and 87% when the sequence comparison was done to the full sequence of wFKBP73 (Table 2). Sequence comparison among the large FKBPs of wheat, *Arabidopsis* and rice revealed high homology among the large FKBPs of these organisms (Table 2). The homologue of the wFKBP73 [24] and *Arabidopsis* ROF1 [21] is the rFKBP64 showing 87 and 77%

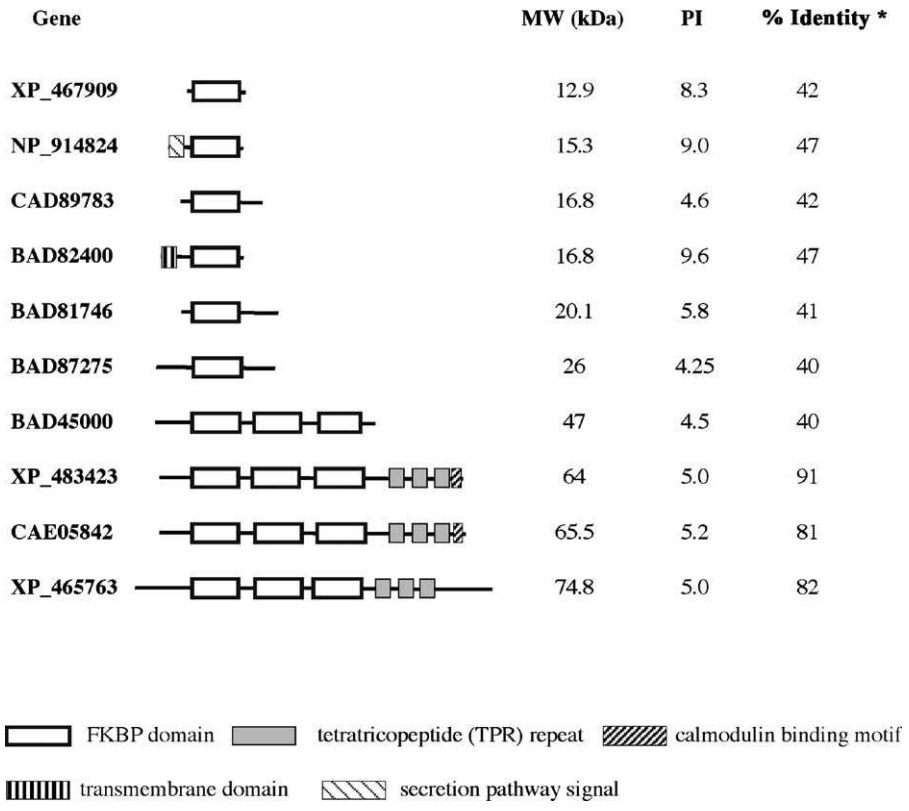


Fig. 1. Domain architecture of the rice FKBP family immunophilins. The molecular weight (MW), the isoelectric point (PI) and % identity (*) to the wheat FKBP73 (X86903) first FKBP12-like domain (42–148 a.a.) are indicated. The National Center for Biotechnology Information database was used to search for rFKBPs (http://ncbi.nlm.nih.gov).

Table 2
Comparison of identity (%) of the deduced amino acid among the rice rFKBP 64/65/75, wheat wFKBP 73/77 and *Arabidopsis* ROF1/2

Protein	wFKBP73	wFKBP77	ROF1	ROF2
rFKBP64 (XP_483423)	87	72	77	70
rFKBP65 (CAE05842)	74	79	73	69
rFKBP75 (XP_465763)	74	79	70	66

identity, respectively (Table 2). Both the rFKBP65 and rFKBP75 show 79% identity to the wheat heat stress induced wFKBP77 [10] and to the ROF2 (above 66%) which is also a heat stress induced FKBP (manuscript in preparation).

In this study we have further characterized the three rice genes showing the highest homology to the FKBP12-like domain characterized in human and plants.

Chromosomal mapping of these sequences was done by using the Knowledge-base Oryza Molecular Biological Encyclopedia (KOME) [34]. Their chromosomal location as defined by the mapping of the cDNA clone to the Japonica rice genome sequence was found to be 75219–79699 bp in chromosome 8 for rFKBP64 (XP_483423, nucleotide accession AK103172), 74507–78577 bp in chromosome 4 for rFKBP65 (CAE05842, nucleotide accession. AK100844) and 28982–33361 bp in chromosome 2 for rFKBP75 (XP_465763, nucleotide accession AK073233).

The predicted molecular weight of the identified rice sequences as determined from their deduced amino acid sequences using Expasy program (http://au.expasy.org/tools/pi_tool.html) were found to be 64.1, 65.5 and 74.8 kDa for rFKBP64, rFKBP65 and rFKBP75, respectively.

This designation was based on the fact that in previous publications involving both animal and plant FKBPs, there is consistency in naming, where generally the proteins are named FKBP with prefix letters to indicate the species origin and a suffix number to indicate the molecular weight. However, in some studies apparent molecular weight is used while in others the calculated molecular weight of the full protein predicted from cDNA deduced amino acid sequence is used, as in this study.

The alignment of the deduced amino sequence of the three putative rice FKBPs with those of wheat FKBP73, wheat FKBP77 and human FKBP59, shows high homology between them (Fig. 2). The three rFKBPs have two FKBP12-like domains (a.a. 46–152, 162–269 for rFKBP64; 50–156, 166–273 for rFKBP65 and 95–201, 211–318 for rFKBP75) similar to those found in wFKBP73, wFKBP77 and hFKBP59 and a third one (a.a. 279–389 for rFKBP64; 283–392 for rFKBP65 and 328–437 for rFKBP75) similar to that found only in wFKBP73 and wFKBP77 [10,24] (Figs. 1 and 2). The first domain of each of the three FKBPs was found to contain all the 10 amino acids of FKBP12 shown to be involved in FK506 binding and in maintaining its hydrophobic core [35]. These amino acids are known to be highly conserved in the FKBP family [35,36] and hence the first domain is a classical FKBP domain responsible for FK506 binding and PPIase activity. The third domain has conserved only five amino acids essential for FK506 binding as their wheat homologues whereas the second domain has maintained only 1 critical amino acid out of 10 [35,24].

Further comparison of the wheat and rice FKBPs showed that the three rFKBPs genes contain a tetratricopeptide (TPR) motif that exhibited high homology (>90%) with those identified in wheat FKBPs [10,24]. The motifs are located toward the C-terminal of genes in the following residues: rFKBP64 (K_{409}–F_{442}, N_{458}–A_{491}, L_{492}–K_{536}), rFKBP65 (K_{414}–F_{447}, N_{463}–A_{496}, L_{497}–K_{541}) and rFKBP75 (K_{457}–F_{492}, N_{508}–A_{541}, F_{542}–K_{586}). Since a high homology between the wheat and rice TPR domains is observed, we can assume that the rice FKBPs interact also with HSP90 as shown previously for wFKBP73 and wFKBP77 [29,37].

The rice FKBP64 and 65 show identical amino acid with the wheat FKBP73 on the calmodulin binding domain motif (CaMBD) spanning 17 a.a. K_{530}–F_{546} (Fig. 2b) [37], whereas the rFKBP75 shows no such identity.

A calmodulin-binding domain (CaMBD) was shown to be located at the C-terminal of wFKBP73 [24] and conserved in the FKBPs of *Arabidopsis* ROF1, ROF2 and rice FKBP64 and FKBP65 (Fig. 2b). The enzymatic activity of this domain was demonstrated in an *in vitro* assay and further proved by a deletion of the motif region resulting in abrogation of calmodulin binding in wheat and maize [25,37]. A variety of CaMBD are found in plant proteins. Yet, a conserved and unique CaMBD motif is found within members of the same family such as the glutamate decarboxylase (GAD) [38].

It was interesting to note that rFKBP75 has a unique sequence (amino acids 22–79) at the N-terminal, which is glycine rich. The glycine rich peptide shows a glycine repeat sequence GGx (where x is any other amino acid). Similar repeat sequences have been observed in other plant species including *Cucumis sativas* [39] and *Arabidopsis* [40,41]. In all these cases the glycine repeat has been associated with consensus targeting for endoplasmic reticulum. This strongly suggests that rFKBP75 is localized at the endoplasmic reticulum and this is further supported by KOME analysis [34], which predicts it to be an ER protein.

3.2. mRNA expression pattern of the rice FKBP64, FKBP65 and FKBP75

In order to determine the expression pattern of the three rice FKBP genes, specific primers which can differentiate between them were designed (Table 1). The expected PCR products: 329 bp (rFKBP64), 321 bp (rFKBP65) and 454 bp (rFKBP75) were sequenced and proven to correspond to the expected data base sequences.

The expression pattern was determined at the mRNA level and the β-actin primers were used as internal standard for semi-quantification purposes (see Section 2).

The RT-PCR analysis performed on the three rice cultivars: Bengal, Lebonnet and Cypress showed a similar pattern being differentially expressed and affected by heat stress. Only the rice cultivar Bengal is represented in Fig. 3. In shoots, rFKBP64 and rFKBP65 transcripts are in very low amounts before the heat treatment (Fig. 3, lanes 2 and 3). However, after exposure

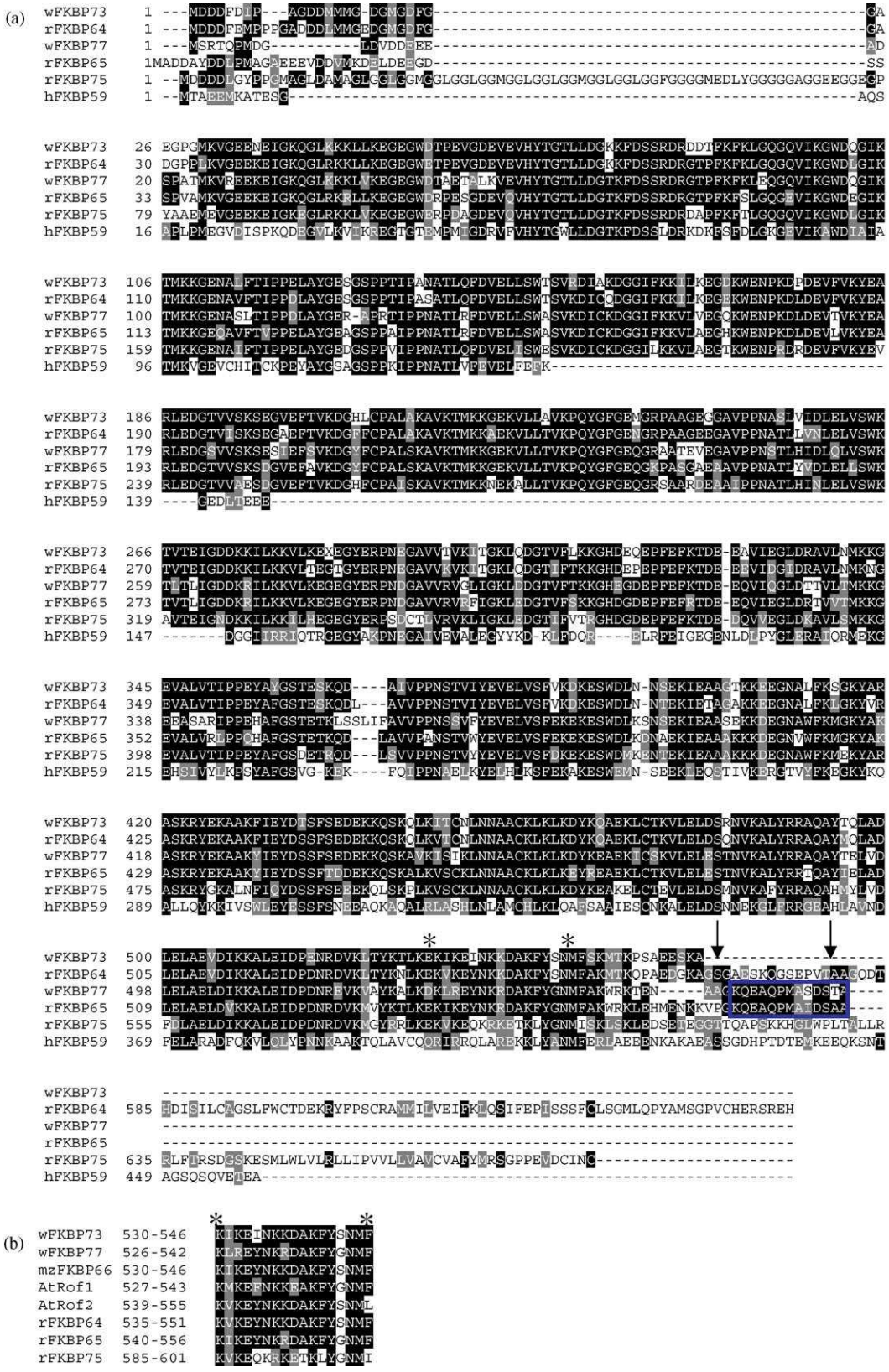
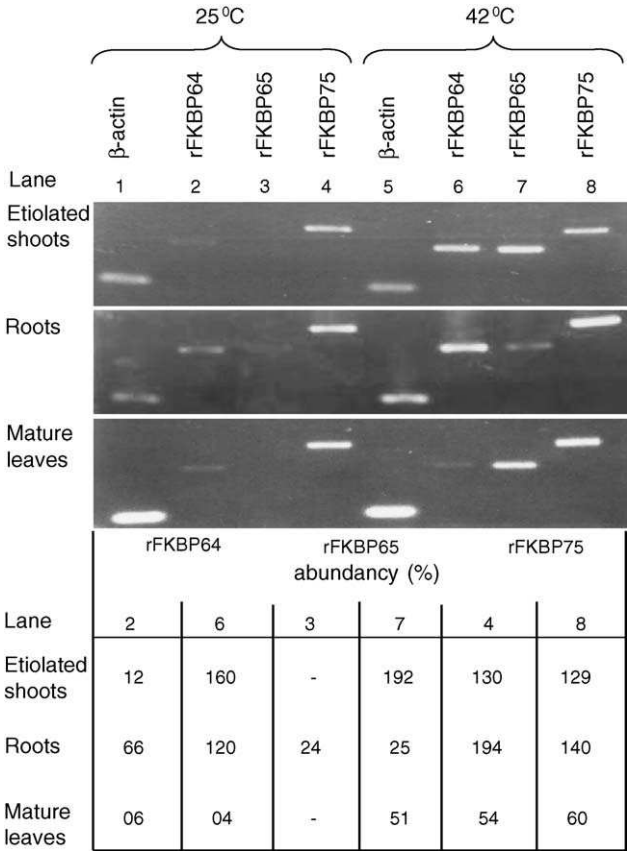


Fig. 2. Alignment of rice FKBPs amino acid sequences with wheat large FKBPs and human FKBP59. (a) The amino acid sequences of rFKBP64 (XP_483423, AK103172), rFKBP65 (CAE05842, AK100844) and rFKBP75 (XP_465763, AK073233) are aligned with wFKBP73 (X86903), wFKBP77 (Y07636) and hFKBP59 (BC007924). Black and gray boxes indicate residues that are identical or similar. Dots indicate gaps introduced to allow optimal alignment of the sequences. Arrow indicates the region of identity between wFKBP77 and rFKBP65 and (*) the region against which the synthetic antibodies of wFKBP77 were raised. (b) Multiple alignment of the conserved CaMBD amino acid sequences of plant FKBPs. The conserved CaMBD amino acids of wFKBP73 (X86903), wFKBP77 (Y07636), maize FKBP66 [25], *Arabidopsis* AtRof1 (NM_113429), AtRof2 (NM_124233) were aligned with those of rFKBP64, rFKBP65 and rFKBP75. Conserved and similarly charged amino acids are indicated with black and gray boxes, respectively.



	rFKBP64		rFKBP65		rFKBP75	
	abundancy (%)					
Lane	2	6	3	7	4	8
Etiolated shoots	12	160	-	192	130	129
Roots	66	120	24	25	194	140
Mature leaves	06	04	-	51	54	60

Fig. 3. Expression of rice FKBPs in shoots, roots and leaves after exposure to 42 °C. Semi-quantitative RT-PCR analysis was performed on rice cDNA for each of the rFKBP genes (Table 1). β-Actin (180 bp) (lanes 1 and 5), rFKBP64 (329 bp) (lanes 2 and 6), rFKBP65 (321 bp) (lanes 3 and 7) and rFKBP75 (454 bp) (lanes 4 and 8). RNA was extracted from shoots, roots and leaves and 25 cycles were run on the PCR. Relative abundances (%) of rice FKBPs transcripts were obtained after quantification (by densitometry using Image Master ID Primer Software) by dividing the observed signal of each gene with that of β-actin. The results are presented for Lebonnet cultivar.

to 42 °C, their level increases significantly (lanes 6 and 7); rFKBP64 increasing by almost 13-fold while rFKBP65 raised from an undetectable level to a level which is two-fold higher relative to β-actin (Fig. 3). The rFKBP75 transcripts are not affected by heat treatment (Fig. 3, lanes 4 and 8).

In roots, the pattern of expression differs slightly as compared to shoots. While rFKBP64 is present before heat shock, the level of rFBP65 is low (Fig. 2, lanes 2 and 3). After exposure to 42 °C, the level of rFKBP64 increases by almost two fold, and that of rFKBP65 remains constant. Therefore, although both rFKBP64 and rFKBP65 were shown to be heat stress induced, they show tissue specificity, rFKBP64 being highly induced in both tissues whereas rFKBP65 is induced only in shoots. The transcripts level of rFKBP75 is similar under the two sets of conditions in shoots and roots.

The expression pattern in mature leaves resembles that observed in shoots. The rFKBP64 level is very low and that of rFKBP65 is undetectable before heat treatment (Fig. 3). After heat shock, the level of rFKBP64 remains low whereas that of rFKBP65 increases significantly.

The semi-quantitative RNA analysis indicates that rFKBP64 is heat induced in shoots and roots but not in leaves. The rFKBP65 is heat induced in shoots and leaves and less in roots. In all three tissues, rFKBP75 is expressed at both temperatures.

3.3. Expression of the rice FKBPs proteins

Protein extracts were prepared from tissues of three rice cultivars, Lebonnet, Cypress and Bengal in order to assess for intraspecific variation among cultivars which are known to grow at different climatic conditions. In order to determine protein expression pattern, we used polyclonal antibodies raised against the wFKBP73. The choice of wFKBP73 antibodies was based on the fact that they are able to recognize the purified recombinant rFKBP64 protein (Fig. 4a and b) and it was expected that they will recognize the other two rice FKBPs due to the high sequence homology between them.

Since the sizes of the rFKBP64 and rFKBP65 are not separable on PAGE-SDS gels, there was a need to use specific antibodies that will distinguish between them. This was achieved by using specific antibodies raised against a synthetic peptide located at the C-terminal of the wFKBP77 protein [10]. Sequence alignment revealed that only the rFKBP65 has an identical amino acids sequence with wFKBP77 at the C-terminus (GKQEAQPMASDSTA, Fig. 2, see arrows starting from amino acid 555 of rFKBP65). As can be seen, the anti-FKBP77 could not recognize the rFKBP64 (Fig. 4c). At the calculated size of ~65 kDa there two proteins (rFKBP65 accession CAE05842) and rFKBP64 accession XP_483423 (Fig. 1). As it was shown that the antibodies do not recognize the rFKBP64 (Fig. 4c) we assume that the specific antibodies raised against the synthetic peptide of the wFKBP77 are recognizing the rFKBP65.

The protein expression pattern of the rFKBPs appeared similar in the Cypress and Lebonnet cultivars but different in the Bengal cultivar. The Lebonnet cultivar is also representative of the Cypress cultivar (Fig. 4).

The rFKBP75 which is the rFKBP with the largest molecular mass is expressed in shoots and its abundancy increase after heat stress. In young shoots, the pattern of expression is similar in the cultivars tested (Fig. 4d). The rFKBP65 is detected only after heat stress as can be seen by using antibodies which recognize the rFKBP65 (Fig. 4d, lanes 2 and 4). Since the rFKBP64 and rFKBP65 are not separable under these conditions it is hard to estimate the effect of heat on accumulation of rFKBP64 protein. However, since at the RNA level there is accumulation of rFKBP64 after heat stress (Fig. 3) it is reasonable to assume that there may be also an effect on the protein level.

In roots, the rFKBP75 accumulates after heat stress (Fig. 4e). The rFKBP65 could be detected only in the Bengal cultivar after heat stress (Fig. 4e, lane 4). It is possible that expression of rFKBP65 in roots can be used to differentiate between the Bengal and Lebonnet cultivars. The rFKBP64 is expressed at 25 °C and at 42 °C in the Lebonnet cultivar. In the Bengal cultivar it is poorly expressed before heat stress and possibly slightly affected by heat stress.

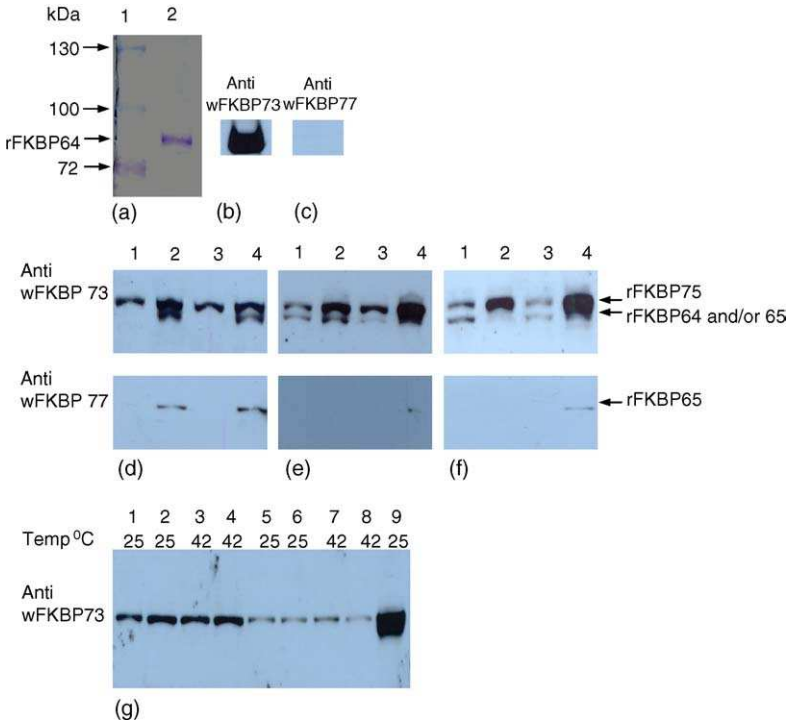


Fig. 4. Tissue specific protein expression of rFKBPs in rice cultivars after exposure to 42 °C. (a) Analysis of rFKBP64 recombinant protein on 7.5% SDS-PAGE stained with Coommassie blue: protein ladder (lane 1), rFKBP64 protein after cleavage with thrombin (lane 2). (b) Western blot of rFKBP64 decorated with anti-wFKBP73. (c) Western blot of rFKBP64 decorated with anti-wFKBP77 antibodies [10]. Western blot of shoots (d), roots (e) and leaves (f) proteins: cv. Lebonnet (lanes 1 and 2) and cv. Bengal (lanes 3 and 4). The protein loaded on lanes 1 and 3 were extracted from plants grown at 25 °C or from plants exposed to 42 °C (lanes 2 and 4). (g) Isolated embryos (lanes 1 and 3) and endosperm (lanes 2 and 4) of dry seeds; imbibed seeds embryos (lanes 5 and 7) and endosperm (lanes 6 and 8); etiolated shoot protein extract (lane 9). The plants were grown at 25 °C (lanes 1, 2, 5, 6 and 9) or the plants were exposed for 3 h at 42 °C (lanes 3, 4, 7 and 8). The dry seeds and imbibed seeds were from the Lebonnet cultivar.

In leaves, the rFKBP75 is expressed in all cultivars and is accumulated after heat stress. rFKBP65 could be detected after heat stress in leaves only in the Bengal cultivar (Fig. 4f). Since the rFKBP64 and rFKBP65 are not separable under these conditions it is hard to estimate the effect of heat on accumulation of rFKBP64 protein.

The expression pattern was tested in embryos and endosperm separated from dry seeds after exposure to heat stress. Rice seeds were found to express the rFKBP75 (Fig. 4g). It appears that the endosperm and the embryo possess comparable amounts of FKBP75. When the seeds were imbibed, the amount of rFKBP75 decreased in both endosperm and embryos. It appears that the rFKBP64 and rFKBP65 are not expressed in the dry and imbibed seeds as can be deduced from the comparison with protein extract of etiolated shoots which show the presence of the rFKBP64 and/or rFKBP65 (Fig. 4g, lane 9).

In rice, similar to the situation in wheat, the FKBPs are probably required in the desiccation stages, the maturization of the embryo and organization of endosperm proteins [42].

In this study we have identified by sequence homology to human, *Arabidopsis* FKBP12 and the wheat FKBP73, 10 rice genes belonging to the FKBP family. Four genes can be classified as low mw (12.9–26 kDa) and six high molecular weight (47–75 kDa). The three genes which show the highest homology to wheat and *Arabidopsis* large FKBPs have been characterized in the paper. All three genes have shown differential expression in the various tissues and their expression was affected by heat.

The largest rFKBP75 which is expected to be localized in the ER, is present in all tissues in relatively high amounts compared to the other two genes before or after heat shock. The observation that the protein accumulates after heat shock in roots and leaves, suggests a possible role in heat stress. Other studies have implicated endoplasmic localized FKBPs such as the bean FKBP15 mRNAs to be elevated by heat shock [11] The observed discrepancy between the relative similar amount of RNA before and after heat stress as compared to significant accumulation of the rFKBP75 protein after heat stress can be explained in several ways such as post transcriptional regulation and/or high stability of the rFKBP75 protein similar to what was documented for the wheat FKBP73 which has a half-life of 45 h [42].

The level of rFKBP65 mRNA was elevated by heat in all rice cultivars in shoots and leaves. However, at the protein level, it was detected in shoots of all cultivars and in roots and leaves only of Bengal cultivar. The rFKBP65 is heat stress induced and almost undetectable when plants are grown at 25 °C. It is noteworthy that wheat FKBP77 which shares 79% identity with rFKBP65 including its unique C-terminal (GKQEAQPMASD-STA) (Table 2) has also been shown to be heat-stress induced and not detectable at 25 °C [10]. This points to the possibility that these two genes have evolved from a similar origin and have their function conserved.

The observation that rFKBP65 protein is not detected in the leaves and roots of Lebonnet and Cypress cultivars, despite presence of their respective mRNAs, could be explained by speculating that probably the protein in the two cultivars requires more time to accumulate to a detectable level as compared to Bengal. This may be attributed to their different ecological zones where they are grown, the hot tropical conditions for Lebonnet and Cypress and relatively low temperature for Bengal.

The rFKBP64 shows 87%identity to the wFKBP73 (Table 2) and most probably they have evolved from the same ancestor gene.

The rFKBP64 was poorly transcribed before heat stress and after heat shock it accumulated only in shoots and roots (Fig. 3). A discrepancy was observed between the very low levels of RNA in leaves and the detectable amount of rFKBP64 protein in leaves of plants grown at 25 °C which decrease in plants exposed to 42 °C. A differential stability or post-translational regulation of the mature protein can be suggested as a plausible explanation to the observed discrepancy. It is conceivable that the low amounts of RNA are translated and the protein accumulates at 25 °C, whereas at 42 °C either the mRNA is not sufficient to be translated to detectable amounts or the protein translated is labile and cannot be detected.

As recently published there are 52 immunophilins which include the FKBPs and the cyclophilins in *Arabidopsis* [2]. Most of the genes studied appeared to be expressed in all tissues. However, more detailed analyses show a tissue and individual expression pattern for each member as demonstrated in this and other studies. The conservation of the large immunophilin families in all species and the large number of members in each family, imply their importance in the life stages of the organisms. Only recently specific roles of individual immunophilins start to emerge. Future studies on each individual member expressed under specific conditions will reveal its specific protein partners and its function.

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References

[1] A. Galat, A note on clustering the functionally-related paralogues and orthologues of proteins: a case of the FK506-binding proteins (FKBPs), Comput. Biol. Chem. 28 (2004) 129–140.
[2] Z. He, L. Li, S. Luan, Immunophilins and parvulins. Superfamily of peptidyl prolyl isomerase in *Arabidopsis*, Plant Physiol. 134 (2004) 1248–1267.
[3] G. Fisher, T. Tradler, T. Zarnt, The mode of action of peptidyl prolyl isomerase in vivo: binding versus catalysis, FEBS Lett. 426 (1998) 17–20.
[4] J. Fanghanel, G. Fischer, Insights into the catalytic mechanism of peptidyl prolyl cis/trans isomerases, Front. Biosci. 9 (2004) 3453–3478.
[5] S.L. Schreiber, Chemistry and biology of immunophilins and their immunosuppressive ligands, Science 251 (1991) 283–287.
[6] D.A. Fruman, S.J. Burakoff, B.E. Bierer, Immunophilins in protein folding and immunosuppression, FASEB J. 8 (1994) 391–400.
[7] Y. Harrar, C. Bellini, J.D. Faure, Review: FKBPs: at the crossroads of folding and transduction, Trends Plant Sci. 9 (2001) 426–431.
[8] A. Breiman, I. Camus, The involvement of mammalian and plant FK506-binding proteins (FKBPs) in development, Transgenic Res. 11 (2002) 321–335.
[9] R.F. Standaert, A. Galat, G.L. Verdine, S.L. Schreiber, Molecular cloning and overexpression of the human FK506-binding protein FKBP, Nature 346 (1990) 671–674.
[10] I. Kurek, K. Aviezer, N. Erel, E. Herman, A. Breiman, The wheat peptidyl prolyl *cis–trans*-isomerase FKBP77 is heat induced and developmentally regulated, Plant Physiol. 119 (1999) 693–703.
[11] S. Luan, J. Kudla, W. Gruissem, S.L. Schreiber, Molecular characterization of a FKBP-type immunophilin from high plants, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 6964–6969.
[12] S.K. Nigram, Y.T. Jim, B.A. Bierer, K.T. Bush, S.J. Burakoff, Localization of the FK506 binding protein, FKBP 13, to the lumen of the endoplasmic reticulum, Biochem. J. 294 (1993) 511–519.
[13] X. Zhang, Y. Wang, H. Li, W. Zhang, D. Wu, H. Mi, The mouse FKBP23 binds to BiP in ER and the binding of C-terminal domains is interrelated with Ca2+ concentration, FEBS Lett. 559 (1–3) (2004) 57–60.
[14] S. Luan, W.S. Lane, S.L. Schreiber, pCyP B: a chloroplast-localized, heat shock-responsive cyclophilin from *fava bean*, Plant Cell 6 (1994) 885–892.
[15] R. Gupta, R.M. Mould, Z. He, S. Luan, A chloroplast FKBP interacts with and affects the accumulation of Rieske subunit of cytochrome bf complex, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 15806–15811.
[16] H. Fulgosi, A.V. Verner, L. Altschmied, R.G. Herman, B. Anderson, A novel multifunctional chloroplast protein identification of a 40-kDa immunophilin-like protein located in the thylakoid lumen, EMBO J. 17 (1998) 1577–1587.
[17] R. Dwivedi, A. Breiman, E. Herman, Differential distribution of the cognate and heat-stress-induced isoforms of high *M*r *cis–trans* prolyl peptidyl isomerase (FKBP) in the cytoplasm and nucleoplasm, Exp. Bot. 393 (2003) 2679–2689.
[18] Y.-J. Jin, S.J. Burakoff, B.E. Bierer, Molecular cloning of a 25 kDa high affinity rapamycin binding protein, FKBP25, J. Biol. Chem. 267 (1992) 10942–10945.
[19] T. Kuzuhara, M. Horikoshi, A nuclear FK506-binding protein is a histone chaperone regulating rDNA silencing, Nat. Struct. Mol. Biol. 11 (2004) 275–283.
[20] A. Breiman, T.W. Fawcett, M.L. Ghirardi, A.K. Mattoo, Plant organelles contain distinct peptidylprolyl *cis*, *trans*-isomerases, J. Biol. Chem. 267 (1992) 21293–21296.
[21] V.A. Vucich, C.S. Gasser, Novel structure of high molecular weight FK506 binding protein from *Arabidopsis thaliana*, Mol. Gen. Genet. 252 (1996) 510–517.
[22] P. Vittorioso, R. Cowling, J.D. Faure, M. Caboche, C. Bellini, Maturation in the *Arabidopsis* pasticcino-1-gene, a new FKBP-like protein, has a dramatic effect on plant development, Mol. Cell. Biol. 18 (1998) 3034–3043.
[23] M. Geisler, H.U. Kolukisaoglu, R. Bouchard, K. Billion, J. Berger, B. Saal, N. Frangne, Z.K. Kalman, C. Koncz, R. Dudler, J.J. Blakeslee, A.S. Murphy, E. Martinoia, B. Schulz, TWISTED DWARF1, a unique plasma membrane-anchored immunophilin-like protein, interacts with *Arabidopsis* multidrug resistance-like transporters AtPGP1 and AtPGP19, Mol. Biol. Cell 14 (2003) 4238–4249.
[24] O. Blecher, N. Erel, I. Callebaut, K. Aviezer, A. Breiman, A novel wheat peptidyl prolyl isomerase: cDNA isolation, structure, enzymatic activity and expression, Plant Mol. Biol. 32 (1996) 493–504.
[25] G. Hueros, J. Rahfeld, F. Salamini, R. Thompson, A maize FK-506-sensitive immunophilin, mzFKBP66 is a peptidylproline cis-trans-isomerase that interacts with calmodulin and a 36 kDa cytoplasmic protein, Planta 205 (1998) 121–131.
[26] M. Geisler, M. Girin, S. Brandt, V. Vincenzetti, S. Plaza, N. Paris, Y. Kobae, M. Maeshima, K. Billion, U.H. Kolukisaoglu, B. Schulz, E. Martinoia, *Arabidopsis* immunophilin-like TWD1 functionally interacts with vacuolar ABC transporters, Mol. Biol. Cell 15 (2004) 3393–3405.

[27] T. Kamphausen, J. Fanghanel, D. Neumann, B. Schulz, J.U. Rahfeld, Characterization of *Arabidopsis thaliana* AtFKBP42 that is membrane-bound and interacts with HSP90, Plant J. 32 (2002) 263–276.
[28] W.B. Pratt, M.D. Galigniana, Y. Morishima, P.J. Murphy, Role of molecular chaperones in steroid receptor action, Essays Biochem. 40 (2004) 41–58.
[29] R.K. Reddy, I. Kurek, A.M. Silverstein, M. Chinkers, A. Breiman, P. Krishna, High-molecular-weight FK506-binding proteins are components of heat-shock protein 90 heterocomplexes in wheat germ lysate, Plant Physiol. 118 (1998) 1395–1402.
[30] J. Harell, I. Kurek, A. Breiman, C. Radanyi, J.M. Renoir, W.B. Pratt, M.D. Galigniana, The Hsp-90-binding immunophilins of wheat, wFKBP73 and wFKBP77 interact with cytoplasmic dynein via their peptidyl prolyl isomerase domains, Biochemistry 17 (2002) 5581–5587.
[31] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding, Anal. Biochem. 72 (1976) 248–254.
[32] J. Sambrook, E.F. Fritsh, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[33] Y.Q. An, S. Huang, J.M. McDowell, E.C. McKinney, R.B. Meagher, Conserved expression of the *Arabidopsis* ACT1 and ACT3 actin subclass in organ primordial and mature pollen, Plant Cell 8 (1996) 15–30.
[34] S. Kikuchi, K. Sator, T. Nagata, N. Kawagashira, et al., Collection, mapping and annotation of over 28,000 cDNA clones from japonica rice, Science 301 (2003) 376–379.
[35] G.D. Van Duyne, R.F. Standaert, P.A. Karplus, S.L. Schreiber, J. Clardy, Atomic structure of the human immunophilin FKBP-12 complexes with FK506 and rapamycin, J. Mol. Biol. 229 (1993) 105–124.
[36] G.D. Van Duyne, R.F. Standaert, P.A. Karplus, S.L. Schreiber, J. Clardy, Atomic structure of FKBP-FK506, an immunophilin–immunosuppressant complex, Science 252 (1991) 839–842.
[37] I. Kurek, R. Dulberger, A. Azem, B. BenZvi, D. Sudhakar, P. Christou, A. Breiman, Deletion of the C-terminal 138 amino acids of the wheat FKBP73 abrogates calmodulin binding, dimerization and male fertility in transgenic rice, Plant Mol. Biol. 48 (2002) 369–381.
[38] W.A. Snedden, H. Fromm, Calmodulin as a versatile calcium signal transducer in plants, New Phytol. 151 (2001) 35–66.
[39] C. Sakuta, A. Oda, S. Yamakawa, S. Satoh, Root-specific expression of genes for novel glycine-rich proteins cloned by use of an antiserum against xylem sap proteins of cucumber, Plant Cell Physiol. 39 (1998) 1330–1336.
[40] D.E. de Oliveira, J. Seurinck, D. Inze, M.V. Montagu, J. Botterman, Differential expression of five *Arabidopsis* genes encoding glycine-rich proteins, Plant Cell 2 (1990) 427–436.
[41] G. Sachetto-Martines, L.O. Franco, D.E. de Oliveria, Plant glycine-rich protein: a family or just proteins with a common motif? Biochem. Biophys. Acta 1492 (2000) 1–14.
[42] K. Aviezer, I. Kurek, N. Erel, O. Blecher, K. Devos, A. Breiman, Studies on the wheat prolyl isomerase FKBP73 during plant development, Plant Sci. 139 (1998) 149–158.