Characterization of stress and methylglyoxal inducible triose phosphate isomerase (OscTPI) from rice

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As compared with plant system, triose phosphate isomerase (TPI), a crucial enzyme of glycolysis, has been well studied in animals. In order to characterize TPI in plants, a full-length cDNA encoding OscTPI was cloned from rice and expressed in E. coli. The recombinant OscTPI was purified to homogeneity and it showed K_m value of 0.1281 ± 0.025 μM, and the Vmax value of 138.7 ± 16 μmol min⁻¹mg⁻¹ which is comparable to the kinetic values studied in other plants. The OscTPI was found to be exclusively present in the cytoplasm when checked with the various methods. Functional assay showed that OscTPI could complement a TPI mutation in yeast. Real time PCR analysis revealed that OscTPI transcript level was regulated in response to various abiotic stresses. Interestingly, it was highly induced under different concentration of methylglyoxal (MG) stress in a concentration dependent manner. There was also a corresponding increase in the protein and the enzyme activity of OscTPI both in shoot and root tissues under MG stress. Our result shows that increases in MG leads to the increase in TPI which results in decrease of DHAP and consequently decrease in the level of toxic MG.

Introduction

Methylglyoxal (MG), a cytotoxin, is a typical 2-oxoaldehyde byproduct of several metabolic pathways such as threonine catabolism and lipid peroxidation. However, the major source of production of MG is via glycolysis where it is produced from non-enzymatic elimination of phosphate from glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). The interconversion of DHAP and GAP is catalyzed by a crucial glycolytic enzyme, triose phosphate isomerase (TPI) which plays an important role in other metabolic pathways involving triosephosphate, such as gluconeogenesis, fatty acid biosynthesis, pentosephosphate pathway, and photosynthetic carbon dioxide fixation. High resolution crystal structures are available for TPI from chicken,3 yeast,3 and Trypanosoma brucei.4 The extremely high turnover rate of TPI has led to its description as a nearly perfect catalyst.5 Genes encoding TPI have been cloned and sequenced from 11 species, including chicken,6 human,7,8 maize,9 mouse,10 Aspergillus nidulans,11 Coptis japonica,12 Escherichia coli,13 Macaca mulatta,14 T. brucei,15 Saccharomyces cerevisiae,16 and Schizosaccharomyces pombe.†7 The high degree of sequence conservation of TPI suggests a low rate of evolutionary divergence, a common feature of all the glycolytic enzymes.18 Isolation and characterization of a cDNA clone encoding the cytosolic form of TPI from rice has also been reported.19

It has been reported that loss of TPI activity results in elevated levels of DHAP20,21 which in turn, is spontaneously converted to MG that modifies proteins and DNA through the formation of advanced glycation end products (AGES) are deleterious to cells.22 Enhanced AGE production also leads to many disease
The existence of plastid and cytosolic TPI isozymes was first reported in an extensive survey of isoelectric points of the enzyme in numerous species of angiosperms, gymnosperms, ferns, and fern allies. TPI isozyme has also been purified from spinach, lettuce, and celery.

In the present study, we report that rice cytoplasmic TPI is induced in response to MG stress. MG affects the transcript, protein and enzyme activity. This could be a mechanism to bring down the toxic levels of MG under stress conditions defending the plants against abiotic stress.

Identification and chromosomal distribution of TPI genes in rice. We have identified 3 TPI genes within the rice genome (TIGR, version 6.1), encoding three isoforms of TPI proteins. They have been demarcated on the basis of cellular localization of the encoded proteins. The physical distribution of TPI genes on different chromosomes of rice is shown in Figure 1A. Out of three isoforms, two are located on chromosome no I, while the third is situated on chromosome IX. The genes on chromosome I encode cytosolic proteins and show about 70% homology at amino acid level with each other (Fig. 1B). The gene located on chromosome IX encodes proteins localized in chloroplast.

Phylogenetic tree reveals that there is about 60–80% similarity between the TPI sequences of Arabidopsis thaliana, Solanum tuberosum, Hordeum vulgare, Zea mays and Oryza sativa (Fig. 1C).

The cytoplasmic TPI, present on the chromosome no I (LOC_Os01 g62420) was selected for further studies, which showed four splice forms (http://rice.plantbiology.msu.edu/). The CDS coordinates (5’-3’) is from 36129310 – 36132720.
The predicted nucleotide and amino acid length is 768 bp and 255 aa respectively, whereas the molecular weight is 27273.4 D.

For microarray analysis of rice TPI genes, Affymetrix GeneChip Rice Genome Arrays (http://www.ncbi.nlm.nih.gov/geo/) was used. Spatial and temporal gene expression in various tissues/ organs and developmental stages of rice was analyzed to identify the genes differentially expressed during various stages of reproductive development. OscTPI (LOC_Os01g62420) was interestingly expressed at all the stages of development as compared with the other isoforms (Fig. 1D).

Expression, purification and specific activity of recombinant OscTPI. Clone of rice TPI located on chromosome no I was amplified and further cloned into pET28a bacterial expression vector. The pET28a-OscTPI recombinant plasmid was transformed into BL21 (DE3) a specific host strain of E. coli, for expression of the recombinant protein. A highly induced band of 27 kDa was visible on SDS-PAGE, which was not present in the uninduced cells as well as induced negative control (BL21 cells containing empty vector (Fig. 2A). The recombinant protein was purified by anion exchange chromatography and monitored on SDS-PAGE (Fig. 2B). The eluted purified protein fractions were pooled and dialyzed against 20 mM Hepes buffer pH 8.0. Yield of the eluted soluble protein was about 7.0 mg/ml of culture.

Enzyme kinetics of OscTPI. Different kinetic parameters such as K_m and V_max were determined with purified recombinant OscTPI by systematically varying the concentration of glyceraldehyde 3-phosphate (50 µM to 1 mM) in the presence of NADH. The K_m was found to be 0.1281 µM, and the V_max 138.7 µmol min⁻¹ mg⁻¹ (Fig. 2C). The kinetic values were very similar to other TPIs of many organisms such as Potato, Yeast, Rabbit and Chicken muscle.33 (Table 1)

Functional complementation of yeast TPI (TIM1) mutant with OscTPI. To confirm whether the OscTPI can functionally complement the yeast TIM1 (YDR050C) mutant by reverting the sensitive phenotype, OscTPI was cloned in yeast expression vector pYES2 under pGAL1 promoter which is galactose inducible and glucose repressible. S. cerevisiae strain BY4741 and TPI deletion strain in the BY4741 background were transformed with pYES2OscTPI construct or empty vector (pYES2 plasmid). Since pYES2 vector has Ura⁺ as the selection marker, the transformed yeast cells were grown on Ura⁻ SD media containing either glucose or galactose at 37°C while TIM mutant with empty vector was unable to grow at 37°C, the BY4741 WT yeast strain could grow. However, when the TIM mutant was transformed with pYES2OscTPI construct, it was able to complement the growth defect of TIM1 mutant at 37°C (in galactose containing media). This result clearly indicates that the rice OscTPI can functionally complement TIM mutation in yeast (Fig. 3).

Localization of OscTPI. To verify the cellular location, OscTPI cDNA was cloned into a GFP based vector, pMBP11-GFP. In the resulting plasmid pMBGFP-TPI, the expression of gene is governed by the strong constitutive cauliflower mosaic virus 35S promoter (CaMV 35S) and the nopaline synthase terminator (NOS-T). Upon inspection of Nicotiana tabacum protoplast transiently transfected with this plasmid, the protein was localized in the cytosol, and evenly distributed without forming any visible aggregates (Fig. 4A). The fluorescence was not detected in the untransfected control cells, confirming that the green fluorescence indeed originated from the expression of the introduced pMBGFP-OscTPI.

Localization assay for OscTPI was also performed using onion peel bombardment assay (Fig. 4B). The ORF of OscTPI was cloned into pMB-GFP vector and pMB-GFP-OscTPI construct was used for onion peel transformation by particle bombardment using the PDS-1000 particle delivery system (Bio-Rad, USA). When observed under fluorescence microscope at 488 nm exciting wavelengths, it was found to be equally distributed in the cytoplasm.

To further confirm the localization of TPI, cellular fractionation from 12 d old rice seedlings was performed by ultracentrifugation and various fractions such as cytosol, chloroplast and nuclei was collected. All the three fractions were loaded on SDS-PAGE and western blot hybridization was performed using the antibodies raised against TPI which reconfirmed the cytoplasmic nature of TPI.

Transcript analysis of OscTPI in response to various stresses. In order to understand the response of the cytoplasmic TPI under various stresses at different time points, the relative transcript level of OscTPI was determined by quantitative RT-PCR after exposure of 6 h and 24 h in response to high temperature (42°C), drought (RT), wounding, low temperature (4°C), salt stress (NaCl, 200 mM), oxidative stress (H_2O_2, 5 mM), and methylglyoxal (5 mM). The OscTPI transcript was about 2 folds upregulated at 6 h and 24 h following wounding and MG stress. About 1.5 folds upregulation of OscTPI was observed under low temperature and H_2O_2 stress at 6 h, the transcript level dramatically decreased to 0.3 folds when stress persisted for 24 h. Since relative expression ratio of OscTPI was less than 1, it showed a consistent 0.6–0.8 fold down-regulation in case of heat, drought and...
PAGE using total protein isolated from non-stressed and MG stressed shoot tissues (Fig. 6E) which reconfirmed the higher activity of TPI under MG stress as compared with control.

To check if the increase in the enzyme activity is due to the increase in protein level, western blot was performed using the same tissues from which activity was measured. Data given in Figure 6D shows an increase in overall protein level with an increase in MG concentration.

Discussion

TPI, a glycolytic enzyme, is well studied in animal systems. However, not much is known about its role in plants. Earlier cytoplasmic TPI (cTPI) has been particularly characterized and purified from *Solanum chacoense* and *Spinach oleracea*. In this work, we report biochemical characterization of cytoplasmic TPI from rice and its regulation under abiotic stress.

The rice TPI located on chromosome I was cloned and characterized. Amino acid sequences of TPIs from various organisms were found highly conserved and showed about 60–80% homology to plants such as *Zea mays*, *Arabidopsis thaliana*, *Hordeum vulgare* and *Solanum tuberosum*. We also found by Complementation assay that rice TPI can complement the yeast TPI partially. This partial complementation could be because the protein sequence for both the organisms shares only 55–60% homology. However, under oxidative stress, higher TPI activity was observed after 6 h which decreased by 24 h. It was also noticed that enzyme activity declined marginally at both the time points when seedlings were subjected to other stresses such as low temperature, drought and heat. In case of MG stress, it showed a good correlation with the transcript level (Fig. 6A). In case of roots also, similar pattern of TPI activity was observed with higher activity under MG, wounding and NaCl stress while the enzyme activity declined in case of cold, drought and heat stress (Fig. 6B).

To confirm the effect of MG, TPI activity was measured using 12 d old rice seedlings exposed to different concentrations of MG for 24 h. We found that activity linearly increased with the increase of MG concentration (Fig. 6C). The TPI activity assay was also performed on native NaCl after 6 h and 24 h after treatment (Fig. 5A).

Transcript level for OscTPI were also measured in the 12 d old rice seedlings given various MG stress (0, 5, 10, 15 and 20 mM) for 24 h (Fig. 5B) and transcript level was found upregulated increasing concentration of MG.

In planta enzyme activity of OscTPI under various stresses. As TPI shows higher transcript level under stress, we checked if this results in the higher enzyme activity. The specific activity was measured under similar conditions of stress treatment as given for measuring the transcript level in rice seedlings. Both root and shoot tissues were harvested after 6 and 24 h of treatment and native TPI activity was monitored. It was found that TPI activity was higher in shoots under MG, wounding and salt stress at both the time points.

### Table 1. Primer sequences used for cloning and Real Time PCR

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<tr>
<th>Primer Name</th>
<th>Primer sequence (5'-3')</th>
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<tr>
<td>OscTPI-UTR-Fwd</td>
<td>5’-CAG CTT CCA CTC CAC GAA AAC C-3’</td>
</tr>
<tr>
<td>OscTPI-UTR-Rev</td>
<td>5’-GAA CAC TAA GCA GAA GAC TTG ACG G-3’</td>
</tr>
<tr>
<td>OscTPI-BamH1-Fwd</td>
<td>5’-CGC GGA TCC ATG GCC AGG AAG TTC T-3’</td>
</tr>
<tr>
<td>OscTPI-Xho1-Re</td>
<td>5’-CGG CTC GAG CTA AGC AGA AGA TTG CAC G-3’</td>
</tr>
<tr>
<td>OscTPI_Real_Fwd</td>
<td>5’-ATC AGA TGA ACT GAA AGT GCC GTT-3’</td>
</tr>
<tr>
<td>OscTPI_Real_Rev</td>
<td>5’-GAC TAC GAA AAC AAG TAA TCA T-3’</td>
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<tr>
<td>OscTPI_Xba_Fwd</td>
<td>5’-GCT CTA GAC AGC TCC CAC TCC ACG AAA ACC-3’</td>
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<tr>
<td>OscTPI_BamH1_Rev</td>
<td>5’-AGT GGA TTC TCA GCA GAA GAC TTG ACG GTA GCA GA-3’</td>
</tr>
<tr>
<td>OscTPI_HindIII_Fwd</td>
<td>5’-CCC AAG CTT CTA AGC AGA AGA CTG GAC G-3’</td>
</tr>
<tr>
<td>OscTPI_BamH1_Rev</td>
<td>5’-AGT GGA TTC TCA GCA GAA GAC TTG ACG GTA GCA GA-3’</td>
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**Figure 3.** Comparison of growth pattern of *S. cerevisiae* TIM1 mutant expressed under different temperature. *S. cerevisiae* transformed with pYES2 and pYES2 OscTPI on solid SD media with either 20% glucose (upper panel) or 20% galactose (lower panel). Plates were incubated at 30°C and 37°C for 72 h.
of conversion of D-GAP-to-DHAP is different in this direction is close to its maximum. Fusion controlled; meaning that $k_{cat}/K_m$ in pea, 44 spinach, lettuce, celery, and isoforms have been separated and studied. A low $K_m$ value means that the enzyme has a high affinity for the substrate i.e., less concentration of substrate is enough to run the reaction at half its max speed. TPI is a non-allosteric glycolytic enzyme which performs isomerization of DHAP and GAP at pH 8. It has been reported that the key component of the active site of TPI is the catalytic base (Glu 167) as well as electrostatic stabilization.

It has also been reported that TPI is a very efficient enzyme. For example, the rate of conversion of D-GAP-to-DHAP is diffusion controlled; meaning that $k_{cat}/K_m$ in this direction is close to its maximum possible value of $10^9 \text{ M}^{-1} \text{s}^{-1}$.

Previous reports have shown that TPI in plants occurs in the cytosol and in the plastid fraction. In fact both the isoforms have been separated and studied in pea, spinach, lettuce, celery, and broccoli. To confirm that the OscTPI is cytoplasmic in nature, localization assay was performed using the PEG mediated protoplast transfection which clearly indicated that it is evenly distributed in the cytosol. Localization of OscTPI was also confirmed using onion peel bombardment assay and fluorescence microscopy. We also performed cellular fractionation from rice seedlings and reconfirmed the cytoplasmic nature of OscTPI by western blot hybridization.

Rice cytosolic TPI has been reported to show higher transcript level under submergence and salt stress. Most of the other glycolytic enzymes also show higher activity under hypoxia and anoxia stress in rice. Even water stress induces TPI in maize while no significant change has been observed in activity of TPI under salt stress in pea. In the present study, we did not see significant upregulation in TPI transcript under NaCl (200 mM) stress. This difference could be due to the use of rice seedlings at different age, stress treatment schedule and the genotype etc. (As we have used IR64, a salt sensitive variety while Kawasaki et al. used Pokkali, which is a salt tolerant variety) under different conditions.

Our data confirms the upregulation of OscTPI under wound and MG stress, however, it was most prominent in case of MG where about 2-folds increase in the transcript level at 6 and 24 h of stress was recorded (Fig. 5A). Interestingly, when transcript was checked from the rice seedling given various MG stress, TPI showed increase in transcript level from 0 mM to 20 mM MG (Fig. 5B). Earlier, cytoplasmic TPI in yeast is reported to increase 7-folds under MG stress. In humans also, it has been shown that MG causes a significant increase in transcript and protein expression of the nervous growth factor (NGF) as well as of the pro-inflammatory cytokine IL-1β, a major inducer of the acute phase response. Though we could not find any reports where MG induces TPI particularly, it has been shown that MG induced a dose- and time-dependent increase in aldose reductase (AR) mRNA level in rat aortic smooth muscle cells.

Western blot hybridization and enzyme activity of TPI also showed a linear correlation between the TPI transcripts, protein level and enzyme activity under MG stress. This correlation was also observed under drought, heat and wound stress. However, in case of salinity stress, a downregulation was observed at the transcript level while the TPI enzyme activity was found to be slightly higher. The difference between transcript level and the enzyme activity is possibly due to the post-translational modification. Studies have shown that the correlation between protein and transcript expression levels can vary significantly based upon the profiling approach used and the system being analyzed. Many specific examples of discordant protein/transcript expression patterns have also been detected recently revealing examples of posttranslational regulation.

We have previously shown that in response to stress, there is an increase in MG level which is quite deleterious to plant growth. Our earlier studies also shows that seed germination, shoot and root growth is severely affected by exogenous application of MG. Inhibitory effect of exogenous MG on seed germination in barley has also been demonstrated in 1970s by Mankikar and Rangekar. Our present data now indicates that there is an upregulation of TPI transcript, protein and enzyme activity under various
concentration of MG. We perceive that in order to rescue the stress, the increase in MG activates the TPI which shifts the reaction toward the formation of GAP, thus decreasing the concentration of DHAP. Once DHAP level goes down, the amount of MG present in the system will decrease and hence the plant could survive under stress. Higher level of MG has been previously shown to induce several glycolytic enzymes such as, phosphoglucone isomerase (PGI), phosphofructokinase (PFK) and triosephosphate isomerase (TPI) in yeast. It is also possible that due to increase in TPI, the amount of GAP will increase in the system which can positively affect the energy level in plant and thus support the plant survival.

Our present results elucidate that MG could act as a signal molecule to induce the expression of TPI in rice. The increase of TPI under MG stress, on one hand, decreases the level of MG by feedback mechanism, and on the other hand increases the concentration of GAP, and thus increasing the energy status of the plant against the stress consequences. This could be one of the mechanisms to defend the plant system from abiotic stresses.

Materials and Methods

Genomic distribution of TPI genes on rice chromosomes. Position of each of the TPI genes on rice chromosome pseudomolecules available at TIGR version 6.1 (http://rice.plantbiology.msu.edu/cgi-bin/ORF_infopage.cgi) was determined by BLASTn search.

Multiple alignment and phylogenetic analyses of TPI. Multiple alignments of all the isoforms of rice TPI proteins were performed using ClustalW2 program. For phylogenetic analysis, sequence from different organisms such as Arabidopsis thaliana, Solanum tuberosum, Hordeum vulgare, Zea mays Oryza sativa, Oryctolagus cuniculus, Homosapiens sapiens, Saccharomyces cerevisiae and Escherichia coli were taken and unrooted tree was generated for cytoplasmic TPI using neighbor joining method employing Mega4 software.

Expression analysis using Rice Genome database. For microarray analysis of rice TPI genes, Gene Expression Omnibus platform (http://www.ncbi.nlm.nih.gov/geo/) accession number GSE6893 was used and heatmap was generated using the Institute for Genomic Research MeV software package. Here, different stages of panicle and seed development have been categorized according to panicle length and days after pollination, respectively, based on landmark developmental events as follows: up to 0.5 mM, shoot apical meristem and rachis meristem (SAM); 0–3 cm, floral transition and floral organ development (P1); 3–10 cm, meiotic stage (P2 and P3); 10–15 cm,
and sonicated in 20 ml sonication buffer and centrifuged. The clear lysate was collected, mixed with 2 ml Ni-NTA agarose slurry by shaking gently (200 rpm on a rotary shaker) at 4°C for 60 min. The lysate–Ni-NTA mixture was loaded into a column and the protein was eluted 10 times with 1 ml elution buffer (50 mM sodium phosphate buffer pH 8.0, 10% glycerol, 300 mM NaCl, 1 mM PMSF, 100 mM Imidazole). The eluted fractions were analyzed by SDS-PAGE.

**Determination of Specific activity and kinetic parameters of OscTPI.** Specific activity of OscTPI was assayed with glyceraldehyde 3-phosphate as substrate. As previously reported, dihydroxyacetone phosphate productions was coupled to NADH oxidation via α-glycerol phosphate dehydrogenase. Reaction mixture containing 100 mM triethanolamine hydrochloride, 10 mM EDTA, 1 mM dithiothreitol, pH 7.4, containing 0.2 mM NADH, 20 ng α-glycerol phosphate dehydrogenase and 0.1-2 mM D-glyceraldehyde 3-phosphate was initiated by the addition of 50 ng of purified recombinant protein. The initial rate was calculated from the change in NADH absorbance at 340 nm with spectrophotometer. All experiments were performed at 25°C.

Kinetic studies for rice TPI were performed using the reaction mixture containing 0.03 mM NADH, 0.4 mM D-glyceraldehyde-3-phosphate and variable concentrations of glyceraldehyde 3-phosphate between 0.05 mM and 2.0 mM and 600 ng of purified triosephosphate isomerase in a final volume of 1 ml.

**Functional complementation of yeast TPI (Tim) mutant with OscTPI.** OscTPI gene was cloned in the yeast expression vector pYES2 downstream to GAL1 promoter. S. cerevisiae cells BY4741 (Mat a, his3Δ1 leu2Δ0 met15Δ0 ural3Δ0), BY4741ΔTIM1 (Mat a, his3Δ1. leu2Δ0, met15Δ0. ural3Δ0. YHR057c::kanMX4) were transformed with either pYES2 alone or pYES2OscTPI and transformants were picked up by Ura prototrophy. For comparing the growth profile of these strains, cells were growing on solid YPD plates supplemented with either 20% glucose or 20% galactose and grown at 30°C and 37°C for 48 h-72 h.

### Table 2. \( K_m \) values for TPI in various organisms

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<tr>
<th>Source of enzyme</th>
<th>( K_m ) (mM)</th>
</tr>
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<tr>
<td>Oryza sativa</td>
<td>0.12 ± 0.025</td>
</tr>
<tr>
<td>Solanum tuberosum</td>
<td>0.13 ± 0.014</td>
</tr>
<tr>
<td>Trypanosoma brucei</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>Saccharomyces cerevisae</td>
<td>1.22 ± 0.05</td>
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<tr>
<td>Chicken muscle</td>
<td>0.47 ± 0.05</td>
</tr>
<tr>
<td>Orycto laguscinusculis</td>
<td>0.39 ± 0.05</td>
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microprojectile bombardment, using a Bio-Rad PDS/1000 helium-driven particle accelerator, as per manufacturer’s instructions. Plate was incubated at 28°C for 18 h in dark before microscopic analysis. Transformed epidermal onion peels were observed under fluorescence microscope. GFP signals were detected between 505–550 nm after exciting with 488 nm.

Native protein isolation and TPI Activity in plants. TPI activity was assayed with D-glyceraldehyde-3-phosphate and glycero phosphate dehydrogenase as a coupling enzyme followed by measuring the decrease in OD at 340 nm. Specific activity of TPI was also measured in 12 d old rice seedlings (IR64) exposed to stress conditions. Native protein was assayed following extraction with buffer containing 0.1 M sodium phosphate buffer, pH 7.0, 50% glycerol, 16 mM MgSO₄, 0.2 mM PMSF and 0.2% PVPP. Protein was quantified using Bradford method and the supernatant was immediately used for TPI assay. Two microgram of crude protein was added to the reaction mixture containing 0.03 mM NADH, 0.4 mM D-glyceraldehyde-3-phosphate and one unit of glycero phosphate dehydrogenase. A time scan was performed spectrophotometrically, taking the OD₃₄₀ at every 10 sec within a time scale that showed a linear increase in absorbance. The molar absorption coefficient of NADH at 340 nm is 6.3 x 10³ M⁻¹ cm⁻¹. The specific activity of the enzyme was expressed in μm/ min/mg of protein.

Native PAGE was performed on 10% acrylamide gels and TPI activity was assayed. The gel was stained as described by Pichersky and Gottlieb, briefly, after native electrophoresis, gels were incubated for 20 min in equilibration buffer (0.1 M Tris–Cl pH 7.5, 0.5 mM EDTA and 0.2 mM NADH) with gentle agitation. The gel was then transferred to developing buffer containing 0.1 M Tris–Cl pH 7.5, 1 mM GAP, 0.5 mM EDTA, 0.2 mM NADH and 3 U/ml GPDH. When viewed over a UV transilluminator, TPI isozymes appeared within 2–5 min as dark bands over a fluorescent background. Control gels were incubated without GAP that did not show any activity band even when incubated for up to 60 min.

Cell Fractionation. Preparation of nuclear extract. Nuclear extracts were prepared according to the method described previously. 12 d old rice seedlings were homogenized and extracted using cold buffer containing 0.55 M sucrose, 50 mM Tris ± Cl pH 8.0 and 10 mM MgCl₂ and the homogenate was passed through two layers of cheesecloth and two layers of Miracloth. The filtrate was then centrifuged at 1000 g for 10 min at 4°C and the pellet was slowly resuspended in STEM containing 2.5% TritonX100, and incubated at 4°C with slow shaking followed by centrifugation at 2000 g for 30 min at 4°C. The resulting nuclear pellet was then resuspended in a buffer containing 600 mM KCl, 50 mM Tris ± Cl pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.5 mM PMSF, 50 mM leupeptin and 1 mM pepstatin, and the homogenate was spun at 12 000 g for 30 min at 4°C, and the clear supernatant (nuclear extract) was dialysed against buffer containing 50 mM KCl, 50 mM Tris ± Cl pH 8, 20% glycerol and protease inhibitors, and stored at ± 80°C.

Preparation of chloroplast lysate. Chloroplast lysate was prepared by the method earlier described earlier. Briefly, 12 d old rice seedlings were homogenized and extracted in buffer containing 0.5 M sucrose, 50 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 10 mM 2- mercaptoethanol, 0.3 mM phenylmethylsulfonyl fluoride, 1 mM sodium bisulfite and 1 mg/ml benzamidine . The homogenate was filtered and filtrate was centrifuged for 10 min at 1000 g and the pellet suspended in 120 ml of the above buffer. To disrupt the chloroplast, 30 ml 12.5% Triton X-100 was added to the chloroplast suspension. The mixture was kept on ice for 45 min, stirred occasionally and centrifuged at 3000 g for 15 min. Chloroplast lysate was used for western blot hybridization.

Western Blot Hybridization. Western blot assay was performed as described previously. Proteins separated on SDS-PAGE were transferred to Hybond-C membrane (GE Healthcare) using Mini Transblot Electrophoretic cell (Biorad, USA). Electroblotting buffer consisted of 150 mM glycine, 20 mM Tris and 20% methanol (pH 8.0). The blot was incubated in blocking solution (5% non-fat dried milk dissolved in PBS) for 45 min and then washed with 0.1% PBST. Blot was further incubated with primary antibodies at specific dilution with blocking buffer for 1 h followed by incubation in Alkaline-Phosphatase conjugated secondary antibodies (diluted in blocking buffer) for 1 h. After washing, The protein-antibody complex was developed in 5-bromo-4-chloro-3-indolyl phosphate (BCIP) / nitroblue tetrazolium (NBT) containing solution (0.1 M TRIS-HCl pH 9.5, 0.1 M NaCl, 5 mM MgCl₂, containing 150 μg/ml of NBT and 75 μg/ml of BCIP). The reaction was stopped by rinsing the blot with water.

Real time PCR. Real Time PCR was performed as previously standardized and described. Total RNA was isolated from the shoot tissues of control and stressed seedlings using RaFlex™ solution I and solution II (GeNei, India), and cDNA synthesis was performed with RevertAid™ RNase H minus cDNA synthesis kit (Fermentas Life Sciences, USA). Manufacturer’s protocol was strictly followed in both the above procedures. Real time PCR primers for OsTPI were designed from 3’ UTR regions using Primer3 software. cDNA was further diluted 50 times and reaction mixture was prepared adding 5 μl of diluted cDNA, 12.5 μl of 2 x SYBR Green PCR Master Mix (Applied Biosystems, USA) and 200 nM of each gene-specific primer in a final volume of 25 μl. ABI Prism 7500 Sequence Detection System was used for qRT-PCR for 2 min a 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C, 1 min at 57°C, and 30 sec at 72°C in 96-well optical reaction plates (Applied Biosystems). Three technical replicates were analyzed and amplification was checked using dissociation graph, and relative expression ratio was calculated using comparative Ct value method.

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19. Xu MQ, Southworth MW, Mertha FB, Hornstra LJ,
31. Tomson JD, Turner JF. Pea seed triosephosphate
29. Artavanis-Tsakonas S, Harris JI. Primary struc-
27. Ting SM, Miller ON, Sellinger OZ. The metabolism
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