Evidence for the role of FK506-inhibitable peptidyl-prolyl *cis/trans* isomerase activity in seed germination

FK506-binding proteins (FKBPs) are peptidyl prolyl *cis–trans* isomerases (PPIase) which bind to the immunosuppressive drug FK506 or rapamycin due to which they are also called immunophilins. FK506-binding proteins are highly conserved in nature and show ubiquitous distribution among various organisms, thus implying a critical role for these proteins in the cellular processes[1]. Many different FKBPs have been isolated, purified and cloned from several plants. Although recent studies suggest that some of the plant FKBPs may be involved in cytokinin-mediated cell division and elongation[2], and control of rice fertility[3], their *in vivo* role in different developmental processes of plants is still a matter of conjecture. In an attempt to understand the *in vivo* role of FKBPs in germination, a key step in plant development, we investigated the effect of immunosuppressive drug FK506, which specifically inhibits the FKBP-associated PPIase activity, on germination of sorghum seeds.

Seeds of *Sorghum bicolor* (L.) Moench cvs. ICSV-272 (tall) and SPRU-94008B (dwarf) were procured from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. Before plating, the seeds were surface sterilized with 1% (w/v) mercuric chloride for 1 min and 70% ethanol for 3 min followed by rinsing with double distilled water. Treatment with FK506 was carried out by imbibing the seeds for 24 h in double distilled water (ddH_2O) containing FK506 (150 µM) while control seeds were imbibed in ddH_2O containing equal volume of the solvent (dimethyl sulfoxide). The seeds were plated (in triplicate) on water saturated Whatman no.1 sheets in petri plates and incubated at 37°C in a seed germinator. The FK-506 treated seeds were washed in double distilled water before plating.

For biochemical analyses all the fine chemicals and reagents used in this study were purchased from Sigma, USA. FK506 was from Fuzisawa, Japan. Total soluble proteins from three replicates of seeds, embryos and endosperms were extracted in ice cold extraction buffer (5 ml g^{-1} FW) [5 mM Tris-Cl (pH 7.8), 12 mM phenyl methyl sulfonyl fluoride, 20 µm leupeptin, 10 µg/ml chymostatin, 50 µg/ml *N*-tosyl-L-phenyl alanine chloromethyl ketone, 20 µg/ml pepstatin-A, 0.015% Triton X-100] after homogenizing with pestle and mortar. The extracts were centrifuged at 10,000 *g* for 15 min at 4°C. The proteins in the crude extracts were concentrated with two volumes of ethanol at 4°C. The ethanol-precipitated proteins were dissolved in HEPES buffer (50 mM, pH 8.0) and used for determining the PPIase activity. Protein concentration of the ethanol-precipitated extracts was determined by Lowry's method[4]. Peptidyl-prolyl *cis–trans* isomerase activity of the ethanol precipitated extracts was assayed in a coupled assay with chymotrypsin using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilidine as the test peptide[5]. The assays were performed at 4°C for 360 s under N_2 environment and monitored at 390 nm with a spectrophotometer (Perkin-Elmer Lambda Bio20) equipped with Peltier temperature control system. The one ml assay mixture contained test peptide (40 µM), assay buffer [50 mM HEPES (pH 8.0), 150 mM NaCl, 0.05% Triton X-100] and 300 µg of the proteins. The reaction was initiated by the addition of chymotrypsin (300 µg/ml) and the change in absorbance was monitored. The FKBP-associated PPIase activity was determined by the extent of inhibition of reaction in the presence of FK506 (30 µM) which was added to the assay mix 30 min before the start of the reaction and incubated at 4°C. The PPIase activity was calculated as described in ref. 5.

The control seeds recorded more than 80% germination and showed high levels of PPIase activity in embryos and endosperms (Table 1). The FK-506 inhibitable PPIase activity in the embryos and endosperm of cv. SPRU-94008B was similar (62% and 69%, respectively) whereas in the cv. ICSV-272 the extent of FK-506 inhibitable activity in the endosperm was appreciably higher (88%) as compared to the embryos (55%). The high levels of PPIase activity observed in the germinating seeds is in accordance with earlier studies reporting maximum expression of FKBP genes in the initial stages of seedling development in wheat[6]. On the contrary, treatment of seeds with FK506 resulted in complete inhibition of germination in both the sorghum culti-

Table 1. Effect of FK506 on germination and peptidyl prolyl *cis–trans* isomerase (PPIase) activity of seeds of two different cultivars of sorghum. The values are mean of three independent experiments ± SE

Cultivar	Treatment	Specific PPIase activity (pmol s^{-1} mg $protein^{-1}$)		Total PPIase activity per seed (nmol s^{-1})		Seed germination (%)
		Embryo	Endosperm	Embryo	Endosperm	
ICSV-272	Control	322.7 ± 21.2 (55%)	1786.6 ± 35.0 (88%)	42.9 ± 3.5	351.6 ± 21.3	87 ± 5
	FK506	ND	ND	ND	ND	Nil
SPRU-94008B	Control	353.5 ± 23.2 (62%)	965.5 ± 31.3 (69%)	48.1 ± 3.1	126.0 ± 20.1	86 ± 4
	FK506	ND	ND	ND	ND	Nil

ND, not detectable.
Values in parenthesis signify FK506-inhibitable PPIase activity.

vars and the FK506-treated seeds depicted lack of PPIase activity (Table 1). To rule out the possibility that the lack of PPIase activity in the extracts of inhibitor-treated seeds was not due to co-precipitation of FK-506, the ethanol-precipitated protein extracts of control seeds were incubated with 450 μM of FK506 followed by re-precipitation with ethanol. There was no significant change (data not shown) in the PPIase activity of the control extracts after re-precipitation, thus confirming that lack of PPIase activity in the extracts of inhibitor treated seeds was due to absence of PPIase enzymes rather than due to the presence of co-precipitated FK506. Since FK-506 binds specifically to FKBPs[7], the FK506-induced inhibition of seed germination is likely to be due to inhibition of PPIase activity of FKBPs whose contribution to total PPIase activity is substantial (55–88%) in the control germinated seeds. The FK506-insensitive PPIase activity observed in the control germinated seeds can be attributed the parvulins and another class of immunophilins, cyclophilins, which bind to the immunosuppressive drug, cyclosoprin-A[8].

Germination is associated with changes in gene expression which lead to synthesis of new proteins[9]. By virtue of their chaperonic and *cis–trans* isomerase activity[1,3] the FKBPs may be facilitating the correct folding of the newly synthesized proteins required for germination. It is also likely that as reported for mammalian cells[1], the FKBPs through their interaction with other proteins may be playing an important role in the signal transduction pathway(s) required for seed germination. This speculation is supported by the fact that binding of FKBPs to other proteins by tetratricopeptide repeat domains is a conserved interaction between plants and animals[10].

During the past few years, a growing number of immunophilins have been characterized from mammalian and also from higher sources ranging from bacteria to higher plants[1,11]. However, their role *in vivo* is still a matter of conjecture. Our studies are the first to reveal that inhibition of FKBP-associated PPIase activity results in total inhibition of seed germination in different cultivars of sorghum, thus implying that FKBPs are playing a critical role in seed germination. Further studies are in progress to identify the specific FKBP(s) which regulate the germination process.

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Germinability, productivity and economic viability of *Rheum emodi* Wall. ex Meissn. cultivated at lower altitude

Current estimates by the Threatened Plants Species Committee of the Survival (TPSSC) of IUCN indicate that 1 in 10 species of vascular plants on earth is endangered or threatened due to commercial exploitation and international trade. It has been pointed out that nearly 60,000 plant species may be in danger of extinction leading to gene erosion during the next 30–40 years[1]. *Rheum emodi* is among the top of that list, particularly for Garhwal Himalaya; it has been identified as a top-priority species for conservation and cultivation.

Rheum emodi Wall. ex Meissn. is a perennial stout herb, distributed in the temperate and subtropical regions of Himalaya from Kashmir to Sikkim, between an elevation of 2800 and 3800 m (Figure 1). In Garhwal Hima laya it is generally found between 2800 and 3600 m in an alpine zone on rocky soil, between boulders and near streams.

The history of rhubarb dates back to ancient China and the Mediterranean region as a highly popular laxative drug and a general tonic[2]. Indian rhubarb is used as purgative and astringent tonic; its stimulating effect combined with apparent properties renders it especially useful in atonic dyspepsia. Powdered roots are sprinkled over ulcer for healing and also used for cleaning teeth. Leaf stalks are eaten either raw or boiled, sprinkled with salt and pepper. Leaves and flowers are