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Stress-induced changes in peptidyl-prolyl *cis-trans* isomerase activity of *Sorghum bicolor* seedlings

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Abstract

Developmental changes and effects of various abiotic stresses on peptidyl prolyl *cis-trans* isomerase (PPIase) activity were studied in the seedlings of sorghum [*Sorghum bicolor* (L.) Moench cv. CSH-6]. The PPIase activity of sorghum seedlings markedly decreased after two days of germination. Up to 90 % of the PPIase activity was inhibited by cyclosporin-A. Maximal increase in specific PPIase activity in the 3-d-old seedlings was observed in response to osmotic stress and it was transient in nature. The stress-induced enhancement in PPIase activity, depending upon tissue and stress treatment, was due to induction of cyclophilins as well as other PPIases. Osmotic stress-induced enhancement in PPIase activity in the drought susceptible cv. SPRU-94008B was maximal in roots, as compared to shoots in the drought tolerant cv. ICSV-272.

Additional key words: abiotic stresses, cyclophilin, sorghum.

Introduction

Peptidyl prolyl *cis-trans* isomerases (PPIases) or rotamases are enzymes that catalyze the reversible conversion of peptidyl prolyl bond from *cis* to *trans* which is a rate limiting step in the folding of proteins (Fischer and Schmid 1999). The three major classes of rotamases comprise of cyclophilins, FK-506-binding proteins (FKBPs) and parvulins. Cyclophilins and FKBPs bind specifically to the immunosuppressive drugs cyclosporin-A and FK-506 or rapamycin, due to which they are also referred to as immunophilins. Parvulins are rotamases of small molecular mass and compared to immunophilins are relatively less abundant (Landrieu *et al.* 2000, Metzner *et al.* 2001, Yao *et al.* 2001). Cyclophilins are highly conserved across species from bacteria to human, and are found in multiple cellular compartments. The conserved nature of these proteins suggests an important and indispensable function for them in the cell.

Many different cyclophilins have been isolated and cloned from various plants (Chou and Gasser 1997 and references therein). Expression of many of the cyclophilin genes is enhanced in response to different abiotic stresses (Marivet *et al.* 1992, Chou and Gasser 1997, Leonardo *et al.* 1998, Kullertz *et al.* 1999, Godoy *et al.* 2000). Since cyclophilins have both protein folding as well as chaperonic properties (Boston *et al.* 1996), it is speculated that these may be stress-related proteins and are required in higher amounts to accelerate the folding step and, therefore, the maturation of newly synthesized proteins under stress. However, the biological function of these proteins *in vivo*, is still a matter of conjecture.

To gain better understanding about the role of cyclophilins in stress adaptation of plants it is imperative that the regulation of cyclophilin-associated PPIase activity should be investigated in a crop like sorghum which is well adapted to hot dry environments and regarded as a model for studying stress tolerance (Sanchez *et al.* 2002). To our knowledge, studies on the effect of different abiotic stresses on peptidyl prolyl *cis-trans* isomerases in general and cyclophilins in particulars have not been carried out in sorghum as yet. The present study reports for the first time the developmental and stress induced changes in the PPIase activity in the sorghum seedlings.

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Abbreviations: CsA - cyclosporin-A; FKBP - FK-506 binding protein; PPIase - peptidyl prolyl *cis-trans* isomerase.

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Materials and methods

Plants and treatments: Seeds of *Sorghum bicolor* (L.) Moench cv. CSH-6 were purchased from National Seed Corporation, Pusa, New Delhi, India. The seeds of drought tolerant (ICSV-272) and drought susceptible (SPRU-94008B) cultivars of sorghum, were procured from ICRISAT, Patancheru, Andhra Pradesh, India. The seeds were surface sterilized with 1 % (m/v) mercuric chloride and 70 % ethanol followed by rinsing with deionized water twice and plated on wet sterile filter papers in the Petri plates. The seeds were germinated at 37 °C in a seed germinator. The developmental changes in PPIase activity were studied in different parts of the seedlings, grown in acid washed sand in pots, for upto 12 d after germination whereas the effects of abiotic stresses on PPIases were examined on the 3-d-old seedlings grown in Petri plates.

For studying the effect of osmotic- and salt-stress the seedlings were irrigated with mannitol (0.75 M) and NaCl (0.41 M) solutions, respectively, for 24 h. The respective solutions, in equal volume, were applied to the filter paper. Osmotic potential (calculated by Van't Hoff equation) of both the solutions was -1.86 MPa. Osmotic stress-induced changes in PPIase activity in different tissues of sorghum seedlings were studied at 8, 16 and 24 h after mannitol treatment. The after-effects were followed by relieving the stress after 16 h by transferring the seedlings to a fresh plate and irrigating with sterile double distilled water and the samples were harvested at 12 h and 24 h. Heat stress-induced changes in PPIase activity were studied by exposing the seedlings to 42 °C for 2 h. The cold stress was imposed for 24 h by transferring the plates to 4 °C. The seedlings were separated into shoot, root and endosperm and, after snap freezing in liquid N_2, were stored at -80 °C till further analysis.

Extraction of proteins: Total soluble proteins from three replicates of shoots, roots, embryo and endosperms from 50 seedlings each were extracted in ice cold extraction buffer 5 cm^3 g^{-1}(f.m.) [5 mM Tris-Cl (pH 7.8), 12 mM phenyl methyl sulfonyl fluoride, 20 μM leupeptin, 10 mg dm^{-3} chymostatin, 50 mg dm^{-3} N-tosyl-L-phenyl alanine chloromethyl ketone, 20 mg dm^{-3} 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. Since PPIase activity was not detectable in the crude extracts, therefore, 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. The ethanol-precipitable PPIase activity of sorghum seedlings was sensitive to temperature with incubation at 65 °C for 5 min resulting in total loss of PPIase activity. Protein concentration of the ethanol-precipitated extracts was determined as described by Lowry (1951).

Peptidyl prolyl *cis-trans* isomerase assay: The fine chemicals and reagents used in this study were purchased from *Sigma*, St. Louis, USA. Cyclosporin-A (*Sandimmune*) (CsA) was puchased from *Sandoz AG*, Basel, Switzerland. Peptidyl-prolyl *cis-trans* isomerase activity of the ethanol precipitated extracts was assayed in a coupled assay with chymotrypsin as described by Breiman *et al.* (1992) using N-succinyl-ala-ala-pro-phe-p-nitroanilidine as the test peptide. The assays were performed at 4 °C for 360 s under N_2 environment and monitored at 390 nm with spectrophotometer (*Lambda Bio20*, *Perkin-Elmer*, Norwalk, USA) equipped with Peltier temperature control system. The assay mixture (1 cm^3) contained test peptide (40 μM), assay buffer [50 mM HEPES (pH 8.0), 150 mM NaCl, 0.05 % Triton X-100] and 600 μg of the proteins. The reaction was initiated by the addition of chymotrypsin (300 mg dm^{-3}) and the change in absorbance was monitored. Cyclophilin-associated PPIase activity was determined by the extent of inhibition of reaction in the presence of 50 μM CsA. The inhibitor was added to the assay mixture 30 min before the start of the reaction and incubated at 4 °C. The PPIase activity was calculated as described by Breiman *et al.* (1992).

Statistical analysis: Data obtained were subjected to analysis of variance.

Results

Specific PPIase activity in the 6-h-old imbibed ungerminated seeds was high and 90 % of this activity was inhibited by CsA (Fig. 1*A,B*). Dissection of the germinated seeds into endosperms and embryos revealed that most of the PPIase activity upto 2 d after germination was localized in the latter and upto 90 % of this activity was inhibitable by CsA. The PPIase activity of both shoot and root was very low in the 3-d-old and older seedlings. The maximum mean PPIase activity was observed in the endosperm whereas the least value was observed in the root (Table 1).

Maximum increase in specific PPIase activity was observed in response to osmotic stress and the response was observed in all the tissues with endosperm being relatively more responsive (Table 1). The salt stress had no significant effect on root PPIase activity but the

PPIase activity of shoot and endosperm increased significantly as compared to the control plants (Table 1). Similarly, significant increase in specific PPIase activity in response to heat stress was observed only in endosperm. Contrary to the endosperm, which showed a significant decrease in specific PPIase activity in response to cold stress, the specific PPIase activity of roots and shoots was relatively insensitive to cold stress.

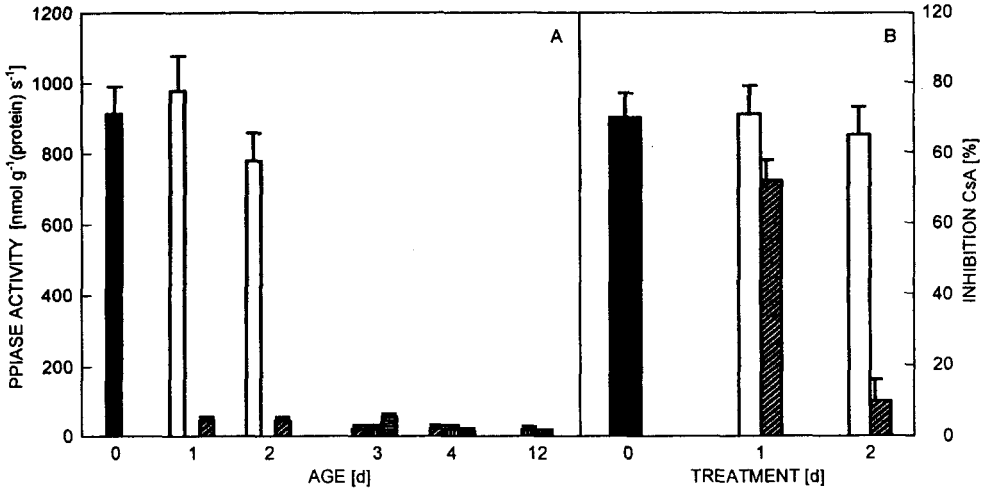

Fig. 1. Developmental changes in specific PPIase activity (*A*) of 6 h imbibed seeds (*full columns*), embryo (*empty columns*), endosperm (*diagonal strips*), root (*vertical strips*) and shoot (*horizontal strips*) of sorghum cv. CSH-6. Panel (*B*) represents the % inhibition of PPIase activity by CsA. Values are mean of three independent experiments ± SE.

Table 1. Effect of different abiotic stresses on specific PPIase activity [nmol g^{-1}(protein) s^{-1}] in different tissues of the 3-d-old seedlings of sorghum cv. CSH-6. Means of three independent experiments ($LSD_{0.05}$: treatment - 9.4, tisuue - 7.3, interaction - 16.2). Values in parentheses represent percentage inhibition of PPIase activity in presence of cyclosporin-A.

Treatment	Shoot	Root	Endosperm
Control	19.9	11.2	34.3
0.41 M NaCl (24 h)	339.9 (75)	11.5	146.6 (45)
0.75 M mannitol (24 h)	239.9 (43)	206.6 (68)	466.0 (59)
42 °C (2 h)	13.3	33.3	133.3 (55)
4 °C (24 h)	18.6	13.3	6.6

Table. 2. Effect of osmotic stress on specific PPIase activity [nmol g^{-1} (protein) s^{-1}] in different tissues of 3-d-old sorghum seedlings of drought tolerant (ICSV-272) and susceptible (SPRU-94008B) cultivars. Means of three independent experiments. Values in parentheses represent inhibition (%) of PPIase activity in presence of cyclosporin-A.

Cultivars	Treatments	Shoot	Root	Endosperm
ICSV-272	control	73.2 (36)	6.6	366.6 (79)
	stress	366.6 (76)	13.3	399.9 (78)
SPRU-94008B	control	19.9	17.3	313.3 (79)
	stress	18.5	139.9 (58)	193.3 (66)
$LSD_{0.05}$	treatment	tissue	interaction	
ICSV-272	16.6	20.4	38.8	
SPRU-94008B	7.6	9.3	23.2	

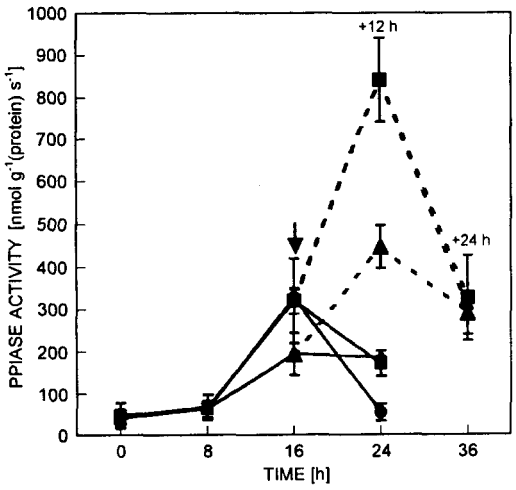

Fig. 2. Kinetic studies on osmotic stress-induced changes in PPIase activity in endosperm (*circles*), shoot (*triangles*), root (*squares*) of the 3-d-old seedlings of sorghum cv. CSH-6. *Arrowhead* indicates relieving of stress and *broken lines* signify the post-stress kinetics. Means of 3 independent experiments ± SE.

Depending upon the stress, the contribution of cyclophilins to the specific PPIase activity in the endosperm varied from 45 - 60 % (Table 1). The CsA-inhibition of shoot PPIase activity under salt- and osmotic stress was 75 and 43 %, respectively. The osmotic stress-induced-enhancement in specific PPIase activity of the roots was also mainly due to CsA-inhibitable activity (68 %). Maximum enhancement in PPIase activity in response to osmotic stress was observed after 16 h of stress exposure. Continued

imposition of stress resulted in a drastic decrease in PPIase activity in all the tissues except shoots (Fig. 2). However, relieving of stress at 16 h resulted in continued increase in PPIase activity for another 12 h in all the tissues before decreasing at 24 h after the stress relief.

The effect of osmotic stress on PPIase activity of drought tolerant (ICSV-272) and susceptible (SPRU-94008B) cultivars was differential and tissue dependent (Table 2). The endosperm PPIase activity of the cv. SPRU-94008B decreased substantially under stress conditions whereas that of the cv. ICSV-272 was unaffected. Maximum increase in osmotic stress-induced specific PPIase activity in ICSV-272 was observed in shoots whereas in cv. SPRU-94008B in the roots. The contribution of cyclophilins to specific shoot PPIase activity in cv. ICSV-272 increased from 36 % under control conditions to 76 % under osmotic stress. Cyclophilin-associated PPIase activity in the stressed roots of cv. SPRU-94008B was 58 %.

Discussion

The determination of total cellular PPIase activity associated with cyclophilins is important for studying the role of these enzymes in stress adaptation since cyclophilins are encoded by up to 20 different genes which are regulated differently (Chou and Gasser 1997). Therefore, in the present study we examined the total cellular PPIase activity in the ethanol-precipitated extracts of sorghum. Since there is no cross inhibition between CsA and FK-506 binding proteins (Kallen *et al.* 1991), therefore, the members of the two classes (*i.e.* cyclophilins and FKBPs) can be differentiated by applying specific inhibitors. In the present study the proportion of cyclophilin-associated PPIase activity was determined by using CsA as an inhibitor. Although regulation of cyclophilin genes in response to different stresses has been studied in various plants (Chou and Gasser 1997) surprisingly no information on PPIases is available in sorghum which is a model crop for studying drought stress tolerance (Sanchez *et al.* 2002).

Our studies revealed that the PPIase activity in the sorghum seedlings remained high for up to 2 d after germination following which it declined to marginal levels. Up to 90 % of the total PPIase activity till 2 d after germination was inhibited by CsA (Fig. 1) thus suggesting that cyclophilins are crucial in the germination and initial growth of seedlings. These results are in accordance with earlier studies reporting maximum expression of cyclophilin genes in the initial stages of seedling development in other crops (Marivet *et al.* 1992, 1994, 1995, Godoy *et al.* 2000). Germination is associated with changes in gene expression which lead to synthesis of new proteins (Colorado *et al.* 1991). High cyclophilin-associated PPIase activity in the germinating seeds and the 3-d-old seedlings may be assisting in the folding and transport of newly synthesized proteins (Owens-Grillo *et al.* 1996, Reddy *et al.* 1998).

The effect of different stresses on PPIase activity was differential and tissue dependent (Table 1). Despite osmotic potential of mannitol and sodium chloride solutions being similar, the change in PPIase activity in different tissues was different for the two stresses thus suggesting distinct regulatory pathways for salt and osmotic stress. Of all the stresses, the PPIase activity of sorghum seedlings was maximally responsive to osmotic stress. The increase in both specific and total PPIase activity (data not shown) in response to different stresses imply specific induction of the PPIase genes under osmotic stress conditions. The continued increase in PPIase activity for another 12 h (Fig. 2) after relieving of osmotic stress lasting 16 h could be due to resumption of the PPIase gene expression. Similar observation was reported for cyclophilin gene expression in bean under heat stress (Marivet *et al.* 1994). The osmotic stress-induced transient increase in PPIase activity observed in this study is consistent with earlier observations reported for different immunophilin genes in response to heat and cold stresses (Marivet *et al.* 1994, Kullertz *et al.* 1999), wounding and methyl jasmonate (Marivet *et al.* 1992, Godoy *et al.* 2000), and salicylic acid (Marivet *et al.* 1995). It is likely, that PPIases, like several other genes which are also transiently expressed in response to salt, wounding (Benedetti *et al.* 1998), cold (Takahashi *et al.* 1997), and drought stress (Yamaguchi-Shinozaki and Shinozaki 1994) are conferring adaptive advantage to the cell under stress conditions. By virtue of their chaperonic and isomerase activity (Boston *et al.* 1996), the stress-induced PPIases may help stress-induced proteins to maturation. PPIases are involved in signal transduction through modulation of Ca^{2+} dependent phosphatase activity (Jackson and Soll 1999), therefore, besides assisting the newly synthesized stress proteins in folding, the stress-induced PPIases may also be involved in signal transduction in response to different stresses. The stress-induced PPIase activity was not completely inhibited by cyclosporin-A (Tables 1, 2), which imply that other PPIases viz. parvulins and FK-506 binding proteins are also playing a role in stress response as reported for wheat FKBP77 (Kurek *et al.* 1999).

The differential osmotic stress-induced changes in PPIase activity of drought tolerant and susceptible cultivars indicate that PPIase activity can be used for studying intercultivar variability with respect to drought tolerance. A broader study with more drought tolerant and susceptible cultivars should be carried out to determine the potential of PPIase activity as a marker for stress tolerance.

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