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Biochemistry

Light-regulated, tissue-specific immunophilins in a higher plant

(fava bean/chloroplast development/FK506-binding protein/cyclophilin/protein folding)

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ABSTRACT **In addition to their application in organ transplantation, immunosuppressive drugs are valuable tools for studying signal transduction in eukaryotic cells. Using affinity chromatography, we have purified immunosuppressive drug receptors (immunophilins) from fava bean. Proteins belonging to both major classes of the immunophilin family identified from animal sources [FK506- and rapamycin-binding proteins (FKBPs) and cyclophilins] were present in this higher plant. FKBP13, the most abundant FKBP family member in leaf tissues, was not detected in root tissues, whereas other FKBPs were present in both tissues. While the abundance of cyclophilin A in leaves was similar to that in roots, cyclophilin B/C was expressed at a much higher level in leaf tissues than in root tissues. Subcellular localization of immunophilins in mesophyll cells showed that chloroplasts contained FKBP13 and cyclophilin B/C but not other members, which explains the preferential expression of these two proteins in leaves over roots. The abundance of chloroplast-localized immunophilins, FKBP13 and cyclophilin B/C, was regulated by light. Although etiolated leaves produced detectable levels of cyclophilin B/C, they did not express FKBP13. Illumination of etiolated plants dramatically increased the expression of both FKBP13 and cyclophilin B/C. The light-induced expression of FKBP13 is closely correlated with the accumulation of chlorophyll in the leaf tissue. Our findings suggest that FKBP13 and cyclophilin B/C may play a specific role in chloroplasts.**

The immunosuppressive drugs cyclosporin A (CsA) and FK506 (1, 2) block a signaling pathway necessary for the activation of resting T lymphocytes (3, 4). FK506 binds to intracellular receptors in T cells named FK506- and rapamycin-binding proteins (FKBPs), whereas CsA binds to distinct intracellular receptors named cyclophilins (CyPs). FKBPs and CyPs are collectively referred to as immunophilins (1). Complexes formed by immunophilins and their ligands are the functional modules for immunosuppression (5–7). The FKBP12–FK506 and CyP–CsA complexes, but not their separate components, bind to and inhibit the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (8). Biochemical and cellular transfection studies have indicated that inhibition of calcineurin activity is necessary for the immunosuppressive effect of CsA and FK506 (9–11). By using FKBP12–FK506 and CyP–CsA complexes as specific inhibitors of calcineurin, an FK506- and CsA-sensitive signaling pathway was recently shown to be responsible for the Ca^{2+}-dependent inactivation of K^+ channels in the guard cells of *Vicia faba* (12).

While studies of FK506 and CsA first recognized the role of calcineurin in T-cell signaling and established a molecular tool for studying the cellular function of calcineurin in other eukaryotic cells, there is as yet no evidence that immunophilins are relevant to immune function in the absence of their immunosuppressive ligands. During the past several years, a growing number of immunophilins have been characterized from mammalian sources. Each immunophilin catalyzes the interconversion of cis and trans rotamers of peptidyl-prolyl amide bonds. These rotamase enzymes have highly conserved sequences in their ligand binding site and enzyme active site. Immunophilins characterized from mammalian sources include CyP A (13) CyP B (14), CyP C (15), FKBP12 (16), FKBP13 (17), FKBP25 (18–20), and FKBP59 (21–23). In contrast to CyP A, both CyP B and CyP C contain a signal peptide at their N terminus. These cyclophilins are more similar to each other than to CyP A (14, 15). All three CyPs form complexes with CsA that inhibit calcineurin activity *in vitro* (8, 24). Among members of the FKBP family, FKBP13 is 52% identical to FKBP12 and binds FK506 and rapamycin with similar affinity (17). FKBP13 also has a signal peptide at its N terminus and is most likely localized in the endoplasmic reticulum of mammalian cells (17). FKBP25 has a much higher affinity for rapamycin than for FK506 and contains a putative nuclear localization sequence (18–20). FKBP59 (or FKBP52) associates with the 90-kDa heat shock protein (hsp90) in untransformed mammalian steroid receptor complexes (21–23). The ligand-binding domain of FKBP59 is distinct from its hsp90 binding site (23).

Organisms other than mammals also express immunophilins. These include the CyP-like ninaA product from fruit flies (25, 26); yCyP, yFKBP12, and yFKBP13 from yeast (27–29); a CyP homologue from *Escherichia coli* (30); and a CyP A homologue from higher plants (31). The ubiquitous distribution of immunophilins among divergent organisms suggests that these proteins participate in important cellular processes. Their attendent rotamase activity led to the suggestion that they facilitate protein folding *in vivo*. For many proteins, *in vitro* folding is a spontaneous process that does not require energy or assisting factors (32). Protein folding *in vivo* is a more complex process that can require ATP and can be aided by several proteins. These proteins fall into two classes: (*i*) folding catalysts, such as disulfide isomerase and prolyl isomerases [rotamases, or peptidyl-prolyl cis–trans isomerases (PPIases)] and (*ii*) molecular chaperones that bind transiently to the protein intermediates, thereby preventing aggregation (33, 34). Recent protein folding studies suggest that rotamases not only catalyze the rate-limiting steps in the folding pathway but also participate in the folding process as a chaperone (35). The chaperone function of immunophilins is exerted on an early folding intermediate, whereas the isomerase activity is required later in the folding pathway (35). This result emphasizes the possibility that immunophilins may play multiple roles during protein folding in organisms ranging from bacteria to mammals.

In higher plants, chaperones were first discovered as ribulose-bisphosphate carboxylase-binding proteins and are now well characterized (for review see ref. 36). However, little was known about immunophilins in plant cells until very recently (12, 31, 37). In an attempt to isolate meristem-

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Abbreviations: CsA, cyclosporin A; CyP, cyclophilin; FKBP, FK506- and rapamycin-binding protein.

specific cDNAs, Gasser et al. (31) isolated a cDNA highly homologous to CyP A. This cDNA is ubiquitously expressed in different plant tissues and its protein product has rotamase activity (31). This activity was also detected in the mitochondria and chloroplasts of pea seedlings; however, its nature has not been characterized at the molecular level (37). Using immobilized immunosuppressants as affinity reagents, we have detected several immunophilins from *Vicia faba* (12). In larger-scale preparations reported herein, we have identified three more FKBPs in this plant. Thus, homologues to all the major immunophilins identified from mammalian cells so far have been purified from *V. faba*. We also find that both plant FKBP13 (pFKBP13) and pCyP B/C are preferentially expressed in leaves. These immunophilins are localized in chloroplasts, and their abundance is regulated by light.

MATERIALS AND METHODS

Plant Materials. Fava bean (*V. faba* L) plants were grown in a growth chamber at 23°C with 14/10-hr light/dark cycles. Leaves and roots were harvested 12 days after sowing. For light/dark treatments, 10-day-old dark-grown seedlings either remained in the dark or were transferred to a growth chamber with constant light. Leaves of the dark-grown and illuminated seedlings were harvested for protein extraction. The 18-day-old plants grown under normal 14/10-hr light/dark cycles were used for isolation of mesophyll protoplasts and cellular fractionation.

Isolation of Mesophyll Protoplasts and Purification of Mitochondria and Chloroplasts. The protoplasts were isolated as described (38) with minor modifications. Briefly, the lower epidermis of fava bean leaves was removed, and the peeled leaves were sliced before enzyme digestion. Enzymes [2% cellulase (Cellulase Onozuka R-10) and 0.5% pectinase (Macerozyme R-10), Yakult Pharmaceutical, Tokyo] were dissolved into digestion medium (0.6 M mannitol/1 mM $CaCl_2$/0.5% dextran sulfate, 0.2% bovine serum albumin, pH 5.5). After the leaf slices were vacuum-infiltrated in the enzyme solution, enzymatic digestion was carried out at 28°C for 90 min. The released mesophyll cells were collected by centrifugation at 300 × *g*. All subsequent isolation procedures were performed at 4°C. Protoplasts were resuspended in buffer A (50 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM phenylmethanesulfonyl fluoride with trypsin inhibitor at 50 μg/ml, and leupeptin and aprotinin each at 5 μg/ml) with 0.3 M sucrose and vortexed at setting 5 on a VWR vortex mixer for 5 sec. The suspension was centrifuged at 700 × *g* for 15 min. The pellet was suspended in 0.3 M sucrose and used as the crude chloroplast fraction. The supernatant was centrifuged at 17,000 × *g* for 15 min to pellet the crude mitochondrial fraction. The supernatant was recentrifuged at 100,000 × *g* for 2 hr to obtain the cytosol fraction. To purify mitochondria, the crude mitochondrial fraction was resuspended in 0.3 M sucrose and centrifuged at 1500 × *g* to pellet most of the chloroplasts and thylakoids. An enriched mitochondrial fraction was pelleted by centrifuging the supernatant at 12,000 × *g*. This fraction was suspended in 0.3 M sucrose and loaded onto a discontinuous sucrose gradient composed of 7 ml of each of the following molarities: 1.8, 1.45, 1.1, and 0.9. After centrifugation in a Beckman SW28 rotor at 20,000 rpm for 50 min, the mitochondrial layer at the 1.45/1.1 M interface was withdrawn by a syringe and diluted into 4 volumes of buffer A. The mitochondria were pelleted at 12,000 × *g* with a Sorvall SS34 rotor for 20 min. The chloroplasts were purified from the crude chloroplast fraction as described (39). The chloroplast bands were also diluted into buffer A and pelleted by centrifugation at 1000 × *g* with an SS34 rotor for 15 min.

Extraction of Proteins from Leaves, Roots, and Organelles. Leaves and roots of 12-day-old seedlings were cut into small pieces and ground separately in a mortar in the presence of liquid nitrogen. The subsequent procedures were done at 0–4°C. The fine tissue powders were homogenized in buffer A and filtered though four layers of Mirocloth (Calbiochem). The filtrates were centrifuged in a Beckman SW40 rotor for 2 hr at 35,000 × *g*. The supernatants were collected and referred to as protein extract from leaves or from roots. To extract the total soluble proteins from chloroplasts and mitochondria, the pelleted organelles were suspended in buffer A and sonicated three times for 20 sec with a macro tip of a Branson Sonifier (VWR Scientific). After centrifugation for 2 hr at 35,000 rpm in an SW40 rotor, the supernatants were used as chloroplast extract or mitochondria extract for immunophilin purification.

Purification of Immunophilins by Affinity Chromatography. The drug affinity matrices were made as described (40). Disposable 1-ml syringes were plugged with glass wool and packed with the drug affinity matrices to a desired volume (usually a bed volume of 50 μl). A 25-ml syringe was connected to the top of the minicolumn by a needle that penetrated a rubber stopper. The FK506 or CsA affinity matrices were washed with buffer A containing 150 mM NaCl (buffer B). The protein extracts from various sources were adjusted to contain 150 mM NaCl before loading into the top syringe. The flow rate was 0.5 ml/min. The matrices were then washed five times with buffer B (1 ml each) and twice with buffer A containing 0.4 M NaCl (1 ml each). The affinity matrices were suspended in buffer A, and the proteins were eluted by 1 mM FK506 or CsA. The eluted proteins were lyophilized and reconstituted in SDS/PAGE loading buffer. The protein samples were analyzed by SDS/PAGE and silver staining. Proteins of interest were transferred to poly(vinylidene difluoride) membrane and submitted for N-terminal sequencing.

RESULTS

Isolation of Immunophilins from Fava Bean Leaves. Preliminary studies revealed that several members of the immunophilin family are present in the leaves of fava beans (12). These include pCyP A, pCyP B, pFKBP13, and pFKBP12 ("p" stands for "plant" to distinguish these proteins from their mammalian homologues). Larger-scale preparations have revealed several more members of the FKBP family in the leaf tissues. As shown in Fig. 1, the predominant FKBP in leaf tissues was pFKBP13. When five times more protein was loaded onto the gel (lane 5), FKBPs of 55, 25, 18, and 12

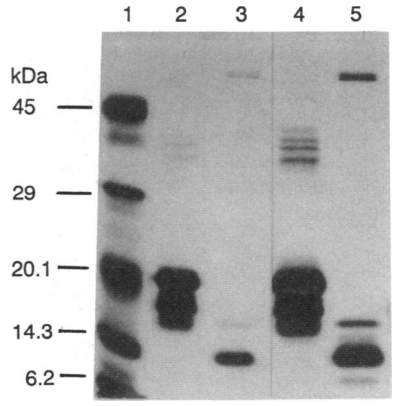

FIG. 1. Immunophilins purified from leaf tissues of *V. faba*. Total protein (40 mg) of fava bean leaf extract was loaded onto columns of 50 μl of FK506 or CsA of affinity matrix. After elution of proteins bound to the matrices by corresponding drugs, 5% (lanes 2 and 3) or 25% (lanes 4 and 5) of eluted protein was analyzed by SDS/PAGE and silver staining. Lane 1, molecular size markers (kDa); lanes 2 and 4, *V. faba* CyPs; lanes 3 and 5, *V. faba* FKBPs.

kDa were detected. Two of these proteins may be homologues of FKBP59 (21–23) and FKBP25 (18–20). The peptide sequence (34 amino acids from the N terminus) of the 18-kDa FKBP is unique and more similar to yFKBP13 (29) than to other FKBPs (data not shown). Determination of the identity for this 18-kDa FKBP awaits the cloning of its cDNA. The FKBP55 is present as a doublet in SDS/polyacrylamide gels, as reported for mammalian tissues (21). The gel mobility shift may be due to phosphorylation, since FKBP59 has been shown to be phosphorylated in mammalian cells (40). The clustered bands of cyclophilins were reported in our earlier study (12) to be CyP A (≈18 kDa) and CyP B (≈21 kDa). Further protein sequence comparisons suggest that the 21-kDa CyP from fava bean has similar homology to both CyP B and CyP C. Therefore, in this report we will refer to the 21-kDa CyP as pCyP B/C. Proteins of ≈40 kDa were detected by silver staining, especially when the gel was overloaded (Fig. 1, lane 4). We found that these proteins were nonspecifically associated with the affinity matrices and that they could be eluted by the elution buffer without drugs (data not shown).

Tissue-Specific Expression of Two Immunophilins, pCyP B/C and pFKBP13. To address the function of the immunophilins in higher plants, we first examined the distribution of these proteins in different plant tissues. The two most distinguishable organs of higher plants are leaves and roots. To compare the protein levels of immunophilins in different tissues, extracts with the same amount of total protein were applied to the same volume of affinity matrices (always in excess of the amount of immunophilins present in the protein extract). The same aliquot (usually 5%) of the purified immunophilins was analyzed by PAGE and silver staining. As shown in Fig. 2, strikingly different patterns of immunophilins were observed in leaf and root tissues. The leaves contained much more pCyP B/C than the roots. pFKBP13, the most abundant FKBP in leaf tissues, was not detected in roots, although other FKBPs were present at a similar level in both leaf and root tissues. To ensure that roots did not have a detectable level of pFKBP13, we loaded the gel with 5-fold more protein and still did not detect pFKBP13 (Fig. 2, lane 8).

Leaf-Specific Immunophilins Are Localized in Chloroplasts. The tissue-specific localization of pFKBP13 and pCyP B/C suggests tissue-specific functions of immunophilins in higher plants. To examine whether the leaf-specific immunophilins are related to the presence of chloroplasts in mesophyll cells of leaves, we isolated mesophyll protoplasts and fractionated the cells into cytosol, chloroplasts, and mitochondria. To extract soluble proteins from chloroplasts and mitochondria, sonication was used to break both the outer and inner membranes of these two organelles. Protein extracts from these fractions were then loaded onto affinity columns of FK506 or CsA to purify immunophilins. The cytosolic fraction contained all the major immunophilins (Fig. 3). Mitochondria contained the 18-kDa FKBP and equal amounts of pCyP A and pCyP B/C. pFKBP13 and pCyP B/C, the two immunophilins preferentially expressed in leaf tissues, were localized in chloroplasts. This is consistent with the hypothesis that leaf-specific immunophilins play a role in chloroplast function.

Levels of pCyP B/C and pFKBP13 Are Regulated by Light. Chloroplast development is controlled by light. Many chloroplast proteins are regulated by a light/dark switch during photomorphogenesis (41). To assess the possibility that chloroplast-localized immunophilins are regulated by a light-mediated signal, we purified immunophilins from both dark-grown and illuminated fava bean plants. As shown in Fig. 4*A*, dark-grown seedlings produced a detectable level of pCyP B/C. However, the abundance of pCyP B/C was increased significantly by illumination. This was seen more easily when the relative abundance of pCyP A and pCyP B/C was examined during the course of illumination. The levels of pCyP A did not significantly change during the greening period. In etiolated leaves, pCyP A was expressed at a higher level than pCyP B/C. After the etiolated seedlings were exposed to light, pCyP B/C protein gradually increased to a level higher than pCyP A (Fig. 4*A*, lanes d and e). In other words, the ratio of pCyP B/C over pCyP A increased while the pCyP A level remained the same upon illumination. Expression of pFKBP13 was dramatically induced by light (Fig. 4*A*). When the same aliquot of purified proteins was loaded to the gel, pFKBP13 was not detected in etiolated leaves, whereas FKBP55 was present at a similar level as in green leaves. We then loaded 5-fold more FKBPs purified from etiolated leaves and from the leaves illuminated for 14 and 38 hr, but the amount of FKBPs from green leaves and from leaves illuminated for 62 hr remained the same. pFKBP13 was still hardly detectable from the etiolated leaves (Fig. 4*A*, lane k). Soon after illumination, pFKBP13 was expressed to a level detectable by silver staining (Fig. 4*A*, lane l) and kept increasing until reaching a level similar to that in green leaves (lanes m–o). We noticed that a rather long

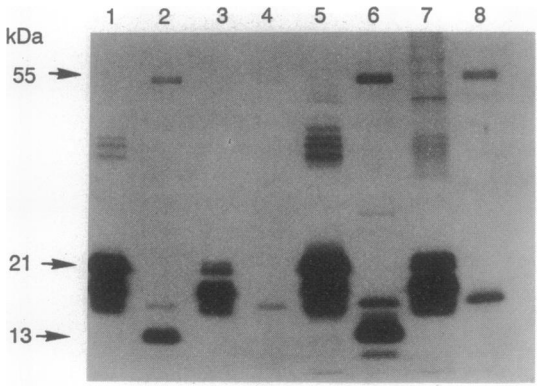

FIG. 2. Tissue-specific expression of immunophilins in fava bean plants. Immunophilins were purified from protein extracts (40 mg of total protein) of both leaves and roots of fava bean seedlings. The eluted protein [5% (lanes 1–4) or 25% (lanes 5–8) aliquot] was analyzed by SDS/PAGE and silver staining. Lanes 1 and 5, leaf CyPs; lanes 2 and 6, leaf FKBPs; lanes 3 and 7, root CyPs; lanes 4 and 8, root FKBPs.

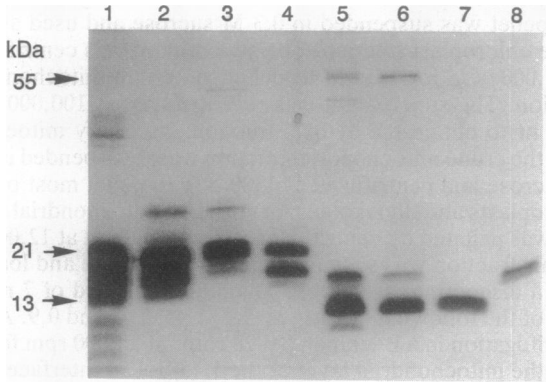

FIG. 3. Subcellular localization of immunophilins in mesophyll cells. Immunophilins were purified from protein extracts (20 mg of total protein) of different fractions of mesophyll protoplasts. Both CyPs (lanes 1–4) and FKBPs (lanes 5–8) were analyzed by loading 10% of eluted protein onto the gel. Lane 1, CyPs of total leaves; lane 2, CyPs of cytosolic fraction; lane 3, CyPs of chloroplasts; lane 4, CyPs of mitochondria; lane 5, FKBPs of total leaves; lane 6, FKBPs of cytosolic fraction; lane 7, FKBPs of chloroplasts; lane 8, FKBPs of mitochondria.

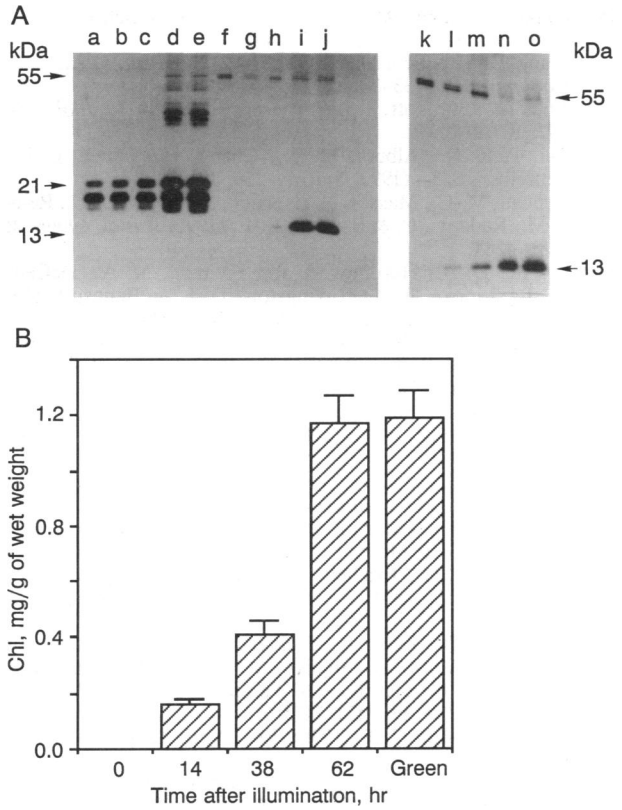

FIG. 4. Correlation of light-induced expression of immunophilins with accumulation of chlorophyll during the greening process. (*A*) Immunophilins were purified from etiolated or illuminated leaves of fava bean seedlings. The etiolated leaves contained a detectable level of pCyP B/C (lane a) and little FKBP13 (lane f). Upon illumination of the dark-grown plants for 14 hr (lanes b and g), 38 hr (lanes c and h), or 62 hr (lanes d and i), both pCyP B/C (lanes b–d) and FKBP13 (lanes e and j) increased to a level similar to that of green leaves. When 5-fold larger aliquots of immunophilins from etiolated (lane k), 14-hr (lane l), and 38-hr (lane m) illuminated leaves were analyzed, low levels of FKBP13 were detected from these samples except for etiolated leaves. (*B*) Chlorophyll contents were measured from etiolated or illuminated leaves. Total chlorophyll (Chl) was extracted by 80% acetone and measured according to Arnon (42).

period of illumination was required before the protein levels of pFKBP13 were significantly elevated (Fig. 4*A*, lanes f–j).

We also analyzed the biosynthesis of chlorophyll in leaf tissues during illumination. No chlorophyll was detected from the etiolated leaves (Fig. 4*B*). Illumination gradually increased the chlorophyll content until the total chlorophyll reached a level similar to that found in young, green leaves. Thus, the development of mature chloroplasts closely correlates with the light-induced increase of pFKBP13 levels. These results indicate that pFKBP13 and probably pCyP B/C may be regulated by chloroplast development in mesophyll cells.

DISCUSSION

We have purified several members of the immunophilin family from the higher plant fava bean. The high affinity of immunophilins for immunosuppressive drugs suggests that their ligand-binding domains are highly conserved during evolution. Although the partial protein sequences we obtained from plant FKBPs (12) are not adequate to verify this notion, sequences of the FKBPs from various organisms ranging from yeast to mammals have shown that these proteins contain a common FKBP12-like domain of about 12 kDa. The alignment of these sequences has been reported (23, 43). The CyP A homologs cloned from higher plants have a high degree of sequence conservation with CyP A proteins from other organisms that diverged hundreds of million years ago (31). We now report another plant CyP homologue with a molecular weight of 21,000. By comparing its peptide sequence to the plant CyP A and other CyPs from other organisms, we find that this 21-kDa CyP is closely related to CyP B and CyP C from mammals. We thus refer CyP 21 from fava bean as pCyP B/C. The N-terminal sequence of pCyP B/C is similar to the N-terminal sequences found in the mature CyP B and C proteins (they are both made as precursors with N-terminal leader sequences). Based on its localization (see next section), we suspect that pCyP B/C may also have a signal peptide at its N-terminus. The sequence of human FKBP13 also contains a signal peptide followed by the mature peptide (17). The N-terminal sequence of fava bean FKBP13 is similar to the N-terminal sequence of mature human FKBP13, suggesting that pFKBP13 may also contain a signal peptide. Chloroplast localization of pFKBP13 in mesophyll cells supports this hypothesis.

Among the immunophilins whose expression pattern has been analyzed in various cell types, only CyP C and ninaA show tissue-specific localization. CyP C and ninaA are specifically expressed in the kidney of mammals (15) and in the retina of fruit flies (25, 26, 44), respectively. In *Drosophila*, at least two cyclophilins are known to be expressed—ninaA and cypl (44). The expression of cypl is ubiquitous, whereas ninaA is expressed only in the retina and is related to specific functions of photoreceptor cells (44). Like its homologues in mammalian cells and cypl from fruit flies, pCyP A from fava bean plants is expressed equally in divergent organs, leaves and roots. Analogous results in tomato plants have been reported (31). pCyP B/C, on the other hand, is preferentially expressed in leaf tissues. The green tissue-specific expression is consistent with the presence of pCyP B/C in chloroplasts. Since both chloroplasts and mitochondria contain pCyP B/C, it is likely that its putative signal peptide can be recognized by both of the organelles. Indeed, a signal peptide for chloroplasts has been shown to occasionally direct a protein to mitochondria (45). We suggest that pCyP B/C may provide a model system for studying protein sorting in plant cells.

The FKBP members reported to date are found in multiple tissues. We have found that pFKBP13 is highly expressed in the leaf tissues but not in the roots. Subcellular localization of immunophilins have shown that pFKBP13 is present in both cytoplasm and chloroplasts in mesophyll cells. In analogy to the subcellular localization pattern of FKBP13 in mammalian cells (17), some pFKBP13 may be localized in the endoplasmic reticulum. However, the absence of pFKBP13 in the roots and the correlation of pFKBP13 expression with the accumulation of chlorophyll during the greening process support the notion that the presence of pFKBP13 is related to the presence of mature chloroplasts.

The ability of immunophilins to catalyze prolyl isomerization suggests that these proteins may be involved in protein folding and possibly translocation processes (1). A role for an *E. coli* CyP homologue in the secretory pathway has been speculated on the basis of its localization in the periplasm (30). Demonstration of mitochondrial forms of *Neurospora crassa* FKBP and CyP led to the hypothesis that these immunophilins may assist in the refolding of proteins following their translocation into the mitochondria (46). The ninaA protein in fruit flies may be responsible for the proper synthesis, folding, transport, and/or stability of rhodopsins in photoreceptor cells (44). The localization of two immunophilins (pCyP B/C and pFKBP13) in the chloroplasts suggests a possible role for these proteins in the assembly of the photosynthetic apparatus. In plants, chloroplast-localized

molecular chaperones have been characterized and shown to be regulated by light (36). Etiolated plants contain a low level of chaperone proteins, but these levels are increased following illumination (36). We have shown that two immunophilins in the chloroplast are also regulated by light. It will be important to determine whether these immunophilins function coordinately with chaperones during protein folding processes in chloroplasts. Our recent studies have shown that FK506 and CsA can be used to dissect the Ca^{2+}-dependent signaling pathways in plant cells (12). Immunosuppressive ligands may also be useful as inhibitors of rotamase activity and for studying the function of immunophilins in higher plants in general.

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