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Molecular characterization of a FKBP-type immunophilin from higher plants

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ABSTRACT **Immunophilins are intracellular receptors for the immunosuppressants cyclosporin A, FK506, and rapamycin. In addition to their use in organ transplantation, these natural products have been used to investigate signaling pathways in yeast, plant, and mammalian cells. We have recently described the identification of an immunosuppressant-sensitive signaling pathway in and the purification of several immunophilins from *Vicia faba* plants. We now report the molecular characterization of a 15 kDa *FK*506- and rapamycin-*b*inding *p*rotein from *V. faba* (VfFKBP15). The amino acid sequence deduced from the cDNA starts with a signal peptide of 22 hydrophobic amino acids. The core region of VfFKBP15 is most similar to yeast and mammalian FKBP13 localized in the endoplasmic reticulum (ER). In addition, VfFKBP15 has a carboxyl-terminal sequence that is ended with SSEL, a putative ER retention signal. These findings suggest that VfFKBP15 is a functional homolog of FKBP13 from other organisms. Interestingly, two distinct cDNAs corresponding to two isoforms of FKBP15 have been cloned from *Arabidopsis* and also identified from rice data base, suggesting that pFKBP15 (plant FKBP15) is encoded by a small gene family in plants. This adds to the diversity of plant FKBP members even with the same subcellular localization and is in contrast with the situation in mammalian and yeast systems in which only one FKBP13 gene has been found. Like the mammalian and yeast FKBP13, the recombinant VfFKBP15 protein has rotamase activity that is inhibited by both FK506 and rapamycin with a K_i value of 30 nM and 0.9 nM, respectively, illustrating that VfFKBP15 binds rapamycin in preference over FK506. The mRNA of VfFKBP15 is ubiquitously expressed in various plant tissues including leaves, stems, and roots, consistent with the ER localization of the protein. Levels of VfFKBP15 mRNA are elevated by heat shock, suggesting a possible role for this FKBP member under stress conditions.**

The immunosuppressants FK506 and rapamycin inhibit different signaling pathways required for T cell activation, yet they bind to the same family of intracellular receptors, the *FK*506- and rapamycin-*b*inding *p*roteins (FKBPs) (1). Several mammalian FKBP members, FKBP12, FKBP13, FKBP25, and FKBP52, have been cloned and characterized (2–9). These proteins share a conserved domain for ligand binding but differ in their flanking sequences and cellular localizations, suggesting that each of the FKBP members may have a unique function in the cell. Indeed, a cytoplasmic member, FKBP12, but not other members mediates the immunosuppressive action of FK506. In the presence of FK506, FKBP12 and FK506 form a complex that binds to and inhibits the activity of calcineurin, a Ca^{2+}, calmodulin-dependent protein phosphatase required for T cell activation (10–14). In the absence of FK506, FKBP12 associates with the Ca^{2+}-releasing ryanodine and IP_3 receptors (15–17). FKBP12 also mediates the action of rapamycin (18). The complex formed by FKBP12 and rapamycin targets the 289-kDa protein FRAP (*F*KBP-*r*apamycin-*a*ssociated *p*rotein) in humans (19) and RAFT1 (*r*apamycin *a*nd *F*KBP12 *t*arget 1) (20) in rat, which are mammalian homologues of TOR1 and TOR2 (*t*arget *o*f *r*apamycin) in yeast that have been genetically shown to be involved in rapamycin sensitivity (21, 22) and more recently shown to be direct target of FKBP–rapamycin in yeast (23–25). These rapamycin targets have been implicated in a signaling pathway leading to G_1-S progression in the cell cycle (21, 22, 26). Studies of other FKBP family members have generated many intriguing findings. For example, FKBP52, the other cytosolic FKBP, has been shown to be associated with two heat shock proteins, hsp70 and hsp90, that are complexed with glucocorticoid receptor (7–9). FKBP13 is an endoplasmic reticulum (ER) protein and responds to heat shock in yeast (27, 28). More recently, FKBP25 has been localized to the nucleus and is associated with casein kinase II and nucleolin (29). The functional significance of these molecular interactions between FKBPs and other proteins is under intensive investigation.

In addition to serving as intracellular receptors for FK506 and rapamycin, all FKBPs identified thus far have peptidyl-prolyl isomerase (PPIase or rotamase) activity (1). This finding led to the speculation that FKBPs may be involved in the intracellular folding of proline-containing proteins (30), although it has proven difficult to distinguish between a folding and chaperone function. Several of the results noted above indicate that these proteins may have completely unrelated functions. To date, FKBP homologs have been found in all the organisms studied so far including bacteria, yeast, and mammals (for reviews, see refs. 1 and 31). By affinity chromatography, we have purified several immunophilins from fava bean plants (32, 33). At least five FKBPs have been isolated from different subcellular compartments (33). Here we report molecular characterization of the first plant FKBP, VfFKBP15, which is from the heavy membrane fraction of fava bean plants. The cDNA-deduced preprotein starts with a typical signal peptide for ER translocation and ends with an ER retention signal. The 15-kDa mature protein represents a homolog of FKBP13 from mammalian and yeast systems. Like the other FKBPs, VfFKBP15 has FK506- and rapamycin-sensitive rotamase activity. Studies of VfFKBP15 homologs from *Arabidopsis* and rice have demonstrated that FKBP15 is encoded by a small gene family in higher plants, whereas only one gene has been found in mammalian and yeast systems. The VfFKBP15 protein and mRNA are detected in all tissues studied, and its mRNA levels are regulated by heat shock, suggesting a possible role for this FKBP member in stress response of higher plants.

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Abbreviations: FKBP, FK506- and rapamycin-binding protein; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; EST, expressed sequence tag; PPIase, peptidylprolyl isomerase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U52045, *Vicia faba* FKBP15 (VfFKBP15); U52046, *Arabidopsis* FKBP15-1 (AtFKBP15-1); U52047, *Arabidopsis* FKBP15-2 (AtFKBP15-2)].

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MATERIALS AND METHODS

Purification of VfFKBP15. Heavy membrane fraction (enriched in mitochondria and ER) was isolated from 14-day-old seedlings of fava bean plants according to the procedure described by Moore and Proudlove (34). Membranous structures were lysed by sonication in an extraction buffer (50 mM Tris, pH 7.5/4 mM EDTA/5 mM 2-mercaptoethenol/1 mM phenylmethylsulfonyl fluoride/5 μg/ml leupeptin). The homogenate was centrifuged at 100,000 × *g* for 45 min in a Beckman model SW40 rotor. To the supernatant 150 mM NaCl was added before loading onto the affinity column packed with FK506 affigel matrix (33, 35). The purified FKBP proteins were separated by SDS/PAGE and transferred to the nitrocellulose membrane for amino-terminal seqence determination by automated Edman degradation.

Construction and Screening of Fava Bean cDNA Library. Total RNA was isolated from leaf tissues. Leaves of 14-day-old fava bean plants were frozen in liquid nitrogen and ground into a fine powder in a mortar. The tissue powder was transferred to a test tube containing an equal volume mixture of phenol and lysis buffer (100 mM Tris, pH 7.5/100 mM LiCl/10 mM EDTA/1% SDS) and mixed for 20 s. Chloroform was then added to the homogenate and the mixture was mixed for another 30 s. After 10 min centrifugation at 6000 × *g*, the upper phase was transferred to a clean tube and total nucleic acid (DNA/RNA) was precipitated with isopropanol. The total RNA was separated from DNA by precipitation in 2 M LiCl. Poly(A)-RNA was purified by the PolyATract mRNA isolation system from Promega. The cDNAs were synthesized using a cDNA synthesis kit from Boehringer Mannheim according to the manufacturer's instructions. The cDNA library was constructed in the LamdaEX*lox* vector (Novagen). A library with 200,000 plaque-forming units was obtained. The procedure described in Sambrook *et al.* (36) was used to amplify and screen the cDNA library. The oligonucleotide probe was designed according to the codon usage in fava bean based on the peptide sequence KNAAPVTELQIGVKYKPA. A 53-mer degenerate oligonucleotide, 5′-AARAATGCAGCAGATGTXACXGARYTXCAAATTGGXGTXAARTATAARCCXGC-3′, was synthesized based on the peptide sequence described above and purified. It was ^{32}P-labeled by T4 polynucleotide kinase and used to screen the fava bean cDNA library. The hybridization was carried out at 42°C for 12–18 h in a buffer containing 5× SSPE (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 5× Denhardt's solution, 20% formamide, and 50 μg/ml of salmon sperm DNA. The filters were washed three times in 0.2× SSC and 0.5% SDS for 30 min each and were exposed to Kodak XAR-50 x-ray .

RNA Blotting and Hybridization. RNA samples (total RNA isolated as described above) were separated by electrophoresis through a 1.2% agarose/formaldehyde gel and blotted onto the Zetaprobe membrane (Bio-Rad) in 0.05 M NaOH. The membrane was neutralized in 0.5 M Na_2HPO_4 (pH 7.2) and prehybridized in 7% SDS, 0.5 M Na_2HPO_4 (pH 7.2), and 1 mM EDTA for 30 min. The randomly labeled cDNA was denatured by 0.2 M NaOH for 15 min at room temperature and added to the prehybridization buffer. The hybridization was maintained at 42°C for 12 h before the membrane was washed in 40 mM Na_2HPO_4 (pH 7.2) and 0.5% SDS. Filters were air-dried and exposed to the x-ray film as described above.

Expression of Recombinant VfFKBP15 Protein in *Escherichia coli*. The cDNA fragment encoding the mature protein of VfFKBP15 was amplified by PCR and cloned in frame with glutathione *S*-transferase (GST) into the pGEX3x vector (Pharmacia). *E. coli* (BL21) cells transformed with the plasmid harboring the fusion protein construct were grown to log phase and expression of the fusion protein was induced by addition of 0.4 mM isopropyl β-D-thiogalactoside (IPTG) to the medium. Cells were harvested 12 h after induction and resuspended into PBS buffer. Purification of the GST-fusion protein was performed essentially as described (37). After lysis by sonication, the suspension was centrifuged at 10,000 × *g* for 15 min. The supernatant was incubated with glutathione Sepharose 4B (Pharmacia) (2 ml bed volume per 1 liter of culture) for 30 min at 4°C on a rotating platform. The glutathione beads were washed three times with PBS and the fusion protein was eluted by 5 mM reduced glutathione in 50 mM Tris buffer (pH 8.0). The eluate was dialyzed in 4 liter of 50 mM Tris buffer (pH 8.0) for 24 h. The cleavage reaction was performed in a buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, and 2 mM $CaCl_2$. Factor Xa (Boehringer Mannheim) was used as the endoprotease at a concentration of 0.5–1% (relative to the amount of fusion protein in the reaction). After incubation for 5 h at 25°C, the reaction mixture was loaded on a glutathione Sepharose column. The GST, uncleaved fusion protein, and other glutathione-binding contaminants were retained on the column and the pure recombinant VfFKBP15 protein was retained in the effluent.

Rotamase (PPIase) Assays. The rotamase activity of the recombinant VfFKBP15 was determined using several synthetic peptides as substrates (see Table 1) in a two-step, coupled reaction involving chymotrypsin as described (30). All assays were performed in 1.5 ml samples that contained 50 mM Hepes (pH 7.9), 60 μM substrate peptide, 100 nM VtFKBP15, and varying concentrations of FK506 or rapamycin as needed. The reactions were initiated by adding 3.5 mg of chymotrypsin (Sigma) and were monitored by measuring absorbance at 408–412 nm for 10 min with a UV-visible spectraphotometer. Data analyses were performed according to the programs written by Standaert (38).

Other Methods. Plant DNA was isolated as described (39). DNA gel blot analysis and hybridizations for *Arabidopsis* library screenings were carried out according to standard procedures (36) using 10% Dextran sulfate (Sigma) in the hybridization solution. High stringency hybridization was performed at 65°C and membranes were washed with 0.1× SSC subsequently. For low stringency hybridizations, the conditions were 55°C and 2× SSC. Two *Arabidopsis* cDNA libraries previously described (40, 41) have been screened to obtain all the different cDNAs for AtFKBP15. DNA sequencing was performed on ALF using the AutoRead Sequencing Kit (Pharmacia) or radioactively using Sequenase II (United States Biochemical). Nucleotide and amino acid sequences were analyzed using the Genetics Computer Group (Madison, WI) program (42) and LASERGENE software (DNAstar, Madison, WI). Databases were searched using the FASTA and BLAST programs. Detailed pairwise comparison of amino acid sequences was done using the BESTFIT program.

RESULTS

A Membrane-Associated FKBP from Fava Bean. In a previous report (33), we purified five FKBP proteins from fava bean leaves, pFKBP12, pFKBP13, pFKBP18, pFKBP25, and pFKBP55. We have since scaled up the purification and obtained amino acid sequences from purified proteins. In the heavy membrane fraction, only one major FKBP running at 18 kDa was detected by SDS/PAGE after affinity chromatography (Fig. 1).

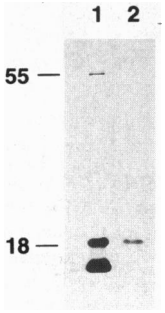

FIG. 1. A 18-kDa FKBP from fava bean. On separation by SDS/PAGE, FKBP proteins purified by a FK506-affinity matrix from whole cell lysates (lane 1) and from soluble protein extract of the heavy membrane fraction (lane 2) were detected by silver staining. On the left, the number indicates the molecular weight of proteins. The FKBP in lane 2 runs at 18 kDa.

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 aga att ctt tca caa tgg ttt tga act tcg tga agt tct gat tga aat agt ttg taa gaa   60

aga aaa aaa cat ctc atc tac tcg ATG AAG CTA TTT TCA ATT TTC CTT ATC TTC ACC ATC  120
                               M  K  L  F  S  I  F  L  I  F  T  I

TTC ATC ATT GCA TCA GCA TTA GTT GCT GCG AAG AGT GCT GCT GAT GTA ACG GAG TTG CAG  180
 F  I  I  A  S  A  L  V  A  A  K  S  A  A  D  V  T  E  L  Q

ATT GGC GTT AAG TAT AAA CCA GCA TCA TGT GAA GTC CAG GCA CAC AAA GGC GAT AAA GTG  240
 I  G  V  K  Y  K  P  A  S  C  E  V  Q  A  H  K  G  D  K  V

AAA GTA CAC TAC CGG GGA AAA CTC ACT GAT GGA ACT GTA TTC GAT TCT AGC TTT GAA AGA  300
 K  V  H  Y  R  G  K  L  T  D  G  T  V  F  D  S  S  F  E  R

AAT AGT CCG ATT GAT TTT GAG CTT GGT GGT GGT CAA GTG ATC AAA GGA TGG GAC CAA GGA  360
 N  S  P  I  D  F  E  L  G  G  G  Q  V  I  K  G  W  D  Q  G

TTA TTG GGA ATG TGT CTT GGT GAG AAG CGA AAA CTG AAA ATC CCA GCA AAA CTT GGC TAT  420
 L  L  G  M  C  L  G  E  K  R  K  L  K  I  P  A  K  L  G  Y

GGA GAG CAG GGT TCC CCA CCA ACT ATT CCA GGT GGT GCA ACA CTT ATA TTT GAC ACT GAG  480
 G  E  Q  G  S  P  P  T  I  P  G  G  A  T  L  I  F  D  T  E

CTT GTG GGA GTG AAC GAC AAA AGC TTA AGC GAA GAA AAA TCA ACC TCG TCC GAA CTG TAG  540
 L  V  G  V  N  D  K  S  L  S  E  E  K  S  T  S  S  E  L  *

aca aat caa cta gac tat tgg caa tct ttg ata gct tac ttt tgt ttc tta ata atg gct  600

ttc aac acc aag gtt tcg ttg tgt aac ctt tga gtg tgg cta gta tag aaa gat ttt gct  660

gtt gta ccc tgt aac tta aat aca ttt att tgg tat ttt tag ctt ttc tga gaa ttt tca  720

ttt gtt aaa aaa aaa aaa aaa aaa aaa aaa
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FIG. 2. Nucleotide sequence and deduced amino acid sequence of faba bean VfFKBP15 cDNA. The predicted amino acid sequence is given below the nucleotide sequence. The coding region of the cDNA is depicted in uppercase letters; noncoding 5′ and 3′ untranslated regions are shown in lowercase letters. An asterisk marks the stop-codon. The amino-terminal sequence obtained by peptide sequencing is underlined indicating the 22 amino acids upstream of this sequence as presequence presumably for protein targeting. The putative ER retention signal on the carboxyl terminus of the protein is boxed.

The amino-terminal sequence of the protein was determined to be KSAADVTELQIGVKYKPASCEVQAHKGDKVKVHY. The underlined region of this sequence (18 amino acid long) was used to generate a long and low-degeneracy oligonucleotide probe for the cDNA cloning of this plant FKBP.

Cloning and Sequence Analysis of the Fava Bean FKBP15 cDNA. After screening the cDNA library with this probe (see *Materials and Methods*), two positive clones were isolated and subcloned into the p*lox* phagemid by automatic transfer according to the manufacturer's instructions (Novagen). The inserts from both clones were analyzed and shown to be identical. Both strands of the insert from one of the clones were sequenced; the resulting sequence is shown in Fig. 2. The 750-bp-long cDNA for the *Vicia faba* FKBP contains an open reading frame of 151 aa, which corresponds to a predicted polypeptide of 17 kDa. Comparing the amino-terminal peptide sequence of purified protein with the amino acid sequence deduced from the cDNA, the preprotein contains a 22-aa signal peptide, presumably for protein targeting. This presequence contains highly hydrophobic amino acids and is typical of signal peptide of proteins translocated across ER membrane. In addition, the carboxyl terminus of the protein ends with SSEL, a putative ER retention signal similar to those found in mammalian FKBP13, an ER-localized FKBP (3, 27). Because the mature protein is a 15-kDa polypeptide, we designate this FKBP member as VfFKBP15. Alignment of the VfFKBP15 amino acid sequence with the sequences of different FKBPs from various organisms shows in every case a striking homology to FKBP13 proteins from several organisms (PAM120 scores of 370 in BLASTP search) and a weaker homology to FKBP52 proteins from human and mouse (PAM120 scores of 220). As shown in Fig. 3, the amino acid sequence of VfFKBP15 is 55% and 53% identical (71% and 68% similar) compared with the FKBP13 from yeast and human, respectively. These findings strongly suggest that pFKBP15 is a functional homolog of the FKBP13 from other organisms.

pFKBP15 Is Encoded by a Small Gene Family in Higher Plants. To genetically approach the function of the plant FKBP15, we decided to clone the homologous gene from *Arabidopsis*. Two different *Arabidopsis* cDNA libraries (both made from the same ecotype Columbia) were screened at low stringency using the VfFKBP15 cDNA as a probe (see *Material and Methods*). This approach resulted in the isolation of several cDNAs encoding two distinct AtFKBP15 (*Arabidopsis* thaliana FKBP15) isoforms (Fig. 3). The predicted amino acid sequences of AtFKBP15-1 and AtFKBP15-2 are highly conserved (72% identity, 86% similarity). Both preproteins start with a similar hydrophobic signal peptide as VfFKBP15 and end with a putative carboxyl-terminal ER retention signal. These two proteins, like VfFKBP15, have the highest homology to FKBP13 from mammalian and yeast (Fig. 3), which have been shown to be localized in the ER. Therefore, we conclude that both genes encode functional homologs of the FKBP13 from other organisms. In contrast to the situation in mammalian and yeast, this is the first observation that FKBP members are encoded by a gene family.

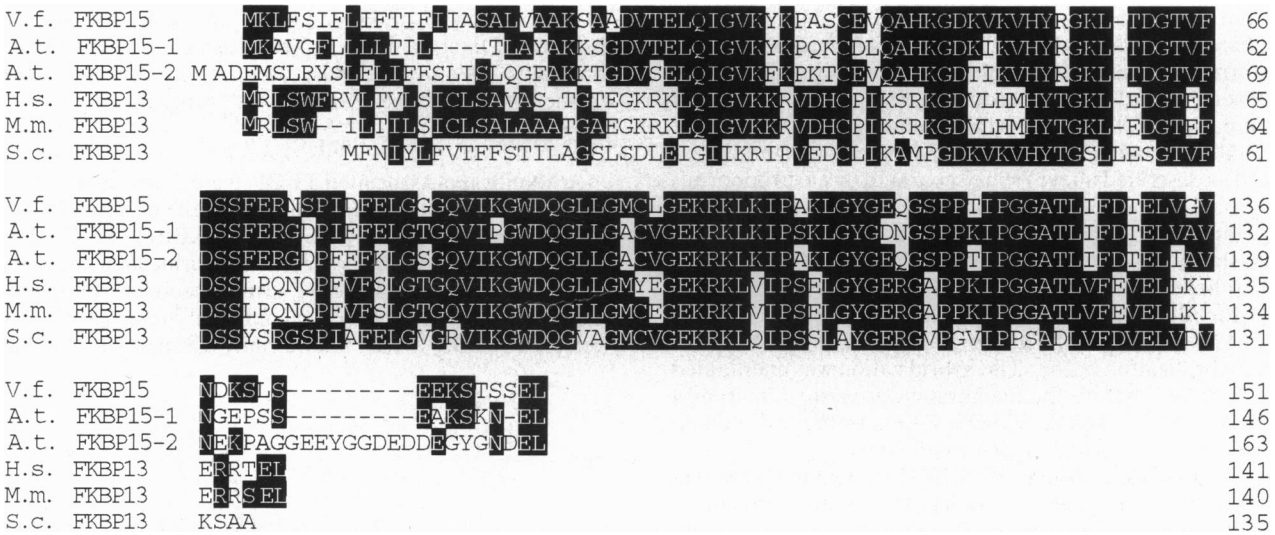

FIG. 3. Genomic analysis of pFKBP15. The pFKBP15 cDNAs encode a protein homologous to mammalian and yeast FKBP13. Alignment of amino acid sequences of the pFKBP15 from faba bean (V.f. FKBP15) and *Arabidopsis* (A.t. FKBP15-1 and A.t. FKBP15-2) with FKBP13 from human (H.s. FKBP13; ref. 29), mouse (M.m. FKBP13; ref. 43), and yeast (S.c. FKBP13; ref. 28) was generated using the CLUSTAL method (44) with DNAstar software. Amino acids identical to the consensus sequence of the alignment are shown on black background. The numbers at right side indicate the amino acid position. Dashes indicates gaps introduced to improve alignment. The highest variability of the proteins is found in the amino-terminal transit peptide.

To further support this hypothesis, we have performed Southern blot analyses using the two cDNA inserts as probes. High stringency blots have shown that each cDNA corresponds to a single copy gene (Fig. 4*A*). Low stringency blots have shown cross hybridization between AtFKBP15-1 and AtFKBP15-2. Comparing the hybridization pattern of the high and low stringency blots, we believe that AtFKBP15 gene family contains only two members. In support of this notion, low stringency hybridization of *Arabidopsis* Southern blots using the VfFKBP15 cDNA only detected the two AtFKBP15 genes (data not shown). Although only one VfFKBP15 cDNA is described in this report, Southern blot analyses at low stringency also revealed at least two related genes in the fava bean genome. This is the first case reported that a FKBP13 protein has two or more isoforms adding the diversity to FKBPs even with same subcellular localization.

After performing data base searches with the cloned cDNAs from fava bean and *Arabidopsis* using the BLASTX algorithm, we identified several expressed sequence tag (ESTs) from *Arabidopsis* (GenBank accession nos. Z29801, H36517, H37484, and T46524), rice (GenBank accession nos. D24714, D23147, and D39710), and *Brassica napus* (GenBank accession nos. L33522 and L33547). An alignment of the predicted amino acid sequences of the identified ESTs is shown in Fig. 4*B*. Because the ESTs represent only partial sequences of cloned cDNAs, it is not possible to determine whether the identified ESTs would encode a protein with an ER retention signal. However, the highly conserved amino acid sequences among the ESTs and the identified pFKBP15 from fava bean and *Arabidopsis* (Fig. 4*B*) strongly suggest that these ESTs yield functional pFKBP15 proteins. All the identified *Arabidopsis* ESTs are identical to the AtFKBP15-2 cDNA we have cloned. The *Brassica napus* ESTs correspond to an identical pFKBP15 protein. Interestingly, the rice ESTs apparently code for two different but highly homologous (72% identity) pFKBP15 proteins. Identification of multiple pFKBP15 isoforms both in dicot and in monocot plants strongly suggests that this protein is generally encoded by a small gene family in higher plants.

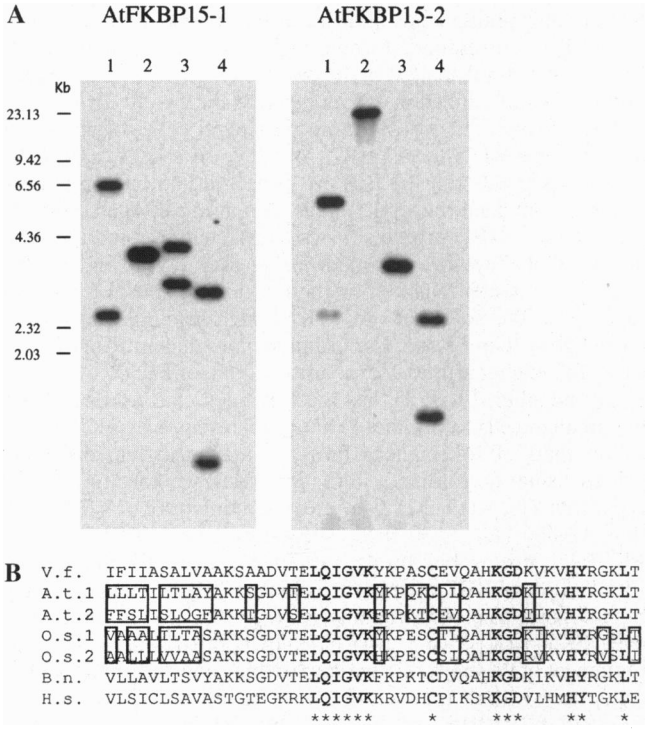

FIG. 4. (*A*) Analysis of the copy number of the AtFKBP15 genes. Genomic DNA extracted from *Arabidopsis* plants was digested with *Bam*HI (lanes 1), *Bgl*II (lanes 2), *Eco*RI (lanes 3), and *Hin*dIII (lanes 4) restriction endonucleases. After agarose gel electrophoresis, DNA was blotted onto nylon membranes and hybridized with ^{32}P-labeled probes for AtFKBP15-1 and AtFKBP15-2. After hybridization and washing, membranes were exposed to x-ray film. *Hin*dIII-digested phage lambda DNA was used to privide molecular size markers. (*B*) pFKBP15 is encoded by a small gene family in higher plants. Alignment of amino acid sequences of the pFKBP15 proteins from fava bean (V.f.) and *Arabidopsis* (A.t.1 and A.t.2) with predicted amino acid sequences of ESTs identified in the data base search. The alignment shows a partial amino acid sequence corresponding to the amino acid position 9–59 of the fava bean gene. The ESTs from rice (O.s.1 and O.s.2) yield two different pFKBP15-like proteins. One EST from *Brassica napus* was also identified (B.n.). The human FKBP13 (H.s.) is shown to verify the high conservation of the amino acid sequence predicted from the identified ESTs. Amino acid differences between isoforms from the same species are boxed. Amino acids identical in all shown proteins are in boldface type and marked with an asterisk.

VfFKBP15 Is a Rotamase Inhibited by FK506 and Rapamycin. All the FKBPs characterized from other organisms so far possess an enzyme activity, namely peptidylprolyl isomerase (PPIase). To characterize the possible PPIase activity of a plant FKBP, we have produced recombinant VfFKBP15 in *E. coli*. After cleavage and purification, VfFKBP15 protein sample was shown to yield a single band on a silver-stained SDS/PAGE gel (Fig. 5*A*). It is worth noting that the recombinant protein again runs at 18 kDa instead of 15 kDa, behaving similarly as the native protein shown in Fig. 1.

Using the purified VfFKBP15 protein sample, we performed rotamase assays. As shown in Fig. 5*B*, VfFKBP15 has rotamase activity comparable with the mammalian and yeast FKBPs and showed strong preference for substrate peptides with a hydrophobic amino acid linked to the proline residue (Table 1). The natural ligands of VfFKBP15, FK506 and rapamycin, inhibit the rotamase activity with a K_i value of 30 nM and 0.9 nM, respectively (Fig. 5*C*). This indicates that rapamycin binds to VfFKBP15 with a much higher affinity than FK506.

Ubiquitous Expression of VfFKBP15 mRNA. To determine the expression pattern of pFKBP15, we isolated mRNA from etiolated and green leaves, roots, and stems. Northern blot analysis has shown that pFKBP mRNA levels are similar in all tissues analyzed (Fig. 6). During the purification of pFKBP15 protein from fava bean plants, we have noticed that leaf and root tissues produced a similar level of pFKBP15 protein (33). The ubiquitous expression pattern is consistent with the possibility that pFKBP15 is localized in the ER.

Transcript Level of pFKBP15 Is Elevated by Heat Shock Treatments. All FKBP proteins identified so far possess PPIase activity that is involved in protein folding processes. Chaperonins are another family of proteins involved in protein folding and their expression is often regulated by environmental stress such as heat shock (45). To test whether expression of VfFKBP15 in fava bean is responsive to heat shock, we transferred the plants to heat shock conditions as described (46, 47). After heat treatments, mRNA levels of pFKBP15 in the control and treated roots were analyzed by Northern blot hybridization. As shown in Fig. 7, heat shock significantly increased the accumulation of the VfFKBP15 transcript (4- to 5-fold). This finding is consistent with the situation in yeast where the transcription of FKBP13 (*FKP2*) gene is also induced by heat shock (28).

DISCUSSION

In the presence of their drug ligands, immunophilins form receptor–drug complexes that target specific molecules such as calcineurin and FRAP/RAFT1, components in a number of signal transduction pathways including those required for T cell activation and cell cycle progression (10, 18). In the absence of the drugs, immunophilins possess an intrinsic PPIase activity involved in protein folding pathways (29). However, recent studies on these proteins have generated a number of intriguing findings, implicating immunophilins in other unknown cellular functions (15–17).

Our recent studies have demonstrated that higher plants produce immunophilins and contain signaling pathways sen-

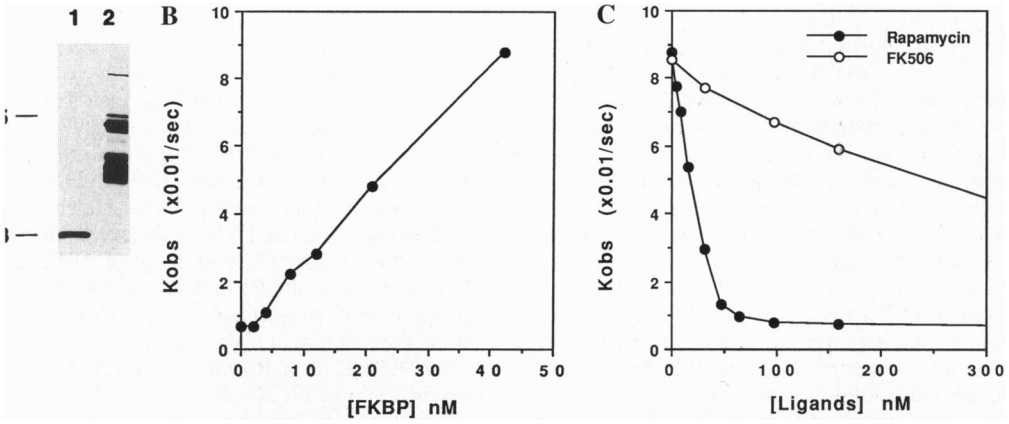

FIG. 5. Biochemical analyses of VtFKBP15. (*A*) Purification of recombinant VfFKBP15 from *E. coli*. GST-VfFKBP15 fusion protein (lane 1) and purified recombinant VfFKBP15 (lane 2) were analyzed by SDS/PAGE and detected by silver staining. (*B*) Rotamase activity of VfFKBP15. Enzyme assays were performed using succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide as substrate in the presence of different concentrations (nM) of pFKBP18 protein. The rate constant is shown as K_{obs}. (*C*) FK506 and rapamycin inhibit the rotamase activity of VfFKBP15. Rotamase activity was determined using the same substrate as in *B* with varying concentrations (nM) of FK506 (○) and rapamycin (●). The concentration of pFKBP18 was 42 nM.

sitive to immunosuppressants (32, 33). As a first step to understanding the cellular function of immunophilins and to further dissect the immunosuppressant-sensitive pathways in higher plants, we have characterized a plant FKBP at the molecular level. This FKBP member, VfFKBP15, was purified by its specific binding to a FK506 affinity column (Fig. 1). A corresponding cDNA was isolated according to the peptide sequence obtained from the purified protein. The cDNA-deduced protein contains a signal peptide for ER translocation and a putative ER-retention sequence at the carboxyl terminus. In addition, the mature protein is most similar to the FKBP13 from mammalian and yeast systems that is localized in the ER compartment. We believe that the plant FKBP15 is a functional homolog of FKBP13 in other organisms and localized in ER. This is consistent with the fact that VfFKBP15 was purified from the heavy membrane fraction (enriched in ER and mitochondria) of fava bean plants.

In contrast to the situation in mammalian and yeast systems in which only one FKBP13 gene has been found, plants contain a small gene family encoding two highly homologous isoforms of pFKBP15. From *Arabidopsis*, we have cloned two homologous, yet distinct, cDNAs for pFKBP15 (Fig. 4*A*). In the EST data base, there are also two different ESTs corresponding to two isoforms of pFKBP15 from rice. In our previous studies (33), at least five different members of FKBP family have been identified from fava bean plants. Each of the family members is different in molecular weight, certain domains of the protein, and subcellular localization, suggesting diverse cellular functions of different members. Finding different isoforms of pFKBP15 in this report adds to the complexity of FKBP structure and function in plant cells. Comparing the two isoforms in *Arabidopsis* with FKBP15 from fava bean and FKBP13 from mammalian and yeast, AtFKBP15-1 has one deletion located at the amino-terminal region and AtFKBP15-2 has an insertion in the carboxyl-terminal domain of the protein. The amino-terminal deletion in AtFKBP15-1 spans four hydrophobic amino acids and the carboxyl-terminal insertion in AtFKBP1-2 corresponds to nine highly hydrophilic amino acids (Fig. 3). Structural analysis of the two proteins may provide insight into the possible functional divergence of the two isoforms.

Table 1. Substrate specificity of VfFKBP15

Xaa*	K_{obs}/K_{unc}†
Ala	2
Val	1
Leu	12
Nle	9
Ile	2
Glu	1
Phe	7

*The substrate peptides for VfFKBP15 were succinyl-Ala-Xaa-Pro-Phe-paranitroanilide.

†K_{obs} is the rate constant in the presence of 42 nM VfFKBP15, K_{unc} is the rate constant without VfFKBP15.

The striking similarity of the amino acid sequence of pFKBP15 to sequences of other FKBPs from diverse sources (40–60% identity) suggests that FKBPs are conserved among organisms ranging from bacteria, yeast, plants, and animals. The conservation of the FKBP proteins is also reflected by the rotamase activity that they have retained during evolution. This enzyme activity is potently inhibited by their natural ligands, FK506 and rapamycin. We note that most FKBP members bind rapamycin more tightly than FK506. For example, mammalian FKBP12 has a two-fold higher affinity for rapamycin than for FK506. A unique FKBP member, FKBP25, has recently been characterized from mammalian cells to preferably bind rapamycin over FK506 with a more than 150-fold higher affinity for rapamycin than for FK506 (4). By using the rotamase inhibition assay, we have found that rapamycin (K_d = 0.9 nM) is a more potent inhibitor of VfFKBP15 than FK506 (K_d = 30 nM), suggesting that VfFKBP15, like FKBP25, preferably binds rapamycin over FK506. Yeast FKBP13, the FKBP member to which pFKBP15 is most highly homologous, also binds rapamycin (K_d = 0.7 nM) in preference over FK506 (K_d = 8.3 nM) (27). In comparison to the core sequences of these FKBPs, we cannot draw conclusions about the sequence(s) that may confer the ligand discrimination to FKBP25, pFKBP15, and FKBP13. Insights into this issue must await structural analyses of FKBPs and of the receptor–ligand complexes formed by the FKBPs and their two ligands.

The fact that FKBPs are present in almost every compartment of the cell is consistent with the possible role of FKBPs in protein folding pathways. The heat shock-responsive expression of VfFKBP15 mRNA also supports this hypothesis.

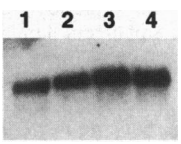

FIG. 6. Ubiquitous expression of VfFKBP15 mRNA. Total RNA (20 μg) from etiolated leaves (lane 1), green leaves (lane 2) stems (lane 3), and roots (lane 4) was analyzed by Northern hybridization using VfFKBP15 cDNA as the probe. Fava bean plants were grown for 14 days in the dark or under normal light-dark cycle. Dark grown leaves and the leaves, stems, and roots from green plants were used for RNA isolation.

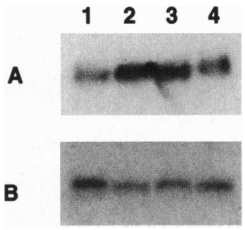

FIG. 7. Heat shock-responsive accumulation of VfFKBP15 transcript. (*A*) Total RNA (20 μg) from plant leaves grown at 23°C (lane 1) or grown at 23°C and then transferred to 37°C for 45 min (lane 2), 2 h (lane 3), and 4 h (lane 4) were analyzed by Northern blotting. (*B*) The mRNA levels of Rubisco small subunit (that is not heat-responsive) is shown as a standard. The relative density of the VfFKBP15 bands were quantified by a laser beam scanner as 1.0 (lane 1), 4.7 (lane 2), 4.6 (lane 3), and 2.5 (lane 4).

Another group of proteins that are regulated by heat shock are molecular chaperones (45). Significant similarities are found in the expression pattern, distribution, and conservation of members of heat shock proteins and FKBP families. Both are highly expressed and are present in virtually all cellular compartments. Each family has members that are induced by heat shock, suggesting a role under stress conditions. Proteins from both families are found in organisms ranging from bacteria to mammals and have amino acid sequences that are highly conserved. In fact, a 59-kDa FKBP has been found to associate with hsp70 and hsp90 in the unactivated estrogen receptor complex (7–9). Studies of the functional significance of this association will provide further information on the relationship between hsp70, hsp90, and immunophilin proteins.

We have previously demonstrated that higher plants contain immunosuppressant-sensitive signaling pathways (32), suggesting the conservation of immunophilins and their targets among eukaryotic systems. Molecular characterization of plant FKBPs will provide useful tools for further dissecting signal transduction pathways including Ca^{2+}-dependent signaling and cell cycle control in higher plants. In addition, results reported here will allow further investigation of FKBP functions in plant cells.

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