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Multiple abiotic stress responsive rice cyclophilin (OsCYP-25) mediates a wide range of cellular responses

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Abbreviations: ABA, abscisic acid; DEAD, aspartate-glutamate-alanine-aspartate; OsCYP-25, *Oryza sativa* cyclophilin; PPIase, peptidyl prolyl isomerase; qRT-PCR, quantitative real-time polymerase chain reaction

Cyclophilins (CYP), a member of immunophillin group of proteins, are more often conserved in all genera including plants. Here, we report on the identification of a new cyclophilin gene OsCYP-25 (LOC_Os09 g39780) from rice which found to be upregulated in response to various abiotic stresses viz., salinity, cold, heat and drought. It has an ORF of 540 bp, encoding a protein of 179 amino acids, consisting of PPIase domain, which is highly conserved. The OsCYP-25 promoter analysis revealed that different ***cis***-regulatory elements (e.g., MYBCORE, MYC, CBFHV, GT1GMSCAM4, DRECRTCOREAT, CCAATBOX1, WRKY71OS and WBOXATNPR1) are involved to mediate OsCYP-25 response under stress. We have also predicted interacting partners by STRING software. In interactome, protein partners includes WD domain containing protein, the 60S ribosome subunit biogenesis protein, the ribosomal protein L10, the DEAD-box helicase, the EIF-2α, YT521-B protein, the 60S ribosomal protein and the PPR repeat domain containing protein. The in silico analysis showed that OsCYP-25 interacts with different proteins involved in cell growth, differentiation, ribosome biogenesis, RNA metabolism, RNA editing, gene expression, signal transduction or stress response. These findings suggest that OsCYP-25 might perform an important function in mediating wide range of cellular response under multiple abiotic stresses.

Introduction

Multiple abiotic stress factors viz., water deficit, water logging, high salinity, extreme temperature, high radiation, chilling injury, heavy metal toxicity and pH cause negative impact on plant growth and productivity by impairing proteins, lipids, DNA function and reducing genome stability.[1] Abiotic stresses are assumed to be the main cause of yield reduction in various crops.[2-4] The estimated potential yield losses due to abiotic stresses, namely, drought, salinity, high temperature, low temperature and other factors were reported as 17, 20, 40, 15 and 8%, respectively.[3,5] Rice production necessitates to be doubled to overcome the increasing demands of the population.[6] In light of these facts, the study of new genes responsive to abiotic stress conditions is still required.

The ability of plants to cope with high stress surroundings in their environment, involves a combination of phenotypic plasticity and genetic adjustment.[7,8] All plants can sense and transmit stress stimuli signals to trigger different cell signaling cascades involved in ion channels regulation, kinase function, hormones like salicylic acid, ethylene, jasmonic acid, and abscisic acid action and pathways related reactive oxygen species (ROS) scavenging. These signaling events altogether induce expression of defense genes that finally lead to the entire defense reaction response.[9] Although multiple reports support the genetic, molecular and physiological bases of how plants act in response to particular stress, it still remains poorly understood.[10]

Rice (*Oryza sativa* L.) is the dominant cereal crop in the world, since it is the most widely consumed staple food for more than three billion people, over half the world's population.[11] Nevertheless, rice plants are highly sensitive to high salt, drought and suboptimal temperature regimes.[12] Since it has a comparatively small genome with respect to other cereals, a huge germplasm collection, broad array of genetic resources, and an effective transformation system, rice considered as a model cereal system.[13] In light of this fact, the immediate attention is urgently required to find out the innovative genes responsive to different abiotic stress conditions to reduce yield loss. Previously, 28 members of cyclophilin gene family in rice have been described.[14]

The cyclophilin, a ubiquitous protein, involved in a wide range of cellular processes viz., cell division, transcriptional regulation, protein trafficking, cell signaling, pre-mRNA splicing, molecular chaperoning and stress tolerance.[14] Cyclophilins, in general, contain a single PPIase domain. Nevertheless, there are few unusual domains viz., WD40 repeat containing domain (in CyP71 from Arabidopsis, LOC_Os08 g44330 from Rice), Leu Zipper and

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Table 1. Rice cyclophilin gene members and their putative orthologous genes from maize, Arabidopsis and Poplar

S.No.	Rice locus ID	Gene name	Sub-cellular location	Maize orthologous	Arabidopsis orthologous	Poplar orthologous
1	LOC_Os01 g02080	OsCYP-1	Chloroplast	GRMZM2G132811	AT1G74070	POPTR_0012s05520
2	LOC_Os02 g02890	OsCYP-2	Cytosol	GRMZM2G162388	AT4G34870	POPTR_0009s13270
3	LOC_Os01 g40050	OsCYP-3	Cytosol	GRMZM2G036720	AT4G33060	POPTR_0018s07590
4	LOC_Os02 g02090	OsCYP-4	Cytosol	Not reported	-	Not reported
5	LOC_Os01 g18210	OsCYP-5	Chloroplast	GRMZM2G063244	AT3G62030	POPTR_0002s18610
6	LOC_Os02 g10970	OsCYP-6	Cytosol	GRMZM2G006107	AT3G63400	POPTR_0006s26710
7	LOC_Os02 g30624	OsCYP-7	Cytosol	GRMZM2G159675	AT2G43810	POPTR_0008s07780
8	LOC_Os02 g52360	OsCYP-8	Cytosol	Not reported	-	Not reported
9	LOC_Os03 g10400	OsCYP-9	Cytosol	GRMZM2G007486	AT5G67530	POPTR_0005s19290
10	LOC_Os03 g59700	OsCYP-10	Chloroplast	GRMZM2G057329	AT2G38730	POPTR_0001s06720
11	LOC_Os05 g01270	OsCYP-11	Chloroplast	GRMZM2G076544	AT5G13120	POPTR_0001s13480
12	LOC_Os06 g04000	OsCYP-12	Cytosol	GRMZM2G397044	AT1G01940	POPTR_0002s15070
13	LOC_Os06 g11320	OsCYP-13	Cytosol	GRMZM2G329306	AT2G15790	POPTR_0004s15170
14	LOC_Os06 g45900	OsCYP-14	Cytosol	Not reported	-	Not reported
15	LOC_Os06 g45910	OsCYP-15	Cytosol	GRMZM2G146190	AT1G53720	POPTR_0005s10020
16	LOC_Os06 g49470	OsCYP-16	Lumen	Not reported	-	Not reported
17	LOC_Os06 g49480	OsCYP-17	Mitochondria	GRMZM2G084521	AT1G56085	POPTR_0001s25880
18	LOC_Os07 g08190	OsCYP-18	Lumen	GRMZM2G070807	AT3G63400	POPTR_0002s04800
19	LOC_Os07 g29390	OsCYP-19	Mitochondria	GRMZM2G139210	AT3G66654	POPTR_0008s10530
20	LOC_Os08 g05050	OsCYP-20	Cytosol	Not reported	-	Not reported
21	LOC_Os08 g19610	OsCYP-21	Chloroplast	GRMZM2G085885	AT5G35100	POPTR_0006s20430
22	LOC_Os08 g44330	OsCYP-22	Cytosol	GRMZM2G049525	AT3G44600	POPTR_0009s14870
23	LOC_Os08 g44520	OsCYP-23	Cytosol	Not reported	AT2G36130	POPTR_0016s07640
24	LOC_Os09 g36670	OsCYP-24	Lumen	GRMZM2G158237	AT4G34960	POPTR_0004s17920
25	LOC_Os09 g39780	OsCYP-25	Lumen	GRMZM2G170397	AT3G56070	POPTR_0078s00220
26	LOC_Os10 g06630	OsCYP-26	Cytosol	GRMZM2G401848	-	Not reported
27	LOC_Os10 g15370	OsCYP-27	Mitochondria	Not reported	-	Not reported
28	LOC_Os11 g38990	OsCYP-28	Lumen	GRMZM2G078638	AT1G26940	POPTR_0010s01660

phosphatase binding domain (in thylakoid lumen cyclophilin TLP40)[15] which play an important role in gene repression or gene silencing via interacting with other proteins.[16,17] The WD40 repeat is a short structural motif of ~40 amino acids, usually terminating in a tryptophan-aspartic acid (WD) dipeptide. Cyclophilin 40 (CyP40) from mammal possesses a tetratricopeptide (TPR) domain that interacts with HSP-90, representing its essential role in signaling.[18] The expression level of cyclophilin gene family under various abiotic stresses viz., salinity, cold, heat and drought and its correlation with different model genomes have also been defined.[14]

Results

Genome sequence analysis of rice cyclophilin (OsCYP-25). Genomic sequence analysis of rice revealed 28 rice cyclophilin gene members. The cellular localization of all predicted rice cyclophilin gene members and their putative orthologous genes from maize, Arabidopsis and Poplar are described in **Table 1**. The genomic sequence of cyclophilin protein (OsCYP-25) (LOC_Os09 g39780) was received from the Rice Genome Annotation Project funded by NSF (http://rice.plantbiology.msu.edu/). It represents an open reading frame (ORF) of 540 bp, to encode for a protein of 179 amino acids. It has an isoelectric point (pI) of the 7.8 and the molecular mass of 19.19 kDa. The protein domain search, performed in the NCBI Conserved Domain Database (NCBICDD; http://www.ncbi.nl m.nih.gov/Structure/cdd/cdd.shtml), revealed entire protein possesses the PPIase activity and so called PPIase domain-2 (9–172) of which few residues of complete protein sequence, highlighted as PPIase domain-1 (57–74), are highly specific for PPIase activity (**Fig. 1A**). This gene is localized on chromosome 9 of rice and putatively expressed in two spliced form (LOC_Os09 g39780.1 and LOC_Os09 g39780.2) (**Fig. 1B**). The human cyclophilin A (PDB-ICWA) was used as template to generate model for cyclophilins from rice (LOC_Os09 g39780) using ExPasy swiss model web server (http://swissmodel.expasy.org). For visualization and editing of PDB models, molecular graphics visualization program PyMoL was used. It found to have a similar structural

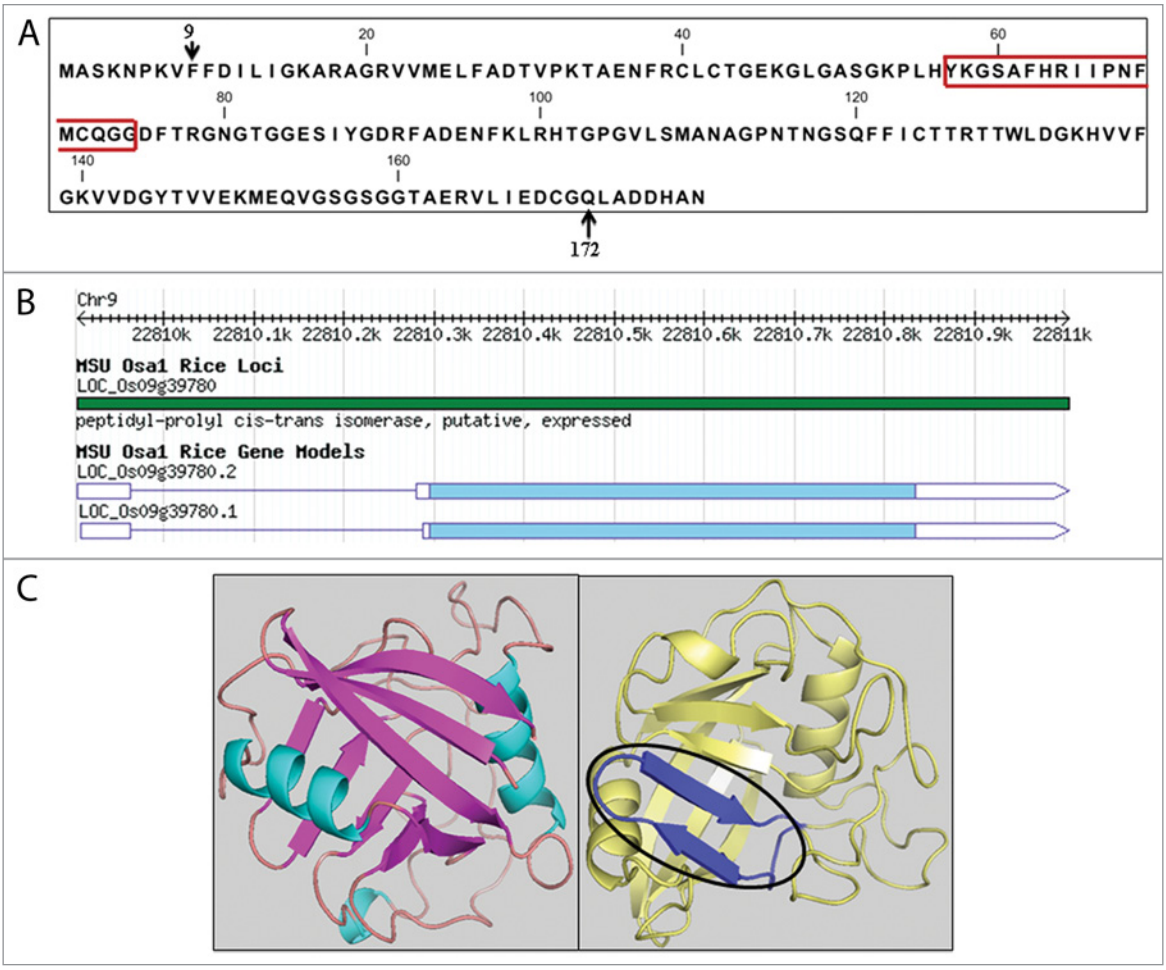

Figure 1. (**A**) Schematic representation of Amino acid sequence of OsCYP-25, showing 18 amino acids internal PPIase domain 1 (highlighted with red rectangle), and PPIase domain 2 from amino acid position 9 to 172, (**B**) genomic localization. (**C**) Homology modeling of OsCYP-25, first model showing 3D structure of OsCYP-25 having 2 α-helices and 8 β-pleated sheets. While the second model represents 3D structure highlighting PPIase 1 domain with 2 β-pleated sheets (black circle).

pattern with 8 β-pleated sheets, 2 α-helices and β-loop region (**Fig. 1C**).

Finding the similarity index of rice cyclophilin (OsCYP-25) with other genera. The comparative study of amino acid sequences of OsCYP-25 was performed using the UniProt BlastP Service (http://www.uniprot.org/blast/), which revealed a 87.36 and 74% similarities with CyP-S from Sorghum sp and CyP-P from poplar (*Liriodendron* sp) (**Table 2**). Moreover, the rice cyclophilin (OsCYP-25) also showed considerable amino acid sequence similarity of 86.78, 85 and 82.84% with the cyclophilin from *Zea mays* (CyP-Z), *Brachypodium* sp (CyP-B), and *Arabidopsis thaliana* (CyP-A), respectively (**Table 2**).

In silico analysis of OsCYP-25 promoter region. The different stress and hormone responsive *cis*-regulatory elements were predicted in 1.0 kb upstream promoter region of OsCYP-25 (**Fig. 2**). The MYBCORE, MYC, CBFHV, GT1GMSCAM4, DRECRTCOREAT, and CCAATBOX1 elements in full length OsCYP-25 promoter region are abiotic stress-responsive which respond to dehydration, salt and heat stress conditions (**Table 3**). On the other hand, few hormone-responsive elements viz., WRKY71OS and WBOXATNPR1 of the promoter were examined to be involved in various stress response mediated through gibberellic acid, ABA and salicylic acid (**Table 3**).

Searching the predicted protein-protein interaction for rice cyclophilin (OsCYP-25). The STRING computer service (http://string-db.org/) was used to find out the predicted protein-protein interaction for OsCYP-25 rice cyclophilin. The results are graphically represented in **Figure 3**. The OsCYP-25 protein was expected to interact with ten different proteins: a WD domain containing protein (G-β repeat domain), the 60S ribosome subunit biogenesis protein, the ribosomal protein L10, the DEAD-box ATP-dependent RNA helicase, the EIF-2 α, the hypothetical Protein, the expressed protein, the YT521-B, the 60S ribosomal protein-related and the PPR repeat domain containing protein (**Fig. 3**).

Transcript profile of OsCYP-25 under stress by quantitative real time PCR. The OsCYP-25 gene transcript is highly upregulated in response to major abiotic stresses like salt, heat, cold, and drought (**Fig. 4A**). The upregulation is also reported in drought and cold stress. The expression analysis of OsCYP-25

Table 2. Percentage of similarity between OsCYP-25 and related cyclophilins of other plant species

Species	UniProt Acc. No.	% similarity	Putative length (aa)	Putative function
Arabidopsis	AT3G56070	82.84	169	rotamase cyclophilin 2 (ROC2) exhibiting peptidyl-prolyl cis-trans isomerase activity involved in signal transduction
Brachypodium	*Bradi 4g38880*	*85*	*175*	*peptidyl-prolyl cis-trans isomerase CYP19-3-like*
Maize	*GRMZM2G170397*	*86.78*	*174*	*Peptidyl-prolyl cis-trans isomerase*
Poplar	*POPTR_0078s00220*	*74*	*172*	*Peptidyl-prolyl cis-trans isomerase*
Sorghum	*Sb02 g012590*	*87.36*	*174*	*Peptidyl-prolyl cis-trans isomerase*

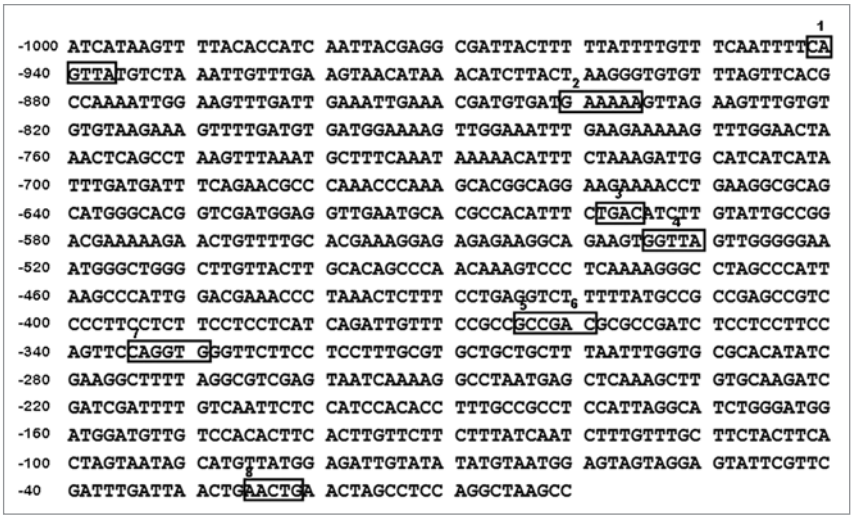

Figure 2. OsCYP-25 promoter sequence. 1) MYB recognition site; 2) GT1GMSCAM4, Salt stress and biotic stress responsive element; 3) WRKY71OS, In response to dehydration; 4) CCAATBOX1, heat stress responsive element; 5) CBFHV, DRE binding proteins element; 6) DRECRTCOREAT, light responsive element; 7) MYC recognition site; 8) WBOXATNPR1, SA-responsive element.

also suggested that in high acidic condition (pH 3.5) the gene is highly expressed as compared with alkaline condition (pH 10.5). The expression analysis under different intensity light conditions revealed that it is found to be increased from white to red light, whereas a sharp peak was observed in case ABA stress (**Fig. 4B**).

Discussion

Cyclophilins, a member of immunophillin group of proteins, are ubiquitous in all genera including plants. It is characterized by diverse forms and wide range of cellular functions viz., cell division, transcriptional regulation, protein trafficking, protein folding, cell signaling, pre-mRNA splicing, molecular chaperoning and stress tolerance.[14] Here we report the response of OsCYP-25 from rice (LOC_Os09 g39780) against various abiotic stresses. The OsCYP-25 domain PPIase-1 contains peptidyl prolyl *cis-trans* isomerase activity that catalyzes the *cis-trans* isomerisation process of proline residues. The amino acid sequence of OsCYP-25 is highly similar (similarity score 71.51%) to OsCYP2 and other cyclophilins of various plant species where seven residues (His-61, Arg-62, Phe-67, Gln-118, Phe-120, Trp-128 and His-33) associated with PPIase catalysis, of which three of these namely His-61, Arg-62 and Phe-120, are found to be extremely important for PPIase activity. Further, phylogenetic distance among plant cyclophilins according to average distance using percentage identity is also evident.[19] Its potent role in protein folding and molecular chaperoning has clearly been illustrated.[20] The rice cyclophilin (OsCYP-25) showed a homology of 60–70% with the human cyclophilin A (PDB-ICWA) indicating OsCYP-25 might be involved in various cellular functions such as mRNA processing, protein degradation, chaperoning arginine kinase folding and signal transduction.[14,21] The CyP-S and CyP-P, possessing peptidyl prolyl *cis-trans* isomerase activity, played an important role in transcriptional regulation and cell signaling.[14] The CyP-Z, CyP-B and CyP-A involved in signal transduction, protein trafficking and molecular chaperoning.[14,22]

A number of *cis*-acting elements in the promoter regions were indispensable for transcriptional regulation of defense-related gene expression during biotic and abiotic stresses. The *cis*-acting elements have been identified from the stress- and hormone response-related gene promoters of different plant species.[23,24] Here, in silico study of OsCYP-25 promoter relies on the fact that predicted *cis*-elements are a like with those showing similar stress response and certainly have a common function. We justified that the function of predicted *cis*-elements which are highly correlated with similar finding of several reports.[25-27] These predicted *cis* elements and their *trans* factors might mediate gene regulatory net work functioning with respect to various abiotic stresses in a similar fashion of previous findings.[28,29] This study suggests that OsCYP-25 promoter is a versatile and stress responsive that functions under multiple abiotic stresses.

Cyclophilins apart from playing an important role in protein folding may perform specific functions via interacting partner proteins in larger multi-component complexes. The search for their interacting partners under high stress plant response and thereby gene interference will provide in-depth understanding of their physiological roles and potential function in stress alleviation. Multiple studies have been focused to identify the interacting proteins and to elucidate their effects on peptidyl prolyl

Table 3. The predicted different stress-responsive *cis*-elements present in OsCYP-25 promoter region using PLACE database analysis

Cis regulatory element	Element number	Core sequence	Role
MYBCORE	2	CNGTTR	In response to dehydration
GT1GMSCAM4	3	GAAAAA	Salt stress and biotic stress responsive element
WRKY71OS	2	TGAC	Involved into Gibberellic acid and ABA mediated pathways
CCAATBOX1	2	CCAAT/GGTTA	In response to heat shock
CBFHV	2	RYCGAC	Binding site of DRE binding proteins; in response to dehydration
DRECRTCOREAT	1	RCCGAC	In response to drought and light
MYC	6	CANNTG	In response to drought
WBOXATNPR1	1	TTGAC/AACTG	In response to Salicylic acid

cis-trans isomerase activity.[20,30] The WD40 domain containing protein plays a key role in gene repression and organogenesis and acts as a histone remodeling factor which ultimately leads to chromatin based gene silencing.[16] The 60S ribosome subunit takes part in ribosome biogenesis, a essential cellular process, via enzymatic modifications along the rRNA. The ribosome biogenesis is linked with nucleocytoplasmic transport ensuring conformity in the maturation process.[31] A 60S ribosomal related protein is required for assembling the 40S to 60S subunits. The ribosomal protein L10 (RPL10) is involved in joining the 40S and 60S ribosomal subunits into a functional 80S ribosome.[32,33] Besides its role in translation, it may also contribute to cell growth[34] and confer protection against oxidative damage.[35] The DEAD-box RNA helicase family comprise enzymes that participate in every aspect of RNA metabolism, signal transduction or stress response to NaCl, dehydration, ABA, blue and red light.[1] The translational control mechanisms are mediated by the specific phosphorylation of the subunit of eukaryotic initiation factor 2 (eIF2a) on serine 51 is evident in all eukaryotes[36] including plant where protein translation is regulated by eIF2α and mediated through the same mechanism of phosphorylation.[37] Its crucial role in cellular homeostasis during growth, differentiation and in response to abiotic and biotic stresses has been defined.[38] The YT521-B is a ubiquitously expressed nuclear protein that can change splice site and is regulated by tyrosine phosphorylation.[39] PPR domain containing proteins mediate specific RNA processing events including RNA editing, transcript processing, and translation initiation, and found to responsible for specific binding to both protein and RNA molecules itself.[40-42] The present in silico analysis explained that OsCYP-25 interacts with essential proteins involved in different cellular pathways and cell signaling network, such as RNA metabolism, RNA editing, ribosome biogenesis, transcription regulation, gene expression, nuclear transport, phytohormone signaling, signal transduction or various stress responsive proteins.

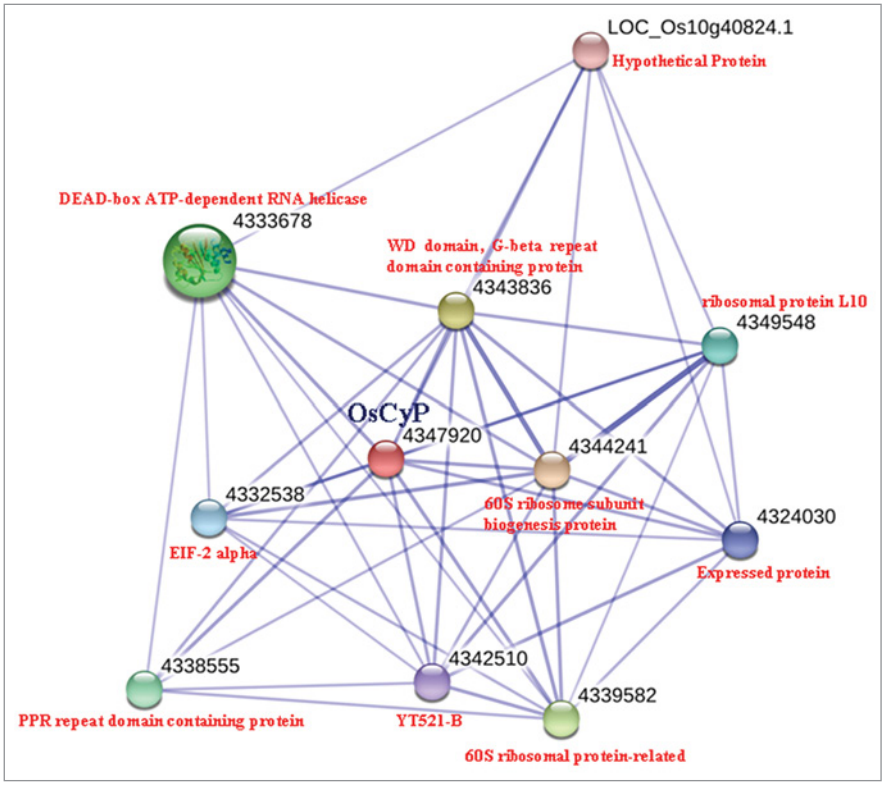

Figure 3. Schematic representation of the putative interaction of OsCYP-25 with other proteins. STRING program (http://string-db.org/) was used for the bioinformatic prediction of protein-protein interaction.

Previously, it has already been reported that cyclophilin plays a significant role in abiotic stress tolerance.[43,44] Cyclophilin (CYP20-3) from *Arabidopsis* was reported to be involved in light as well as other abiotic stress tolerance.[14] It has been also reported that OsCYP20-2 play vital role in response to various abiotic stresses.[45] Recently we have done structural analysis of abiotic stress induced cyclophilin gene from *Piriformospora indica*.[46,47] Here we have shown that OsCYP-*25* gene transcript is upregulated in response to various abiotic stresses ascertaining its various functions directly or indirectly involved in cellular response to multiple abiotic stresses.

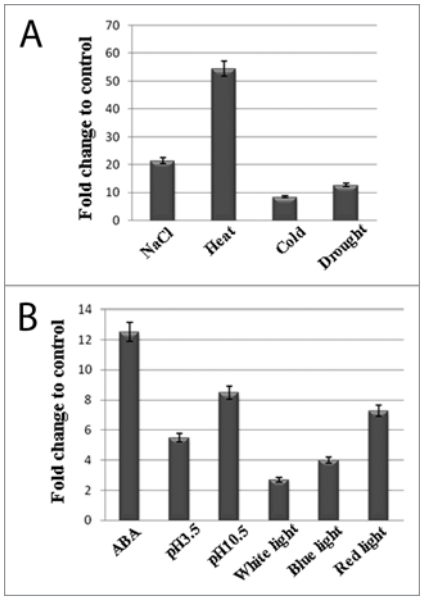

Figure 4. qRT-PCR analysis showing the expression levels of OsCYP-25 in three weeks-old rice plants grown in greenhouse and challenged with different abiotic stresses; (**A**) 200 mm NaCl, heat (42°C), cold (4°C) and drought (**B**) ABA (100 μm), pH 3.5, pH 10.5, blue light, red light and while light. The relative expression is presented as fold change to control. Values are expressed as means ± SD of three biological replicated plants.

The present study establishes that rice cyclophilin do play a cellular response to specific abiotic stress. In silico analysis revealed a positive correlation between rice cyclophilin protein family and plant stress response. Additionally, OsCYP-25 via acting as a component in stress mechanism may assists in understanding different physiological roles in relation to stress alleviation. The underlying mechanism which is targeted by rice cyclophilin protein to bring about multiple abiotic stress protection has not been worked out and further characterization is still needed and currently in progress to fine tune insight into the mechanism of stress tolerance mediating via cyclophilins in plants.

Materials and Methods

In silico analysis of rice cyclophilin (OsCYP-25). The genomic sequence of cyclophilin (OsCYP-25) (LOC_Os09 g39780) was received from the Rice Genome Annotation Project funded by NSF (http://rice.plantbiology.msu.edu/). The deduced amino acid sequence of rice cyclophilin protein (OsCYP-25), was compared with respective subunits of important monocots viz. Arabidopsis, Brachypodium, maize, Sorghum and poplar, by multiple amino acid sequence alignment using Clustal W 2.0 program (http://www.ebi.ac.uk/clustalw). The pairwise amino acid sequence identity between OsCYP-25 and with respective subunits of Arabidopsis, Brachypodium, maize, Sorghum and poplar was calculated using ClustalW2 (EMBL-EBI). The protein domain search, performed in the NCBI Conserved Domain Database (NCBICDD; http://www.ncbi.nl m.nih.gov/Structure/cdd/cdd.shtml) to highlight the specific PPIase region. The human cyclophilin A (PDB-ICWA) was used as template to generate model for cyclophilins from Rice (LOC_Os09 g39780) and were created using ExPasy swiss model web server (http://swissmodel.expasy.org). The comparative study of amino acid sequences of OsCYP-25, performed by using the UniProt BlastP Service (www.uniprot.org/blast/), revealed a 87.36 and 74% similarities with CyP-S from Sorghum sp and CyP-P from poplar (*Liriodendron* sp).

Identification of *cis*-regulatory elements in OsCYP-25 promoter. PLACE (http://www.dna.affrc.go.jp/PLACE/) were used to analyze the *cis*-acting regulatory elements and to analyze the OsCYP-25 promoter sequences (http://rice.plantbiology.msu.edu). To validate stress-responsive nature of OsCYP-25 promoter, predicted *cis*-regulatory elements were analyzed for their different role in various abiotic stress responses.[48]

In silico predictions of protein-protein interactions. The protein-protein interaction for OsCYP-25 rice cyclophilin was made using STRING computer service (http://string-db.org/). The predicted protein-protein interactions for OsCYP-25 were examined individually for their role in various stress response.[14]

Quantitative real-time PCR. The expression profiling of OsCYP-25 under different abiotic stress conditions was determined by quantitative real time PCR. qRT-PCR reactions were performed by methods described previously[49] using 5'-TTACAAGGGT TCTGCCTTCC-3' and 5'-CAAACCTGTC GCCGTAGAT-3' primer pair. Rice α-tubulin gene was used as reference control transcript.[50] The qRT-PCR experiment repetitive three times and in each experiment three technical replicates were used for each time point of treatment. Relative gene expression was calculated using the $2-\Delta\Delta CT$ values following Livaks' method.[51]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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