Comparative studies on drought-induced changes in peptidyl prolyl cis–trans isomerase activity in drought-tolerant and susceptible cultivars of *Sorghum bicolor*

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Drought stress-induced changes in peptidyl prolyl cis–trans isomerase (PPIase) activity were studied in two sorghum (*Sorghum bicolor* (L.) Moench) cultivars, viz. ICSV-272 and SPRU-94008B, which are categorized as drought-tolerant and susceptible respectively. Stress-induced changes in PPIase activity of different tissues were examined at various stages of development. Imposition of water stress resulted in a significant increase in leaf- and root-PPIase activity in the cultivar ICSV-272, whereas, in the drought-susceptible cultivar SPRU-94008B, the PPIase activity of the two tissues decreased appreciably in response to drought. ICSV-272 also showed substantially higher drought-induced increase in the grain PPIase activity. The water stress-induced increase in total PPIase activity in ICSV-272 was due to specific induction of PPIases. The PPIase activity in different tissues was due to presence of both cyclophilins (Cyps) and FK506-binding proteins (FKBPs). The effect of water stress on Cyp- and FKBP-associated PPIase activity was differential and tissue-dependent. The differential effect of drought stress on PPIase activity in the two cultivars was independent of water-potential, thus suggesting different regulatory pathways in the drought-tolerant and susceptible cultivars of sorghum.

PROTEIN folding in vivo is mediated by an array of proteins that act as molecular chaperones, foldases or both. In folded proteins, the peptide bonds occur only in two conformations, cis or trans. The peptide bonds not preceding proline are almost always trans in folded proteins, but 5.7% of all Xaa–Pro peptide bonds show cis confirmation in proteins with known three-dimensional structure. The slow isomerizations about proline imidic bonds are frequently the rate-determining events in folding. In a cell, folding should not be too slow and partially folded intermediates should not be present for an extended time in order to minimize the risk of their aggregation. Peptidyl prolyl cis–trans isomerases (PPIases), also called rotamases or immunophilins, are the only enzymes evolved to stabilize a transition state that is separated from a ground state only by a difference in torsional angle. The term ‘immunophilins’ refers to their propensity to bind immunosuppressive drugs, Cyclosporin-A (CsA) and FK506 or Rapamycin. The immunophilins consist of two major unrelated families: the cyclophilins (Cyps) which bind Cyclosporin-A and the FK506-binding proteins (FKBPs) which bind the macrodile drugs FK506 and Rapamycin.

Peptidyl prolyl cis–trans isomerases are ubiquitous proteins and are found in the cytosol of both prokaryotic and eukaryotic cells and in organelles such as ER, mitochondria and chloroplasts. Various FKBPs and cyclophilins have been cloned from higher plants. Expression of many of the cyclophilin and FKBPs genes is induced by different environmental stresses. Maize cyclophilin gene was reported to be expressed at higher levels in response to salt-, cold-, metal-, heat-, and wounding stress. In addition to heat stress in *Solanum tuberosum* and low temperature, drought, wounding, salicylic acid and pathogen challenge in *Solanum commersonii*, the cyclophilin genes of these species were also induced by the stress hormone, abscisic acid (ABA). The increased freezing tolerance of proembryonic masses of *Digitalis lanata* by cold, ABA and osmotic stress treatment during cryopreservation coincided with enhanced levels of cyclophilins. The thermal stress-induced expression of cytoplasmic Cyp gene was found to be essential for the survival of the *Sacharomyces cerevisiae* cells, because disruption of this gene resulted in decrease in the capacity of the cells to survive at high temperatures. The FKBPs also, are responsive to stress, since levels of FKBPs were reported to increase in response to heat stress in wheat and *Vicia faba*. Due to their protein folding and chaperonic properties, and responsiveness to stress, the immunophilins have been proposed to play an important role in stress tolerance. However, an association between stress tolerance at whole-plant level and expression of PPIases has not been studied. Therefore, to assess their role in stress tolerance, changes in PPIase activity were compared between stress tolerant and susceptible cultivars. Further, the studies carried out so far on stress-induced changes in the immunophilins have focused on individual genes. Since FKBPs

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and cyclophilins⁵ are present in multiple forms and the different PPlase genes are regulated differently in a temporal- and tissue-specific manner, these studies therefore do not throw light on the overall status of total cellular PPlase activity in response to different stimuli. Further, under stress conditions activity of many of the genes is regulated at post-transcriptional and post-translational levels¹⁷,¹⁹-²², thus making it imperative that PPlase expression should be studied at the activity level. In view of these objectives, the present study investigated the effect of water stress on total PPlase activity in two sorghum cultivars which differed in their tolerance to drought.

Materials and methods

Plant material

Seeds of drought tolerant (ICSV-272) and susceptible (SPRU-94008B) cultivars of Sorghum bicolor (L.) Moench were procured from International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India. The drought-tolerant sorghum cv. ICSV-272 exhibits higher growth rate under drought and faster recovery after water stress compared to the susceptible cv. SPRU-94008B. Plants were raised in pots (one plant per pot) in the net house under natural conditions and tagged on the day of anthesis. Single water-stress treatment at different stages of development was imposed (5–6 days prior to sampling) by withholding water supply, while the control plants were watered daily. Leaf samples were collected at 30, 60, 70 and 80 days after sowing (DAS), while the developing grains were collected at 9, 15, 21 and 27 days post anthesis (DPA). Water-potential measurements (in five replicates), using Thermocouple Dew Point Microvoltmeter (Model HR 33T, Wescor Inc., USA) were taken between 9:00 and 10:30 am on the uppermost, fully-expanded leaf before flowering and on the first leaf below the flag leaf during grain filling. Due to rapid senescence, water-potential of the leaves could not be measured after 9 DPA (80 DAS). Freshly-harvested grains (in five replicates) for water-potential studies were taken from the middle portion of the panicles. Equilibration times for leaf- and grain-water-potential were 30 min and 5 h respectively. For biochemical analysis roots and leaves from stressed and control plants, harvested in triplicate at different stages of growth, were frozen in liquid nitrogen until further analysis. Panicles from three pots, representing one replicate, were harvested in triplicate at 9, 15, 21 and 27 DPA and frozen in liquid nitrogen till further analysis.

Extraction of proteins

Total soluble proteins from three replicates of the middle of the panicles for each replicate) harvested at different stages of growth were extracted in ice-cold extraction buffer (5 ml g⁻¹ f wt) [5 mM Tris-HCl (pH 7.8), 12 mM phenyl methyl sulphonyl fluoride, 20 µM leupeptin, 10 µg/ml chymotrypsin, 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 seeds at 9 and 15 DPA were separated into embryo and endosperm for determining the relative contribution of different tissues to the total seed PPlase activity. At later stages of grain development, it was not possible to separate these tissues. The extracts were centrifuged at 10,000 g for 15 min at 4°C. Since PPlase activity was not detectable in the crude extracts, the proteins were precipitated with two volumes of ethanol at 4°C, dissolved in HEPES buffer (50 mM, pH 8.0) and used for determining the PPlase activity. Protein was determined according to Lowry’s method²³.

Peptidyl prolyl cis–trans isomerase assay

The fine chemicals and reagents used in this study were purchased from Sigma Chemical Co, St. Louis, USA. Cyclosporin-A (Sandimmune) and FK506 were from Sandor AG, Basel, Switzerland and Fujisawa Pharmaceutical Co, Ltd, Osaka, Japan respectively. Peptidyl prolyl cis–trans isomerase activity was assayed in a coupled reaction with chymotrypsin, as described in Breiman et al. The assays were performed at 4°C for 360s under N₂ environment. The 1 ml assay mixture contained 40 µM N-succinyl-ala-ala-pro-phe-p-nitroanilidene as test peptide, assay buffer [50 mM HEPES (pH 8.0), 150 mM NaCl, 0.05% Triton X-100] and 300 µg of the protein. The reaction was initiated by the addition of chymotrypsin (300 µg/ml) and the change in absorbance at 390 nm was monitored using a spectrophotometer (Perkin-Elmer Lambda Bio20) equipped with Peltier temperature control system. Cyclophilin- and FKBP-associated PPlase activities were determined by the extent of inhibition of reaction in the presence of CsA (50 µM) and FK506 (30 µM) respectively. The inhibitors were added to the assay mix 30 min before the start of the reaction and incubated at 4°C. For calculating the PPlase activity, the difference between the catalysed and uncatalysed first-order rate constants, derived from the kinetics of the absorbance change at 390 nm, was multiplied by the amount of the substrate in each reaction.

Results

Water-potential

Compared to 30 DAS, leaf water-potential in irrigated and stressed plants of both the cultivars was significantly lower at 80 DAS (Figure 1A). Water stress at all the
stages resulted in significant lowering of leaf water-potential in both the cultivars. The grain water-potential did not show any significant intercultivar differences under control and stress conditions (Figure 1 B). Imposition of water stress resulted in significant decrease in water-potential at all the stages with the effect of drought being more pronounced at 9 and 15 DPA.

**Peptidyl prolyl cis–trans isomerase activity in leaves**

Significant leaf PPlase activity under control conditions was observed only in SPRU-94008B which, following an exponential increase between 30 and 60 DAS, decreased to insignificant levels (Figure 2 A). The effect of water stress on leaf PPlase activity was contrasting in the two cultivars of sorghum. The total leaf PPlase activity of SPRU-94008B decreased dramatically at both 30 and 60 DAS in response to drought stress (Figure 2). On the contrary, the total PPlase activity in the leaves of drought-tolerant cultivar ICSV-272 increased by about 16-fold in response to water stress at 30 DAS (Figure 2 A) and was primarily due to specific induction of PPlases (Figure 2 B). Inhibition studies revealed that drought-induced PPlase activity in the ICSV-272 leaves at 30 DAS was due to similar induction of Cyps (55%) and FKBP5 (45%).

**Peptidyl prolyl cis–trans isomerase activity in roots**

Intercultivar differences with respect to root PPlase activity were observed under both control and stress conditions (Figure 3). Contrary to the irrigated roots of SPRU-94008B which, on both protein and dry weight basis, showed substantial PPlase activity at 60 DAS, the roots of the control plants of ICSV-272 depicted only marginal levels of total and specific PPlase activity at this stage (Figure 3). Imposition of water stress in ICSV-272 resulted in significant induction in the total root PPlase activity at 60 DAS (Figure 3 A), with the increase being mainly due to specific increase in PPlases (Figure 3 B). Inhibition studies with CsA and FK506 showed that the stress-induced increase at 60 DAS in PPlase activity of ICSV-272 roots was primarily due to increase in FKBP5 (70%), as Cyp-associated activity was only about 30%.

Although specific root PPlase activity of SPRU-94008B was unaffected by water stress at 60 DAS (Figure 3 B), the total PPlase activity (Figure 3 A) decreased significantly due to an overall decrease in total protein content (data not shown). Further, stress imposition at 60 DAS in the roots of cv. SPRU-94008B also resulted in change in relative expression of different PPlases. Under control conditions, almost whole of the PPlase activity of SPRU-94008B roots was due to Cyps (40%) and FKBP5 (60%), whereas under stress conditions, only about 45% of the PPlase activity was inhibited by the two inhibitors, with CsA and FK506 causing 15% and 30% inhibition respectively. These observations suggest that besides immunophilins, other PPlases also get induced in the roots of cv. SPRU-94008B under stress conditions.

**Peptidyl prolyl cis–trans isomerase activity in developing grains**

The total and specific PPlase activity of developing grains in both the cultivars after increasing exponentially between 9 and 15 DPA was undetectable at 21 DPA onwards (Figure 4 A). On protein as well as tissue basis, the

![Figure 1](https://i.imgur.com/3QK7Q.png)

**Figure 1.** Water-potential of leaves (A) and grains (B) at different stages of development in sorghum cultivars ICSV-272 (squares) and SPRU-94008B (triangles) under irrigated control (solid) and drought-stress conditions (- - - - -). Values represent mean of three replicates ± SE. Values shown by common letter are significantly different with respect to irrigated control at (P ≤ 0.001).
seed PPlase activity of the cv. ICSV-272 was higher than cv. SPRU-94008B, under both control and stress conditions. Drought stress-induced changes in the seed PPlase activity of the two cultivars varied with the developmental stage. Compared to 9 DPA in cv. SPRU-94008B, the significant increase in drought stress-induced total seed PPlase activity of cv. ICSV-272 was observed at 15 DPA (Figure 4A).

To determine the relative contribution of different tissues to the seed PPlase activity, the PPlase activity of embryo and endosperm was determined separately. Total and specific-embryo PPlase activity in the two cultivars increased between 9 and 15 DPA under irrigated conditions (Figure 4B). Drought-induced changes in embryo PPlase activity in both the cultivars were stage-dependent. Contrary to a dramatic decrease at 9 DPA, impos-
tion of water stress at 15 DPA resulted in substantial increase in embryo PPIase activity in cv. ICSV-272. The reverse was observed for the embryos of SPRU-94008B. The drought-induced decrease at 9 DPA in PPIase activity of ICSV-272 embryos was due to relatively greater inhibition of cyclophilins, since as compared to 45% in control, the CsA-inhibitable activity under stress conditions decreased to 20% (Table 1).

Stress-induced increase in embryo PPIase activity at 15 DPA in cv. ICSV-272 was not accompanied by any significant change in the relative contribution of Cyps and FKBP5s, thus implying that both responded equally to drought stress (Table 1). In SPRU-94008B, the water stress-induced increase in the embryo PPIase activity at 9 DPA (Figure 4B) was mainly due to Cyps, since CsA-inhibitable activity increased from 22% under irrigated

![Figure 4](image)

**Figure 4.** Total and specific PPIase activity in (A) developing grains of sorghum cultivars ICSV-272 (squares) and SPRU-94008B (triangles) under irrigated (——) and drought-stress conditions (••••). **B** and **C**, PPIase activity in embryos and endosperms of ICSV-272 (□ control; ■ stress) and SPRU-94008B (■ control; □□ stress) respectively. Values represent mean of three replicates ± SE. Values shown by common letters are significantly different with respect to irrigated control at P ≤ 0.05 b, P ≤ 0.005 c, P ≤ 0.001 d.
conditions to 40% in the stressed plants, whereas the change in FK506-inhibitable activity was marginal at this stage (Table 1). These results demonstrate that in the 9-DPA sorghum embryos, Cyps are more responsive to stress than FKBP's, and their expression in the two cultivars is regulated differently by drought stress. Drought stress caused a significant decrease in total and specific PPIase activity in 15 DPA embryos of SPRU-94008B (Figure 4B). Under irrigated conditions, most of the embryo activity at 15 DPA in SPRU-94008B was due to FKBP's (94%), which in response to stress conditions was reduced to 36%, while contribution of Cyps increased from 4% under control to 64% in stressed plants (Table 1).

The total and specific PPIase activity of endosperms was higher at 15 DPA than at 9 DPA in both the cultivars (Figure 4C). Stress-induced increase in endosperm PPIase activity of cv. SPRU-94008B was significant at both the stages, whereas in cv. ICSV-272, it was significant only at 9 DPA (Figure 4C). Inhibition of endosperm PPIase activity by FK506 and CsA was almost total at both the stages in irrigated and stressed plants of the two cultivars. Drought stress resulted in significant enhancement in total and specific endosperm PPIase activity at 9 DPA in both the cultivars, which was due to relatively greater increase in FKBP's. This was evident, since FK506-inhibitable endosperm PPIase activity at 9 DPA in ICSV-272 and SPRU-94008B increased from 15 to 45% and from 18 to 52% respectively (Table 1). The Cyp-associated PPIase activity in the endosperm of both the cultivars registered a marginal decline in response to drought stress (Table 1). Relative contribution of Cyps and FKBP's to the total endosperm PPIase activity at 15 DPA was more or less unaffected by stress in both the cultivars.

### Table 1. Drought-induced changes in cyclosporin-A and FK506-inhibitable peptidyl prolyl cis–trans isomerase activity in embryos and endosperms of sorghum cultivars, ICSV-272 and SPRU-94008B, at 9 and 15 days post anthesis (DPA). Values represent mean of three replicates ± SE. Values shown by common letters are significantly different with respect to irrigated control at P ≤ 0.001 d

<table>
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<tr>
<th>Tissue</th>
<th>Cultivar</th>
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<th>15 DPA Inhibition (%)</th>
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<td>CsA</td>
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<tr>
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C, Control; S, Stress.

### Discussion

Imposition of stress to plants results in change in gene expression\(^2\). Many of the proteins which are induced by stress have chaperonic and/or foldase activity\(^1\) which is required for correct folding of the newly synthesized proteins. One such class of proteins comprises PPIases which catalyse the slow rate-limiting conversion of prolyl peptide bond from \textit{cis} to \textit{trans}, which is a rate-limiting step in protein folding. The PPIases are encoded by many different genes\(^11,18\). Effect of different abiotic stresses on the expression of many of these genes has been studied\(^5,12,13,17,24-27\), but information on stress-induced changes in the total PPIase activity under field/nethouse conditions is lacking. Therefore, to examine the role of PPIases in stress tolerance, we focused on drought stress and studied the regulation of PPIases in response to water-stress conditions in the two cultivars of sorghum differing in their tolerance to drought. In the present study, the total PPIase activity represents the ethanol-precipitable activity. The \textit{cis}–\textit{trans} conversion of the peptidyl-prolyl bonds observed in the protein extracts of different tissues of sorghum was due to the presence of PPIases, since first-order rate constant increased with the amount of total proteins in the assay mix and heat treatment for 15 min at 60°C resulted in complete loss of this activity (data not shown).

The PPIase activity in different tissues of sorghum was regulated developmentally and was cultivar-dependent. The tissue- and stage-dependent differential PPIase activity observed in the present study is consistent with the spatial and temporal regulation of different FKBP and Cyp genes reported earlier\(^24,26-31\). Maximum PPIase activity in both the cultivars was observed in the seeds at 15 DPA (Figure 4A), with both embryo and endosperm contribu-
ting to this activity (Figure 4 B and C). Since seeds represent a synthesis and storage site for proteins, increased PPlase activity may be assisting in the folding and transport of newly synthesized proteins\textsuperscript{32}. This is supported by the fact that exponential increase in PPlase activity between 9 and 15 DPA (Figure 4 A) was coincident with the phase of maximum protein accumulation (data not shown). The PPlase-assisted folding of the newly synthesized seed proteins could be through their tetrameric repeat (TPR) domain-mediated association with HSP90 proteins\textsuperscript{33-35} which are abundant in the seeds.

Significant increase in leaf-, root- and seed PPlase activity in response to water stress was observed only in the drought-tolerant cultivar ICSV-272, which was due to specific induction of PPlases. The relative contribution of FKBP\textsubscript{5}s and Cyps to the drought-induced PPlase activity in different tissues of cv. ICSV-272 was determined by studying the inhibition of PPlase activity in the presence of FK506 and CsA respectively. This is possible since there is no cross inhibition between CsA and FK-506 (ref. 36), and thus the members of the two classes can be differentiated by applying these specific inhibitors. Compared to similar induction of Cyps (55\%) and FKBP\textsubscript{5}s (45\%) in the stressed leaves of ICSV-272 at 30 DAS, the drought-induced increase in PPlase activity at 60 DAS in the roots of this cultivar was primarily due to induction of FKBP\textsubscript{5}s (70\%) thus implying differential regulation of Cyps and FKBP\textsubscript{5}s by drought stress in the different tissues. Differential regulation of Cyps and FKBP\textsubscript{5}s under stress conditions was also observed in the embryo and endosperm of the two cultivars. In the embryos, the effect of water stress at 9 DPA was more pronounced on Cyps, whereas in endosperms it was the FKBP\textsubscript{5}-associated PPlase activity which was affected more by drought-stress at this stage (Table 1). The effect of drought stress on PPlase activity of embryo and endosperm was stage- and cultivar-dependent. The drought-induced increase in total PPlase activity at 15 DPA in the cultivar ICSV-272 was primarily due to increase in embryo PPlases (Figure 4 B). These observations indicate that within the seeds the regulatory pathways operating in response to drought stress are distinctly different in the embryo and endosperm.

Apart from Cyp- and FKBP\textsubscript{5}-associated PPlase activity, our studies also revealed the presence of stress-inducible PPlase activity in the roots of SPRU-9400SB at 60 DAS, which was insensitive to CsA and FK-506. This fraction of PPlase activity is likely to be due to another class of PPlases called parvulins. Parvulins are small-molecular weight proteins and have been reported earlier from Arabidopsis thaliana\textsuperscript{37}, Digitalis lanata\textsuperscript{38} and Malus domestica\textsuperscript{39}. However, this study demonstrates that the CsA- and FK506-insensitive PPlase activity is modulated by stress conditions, thus suggesting that it may also be involved in stress adaptation.

Drought-induced changes in expression of several genes have been associated with a decrease in tissue water-potential\textsuperscript{40,41}. However, in the present study, in spite of the water stress-induced decrease in leaf- and grain water-potential being similar in the two cultivars, the drought-induced increase in PPlase activity was observed only in the tissues of drought-tolerant cultivar ICSV-272. These observations suggest that the regulatory pathways which control the PPlase activity may be different in the drought-tolerant and susceptible cultivars of sorghum. Multiple signal transduction pathways between perception and drought-stress signal are involved in stress-induced gene expression due to which the stress response observed after exposure of plants to drought stress is complex\textsuperscript{32}. Some genes are rapidly induced within 10 min of stress while others are slowly induced after accumulation of endogenous ABA. It is likely that the stage- and cultivar-dependent increase in PPlase activity under drought stress conditions may be due to elevated levels of endogenous ABA, since induction of different immunophilin genes by ABA has been reported earlier\textsuperscript{13,15}. Further, studies on stress-induced changes in the endogenous ABA and PPlase induction are required to elucidate the mechanism of hormonal regulation.

In view of their stress-inducibility, different Cyp and FKBP genes have been speculated to play a role in stress adaptation of plants. However, a direct relationship between stress tolerance and expression of total PPlase activity has not been reported as yet. Our study demonstrates that, compared to the drought-susceptible cultivar, the stress-induced expression of total PPlase activity in different tissues of sorghum is dramatically higher in the cultivar which is tolerant to drought. The water stress-induced enhancement in the PPlase levels in different tissues of the cultivar ICSV-272 may be helping the other stress-induced proteins to maturation by virtue of their chaperonic and PPlase activity\textsuperscript{1}. It is also likely that enhanced levels of PPlase activity under stress conditions may be regulating the expression of other genes imparting stress tolerance since they are also implicated in signal transduction\textsuperscript{12}. These results suggest that it will be worthwhile to carry out such investigations with many drought-tolerant and drought-sensitive varieties to determine the potential of the total ethanol-precipitable PPlase activity as a useful marker for breeding stress-tolerant crops.

RESEARCH ARTICLES


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