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ORIGINAL PAPER

Expression of a cyclophilin OsCyp2-P isolated from a salt-tolerant landrace of rice in tobacco alleviates stress via ion homeostasis and limiting ROS accumulation

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Abstract Cyclophilins are a set of ubiquitous proteins present in all subcellular compartments, involved in a wide variety of cellular processes. Comparative bioinformatics analysis of the rice and *Arabidopsis* genomes led us to identify novel putative cyclophilin gene family members in both the genomes not reported previously. We grouped cyclophilin members with similar molecular weight and subtypes together in the phylogenetic tree which indicated their co-evolution in rice and *Arabidopsis*. We also characterized a rice cyclophilin gene, OsCyp2-P (Os02g0121300), isolated from a salinity-tolerant landrace, Pokkali. Publicly available massively parallel signature sequencing (MPSS) and microarray data, besides our quantitative real time PCR (qRT-PCR) data suggest that transcript abundance of OsCyp2-P is regulated under different stress conditions in a developmental and organ specific manner. Ectopic expression of OsCyp2-P imparted multiple abiotic stress tolerance to transgenic tobacco plants as evidenced by higher root length, shoot length, chlorophyll content, and K^+/Na^+ ratio under stress conditions. Transgenic plants also showed reduced lipid peroxidase content, electrolyte leakage, and superoxide content under stress conditions suggesting better ion homeostasis than WT plants. Localization studies confirmed that OsCyp2-P is localized in both cytosol and nucleus, indicating its possible interaction with several other proteins. The overall results suggest the explicit role of OsCyp2-P in bestowing multiple abiotic stress tolerance at the whole plant level. OsCyp2-P operates via reactive oxygen species (ROS) scavenging and ion homeostasis and thus is a promising candidate gene for enhancing multiple abiotic stress tolerance in crop plants.

Keywords Abiotic stress · Cyclophilin · OsCYP2-P · Peptidyl-prolyl-*cis-trans* isomerase · ROS

Abbreviations

At	*Arabidopsis thaliana*
Cyp	Cyclophilin
CLD	Cyclophilin-like domain
PPIase	Peptidyl-prolyl-*cis-trans* isomerase
Os	*Oryza sativa*
WT	Wild type

Introduction

Cyclophilins and FK506 binding proteins (FKBPs) are two subfamilies of immunosuppressant receptors or immunophilins (IMMs) superfamily (Kim et al. 2012; Kumari et al. 2013). Cyclophilins and FKBPs bind to immunosuppressive drugs cyclosporin A (CsA) (Handschumacher et al. 1984) and FK-506/rapamycin, respectively. Despite their lack of structural similarity, these two families share a common peptidyl-prolyl-*cis-trans* isomerase (PPIase) domain, catalyzing the rotation of X-Pro peptide bonds from a *cis* to *trans* conformation, a rate-limiting step in protein folding

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(Wang et al. 2010b). They are highly conserved in prokaryotes as well as eukaryotes (Wang et al. 2005). Cyclophilins are present in all subcellular compartments, involved in a wide variety of processes including protein trafficking and maturation (Shieh et al. 1989; Ferreira et al. 1996; Galat 1999), receptor complex stabilization (Leverson and Ness 1998), apoptosis (Lin and Lechleiter 2002), receptor signaling (Brazin et al. 2002; Yurchenko et al. 2002), RNA processing (Krzywicka et al. 2001), immune response (Wiederrecht et al. 1992), spliceosome assembly (Horowitz et al. 2002), miRNA activity (Smith et al. 2009), and RISC assembly (Iki et al. 2012). They are broadly classified into two groups: single-domain (SD) cyclophilins having a single PPIase or cyclophilin-like domain (CLD) and multidomain (MD) cyclophilins having a PPIase or CLD in conjunction with domains of other functions such as tetratricopeptide repeat (TPR), WD40, coiled-coil domain (CCD), and internal repeats domain (RPT), RRM, Zinc Finger etc. (Kumari et al. 2013).

The availability of high-quality finished genome sequences of rice and *Arabidopsis* and their expression data in the form of massively parallel signature sequencing (MPSS) and microarray makes it possible to analyze and study specific gene families. Bioinformatics-based tools have enabled the discovery of new members of several gene families in *Arabidopsis* and rice such as homeobox gene family (Jain et al. 2008), protein phosphatase 2C family (Xue et al. 2008), nucleocytoplasmic transport receptors family (Huang et al. 2010), etc. Our group has also recently carried out detailed genome-wide analysis and expression profiling of few important gene families, i.e., two-component system (Pareek et al. 2006), CBS domain containing proteins (Kushwaha et al. 2009), and glyoxalase gene family (Mustafiz et al. 2011). Since cyclophilins have multigene members in plants, we have carried out genome-wide analysis to comment on their number and domain organization in rice in comparison to *Arabidopsis*. In addition, comprehensive analysis of cyclophilin genes, including putative domain architecture, genomic distribution on respective chromosomes, subcellular localization, phylogenetic relationship between family members, and MPSS gene expression in *Arabidopsis* and rice, and tiling array gene expression analysis of cyclophilins under various environmental stresses in *Arabidopsis* has also been conducted.

Using subtractive hybridization screening, we previously cloned and identified a salinity-inducible SD cyclophilin member *OsCyp2* from a salinity-tolerant landrace of rice, Pokkali. Although enhanced total PPIase activity has been correlated with stress-tolerant genotypes (Sharma and Singh 2003), effect of individual PPIase members on stress tolerance has not been addressed. With a view to investigate the role of *OsCyp2* in enhancing multi-stress tolerance ability in plants, overexpression studies were extended to model plant tobacco. Transgenic approach was used wherein OsCyp2-P gene was ectopically expressed in tobacco plant using *Agrobacterium*-mediated transformation which resulted in a profound increase in tolerance against a variety of major abiotic stresses. Based on this study, it is proposed that this gene may serve as a "candidate" for enhancing multiple abiotic stress tolerance in crop plants. It is important to mention here that this is the first report where functional characterization of a cyclophilin ortholog from a salt-tolerant landrace, Pokkali, has been attempted, and good correlative evidence between the messenger RNA (mRNA) expression, protein expression, and tolerance towards multiple abiotic stresses in plants has been presented.

Materials and methods

Isolation of cDNA clone

The complementary DNA (cDNA) clone of OsCyp2 (EF576508) was obtained from Pokkali subtractive cDNA library of differentially expressed genes under salinity stress as described earlier (Kumari et al. 2009a).

Identification of CYPs gene families

Putative cyclophilin gene members in *Arabidopsis thaliana* and *Oryza sativa* were identified using protein profiles of cyclophilin (PF00016) from Pfam database (http://pfam.sanger.ac.uk/) using HMMER 3.0 software (http://hmmer.janelia.org/) against genome browser databases TAIR 9 for *A. thaliana* (http://www.Arabidopsis.org/) and TIGR Rice 6.1 (http://rice.plantbiology.msu.edu/), respectively.

Subcellular localization of cyclophilin genes

The subcellular localization of cyclophilins was predicted using programs such as PSORT (http://wolfpsort.org/), Predotar v.1.03 (https://urgi.versailles.inra.fr/predotar/predotar.html), and TargetP 1.1 server (http://www.cbs.dtu.dk/). Signal peptide cleavage sites and chloroplast transit peptides were predicted using SignalP 4.1 and ChloroP 1.1 servers (http://www.cbs.dtu.dk/), respectively, in a protein sequence.

Chromosomal location and gene structure of IMMs

Chromosomal locations of cyclophilin genes were determined using the TAIR *Arabidopsis* genome browser (http://www.Arabidopsis.org/) and *O. sativa* genome browser (http://rice.plantbiology.msu.edu/) along with the ensemble genome browser (http://plants.ensembl.org/index.html), and mapped on their respective chromosomes. The *A. thaliana* IMMs genomic distribution were crosschecked using chromosomal

map tool (http://www.Arabidopsis.org/jsp/ChromosomeMap/tool.jsp). Gene duplication and their presence on duplicated chromosomal segments were also investigated in *Arabidopsis* and *Oryza*. The scaled position of cyclophilin genes on respective chromosomes and segmental duplication have been drawn using gplots package of open source R software. For nomenclature of new members of rice and *Arabidopsis* cyclophilin gene family, we have employed the previously established system for *Arabidopsis*. The cyclophilin genes were named "CYP" with a prefix "*Os*" for rice and "*At*" for *Arabidopsis* and a suffix number to indicate the predicted molecular weight. Members with same molecular weight were designated by Arabic numeral followed by a small case alphabet to indicate alternatively spliced form.

Domain annotations of CYPs

Cyclophilin members of *A. thaliana* and *O. sativa* from database search were analyzed for the presence of cyclophilin-like domain (CLD) using Pfam (http://www.kks/) and SMART (http://www.jjkjs/) databases. Only those sequences having CLD were used for further analysis. Based on domain architecture of these genes, they have been classified into separate groups, and a representative picture of each of these groups has been drawn using gplots package of open source R statistical software.

Phylogenetic analysis

A phylogenetic tree was constructed with the aligned *A. thaliana* and *O. sativa* cyclophilin sequences using Clustal X (version 2.1; http://www.clustal.org/) and using neighbor-joining (NJ) method with default options. For statistical reliability, we conducted bootstrap analysis with 1000 replicates. The constructed tree file was visualized and finally drawn by Iterative Tree of Life (iTOL) (Letunic and Bork 2011). Similarly, the classification of cyclophilin members of rice and *Arabidopsis* into various subtypes was performed using KOBAS 2.0 database. Cyclophilin genes are classified into single-domain (SD) and multidomain (MD) members depending upon the number of domains present. SD members possess only CLD catalytic domain while MD members contain various additional functional domains other than the catalytic CLD, such as tetratricopeptide repeat (TPR), WD40 domain, RNA binding domain (RNP), zinc finger domain, RNA recognition motif (RRM), ATPase (AAA) family, transposase family (tnp2), etc. The membrane-bound cyclophilin members (having a characteristic transmembrane domain) of rice and *Arabidopsis* and those localized in nucleus (possessing nuclear localized signals (NLS)) (Tables 1 and 2) were identified using WOLF PSORT (http://wolfpsort.org/) and Nucpred (http://www.sbc.su.se/).

Expression analysis using MPSS database

Expression evidence from massively parallel signature sequencing (MPSS) tags was determined from the *Arabidopsis* MPSS project (http://mpss.udel.edu/at/) and *Oryza* MPSS project (http://mpss.udel.edu/rice) mapped to *Arabidopsis* and *Oryza* gene models. The signature was considered to be significant if it uniquely identifies an individual gene and shows perfect match (100 % identity over 100 % length of the tag). The normalized abundance (tags per million, tpm) of these signatures for a given gene in a given library represents a quantitative estimate of expression of that gene. The description of MPSS libraries in *A. thaliana* and *O. sativa* is provided in Supplementary Tables 1 and 2. Expression values obtained from MPSS database for respective cyclophilin genes were used for making the heatmap using gplots package of open source R software.

Expression analysis using microarrays

The microarray data showing expression of cyclophilin gene family members under various abiotic stress conditions such as cold, osmotic, salt, drought, genotoxic, oxidative, UV-B, wounding, and heat stress were retrieved from The *Arabidopsis* Information Resource (TAIR) and Rice Oligonucleotide Array Database (http://www.ricearray.org/expression/expression.php). The datasets obtained were corresponding to shoot tissues at different time sets of stress namely 0.5, 1, 3, 6, 12, and 24 h. For rice, the data was obtained for drought, salt, and cold stress. Fold increase in transcript abundance under stress conditions were calculated with respect to their controls. The hierarchical clustering analysis and the heatmaps were made using gplots package of R software as described earlier (Kushwaha et al. 2011).

Plant material and stress treatments for transcript analysis of OsCyp2-P

Rice seeds were rinsed with distilled water and germinated in a hydroponic system for 14 days (28±1 °C; 12 h day/night) in half strength Hoagland medium. Seedlings were exposed to salinity stress by transferring them to Hoagland medium containing 200 mM NaCl. For heat stress, seedlings were transferred to a growth chamber maintained at 42 °C. For drought stress, medium was supplemented with 5 % PEG 6000. The untreated samples were taken as control. Control and treated shoots were harvested after 30 min and 24 h of stress treatment and kept in liquid nitrogen until further use. In the case of tobacco, pot-grown wild type (WT) and transgenic mature plants maintained under greenhouse conditions (28±1 °C; 12 h day/night) were taken for leaf disc assay. RNA was isolated from control (half-strength Hoagland medium) and

Table 1 List of putative cyclophilin gene members in Arabidopsis along with their nomenclature based on molecular weight, isoelectric point, alternative spliced forms, KOBAS subtype, AA length, and predication localization (*MW* molecular weight, *PI* isoelectric point and *AA* amino acid)

TAIR ID	Splice variants	Genome location	Nomenclature	KOBAS	MW/PI	AA length	Target P[a] (mTP; SP)	SignalP	pSORT[b]/ Predotar
AT1G01940		Chr 1: 323027-324917	AtCYP18-1	PPIL2	17.5/8.4	160	(0.077; 0.046)	0.114	*CS*
AT2G36130		Chr 2: 15166773-15168497	AtCYP18-2	PPIL1	18.2/8.6	164	(0.118; 0.060)	0.102	*C*
AT4G38740		Chr 4: 18083395-18084251	AtCYP18-3	PPIF	18.3/7.9	172	(0.101; 0.118)	0.098	*Ch*
AT4G34870		Chr 4: 16614327-16615313	AtCYP18-4	PPIA	18.3/8.9	172	(0.164; 0.100)	0.117	*C*
AT2G16600	AT2G16600.1	Chr 2: 7200807-7201574	AtCYP19-1a	PPIF	18.4/8.6	173	(0.153; 0.102)	0.097	*C*
	AT2G16600.2		AtCYP19-1b	PPIF	15.9/6.5	151	(0.128; 0.059)	0.099	*C*
AT2G21130		Chr 2: 9055398-9056232	AtCYP19-2	PPIF	18.4/8.3	174	(0.074;0.167)	0.126	*C*
AT3G56070	AT3G56070.1	Chr 3: 20806749-20808152	AtCYP19-3a	PPIA	18.9/7.9	176	(0.090; 0.175)	0.164	*C*
	AT3G56070.2		AtCYP19-3b	PPIA	18.9/7.9	176	(0.090; 0.175)	0.164	*C*
AT2G29960	AT2G29960.1	Chr 2: 12769033-12770579	AtCYP19-4a	PPIF	21.5/9.6	201	SP (0.032; 0.945)	0.853	*E/ ER*
	AT2G29960.2		AtCYP19-4b	PPIB	20.5/9.7	191	SP (0.032; 0.945)	0.853	*E/ ER*
AT5G58710		Chr 5: 23717775-23719765	AtCYP20-1	PPIB	21.9/9.6	204	SP (0.056; 0.919)	0.909	*E/ ER*
AT5G13120	AT5G13120.1	Chr 5: 4162503-4164794	AtCYP20-2a[#]	PPIB	28.3/9.8	259	M (0.893; 0.022)	0.128	*Ch/ P*
	AT5G13120.2		AtCYP20-2b[#]	PPIB	28.3/9.8	255	M (0.955; 0.028)	0.178	*Ch/ P*
AT3G62030	AT3G62030.1	Chr 3: 22973004-22975378	AtCYP20-3a	PPIB	28.2/8.7	260	M (0.837; 0.023)	0.126	*Ch/ P*
	AT3G62030.2		AtCYP20-3b	PPIB	34.3/9.5	313	SP (0.191; 0.467)	0.532	*Ch/ ER*
	AT3G62030.3		AtCYP20-3c[#]	PPIB	28.0/8.9	259	M (0.831; 0.023)	0.126	*Ch/ P*
AT4G34960		Chr 4: 16648605-16650897	AtCYP21-1	PPID	24.5/6.8	224	SP (0.015; 0.977)	0.430	*Ch/ ER*
AT3G55920		Chr 3: 20743443-20745083	AtCYP21-2	PPIF	24.5/7.0	228	SP (0.009; 0.990)	0.480	*V/ ER*
AT2G47320		Chr 2: 19427372-19428933	AtCYP21-3	PPWD1	26.0/7.6	230	SP (0.197; 0.462)	0.082	*V/ M*
AT3G66654	AT3G66654.1	Chr 3: 2087980-2090495	AtCYP21-4a	PPWD1	26.4/9.4	236	(0.179; 0.447)	0.103	*Ch/ M*
	AT3G66654.2		AtCYP21-4b	PPWD1	26.4/9.4	236	(0.179; 0.447)	0.103	*Ch/ M*
	AT3G66654.3		AtCYP21-4c	PPWD1	26.4/9.4	236	(0.179; 0.447)	0.103	*Ch/ M*
AT2G38730		Chr 2: 16192356-16194103	AtCYP22	PPIH	21.4/8.2	199	(0.238; 0.074)	0.126	*C*
AT1G26940		Chr 1: 9343126-9345145	AtCYP23	PPWD1	25.5/8.4	226	SP (0.051; 0.903)	0.867	*V/ ER*
AT3G22920		Chr 3: 8122713-8123411	AtCYP26-1	PPID	26.0/4.6	232	(0.071; 0.154)	0.102	*C*
AT1G74070		Chr 1: 27851488-27852976	AtCYP26-2	PPIA	34.3/10.0	317	M (0.404; 0.199)	0.132	*Ch*
AT5G35100	AT5G35100.1	Chr 5: 13360283-13361390	AtCYP28a	PPIB	30.5/7.3	281	M (0.408; 0.174)	0.451	*Ch*
	AT5G35100.2		AtCYP28b	PPIB	26.6/6.5	245	M (0.408; 0.174)	0.451	*Ch*
AT3G15520		Chr 3: 5249599-5252506	AtCYP37	PPIL1	50.4/7.0	466	M (0.906; 0.023)	0.188	*Ch/ P*
AT3G01480	AT3G01480.1	Chr 3: 188384-190851	AtCYP38-1a	PPIL1	47.9/4.7	437	M (0.815; 0.285)	0.170	*Ch/ P*
	AT3G01480.2		AtCYP38-1b	PPIL1	39.1/6.3	355	M (0.815; 0.285)	0.170	*Ch/ P*
AT4G17070		Chr 4: 9595385-9598237	AtCYP38-2[#]	PPIL3	37.6/5.5	343	SP (0.025; 0.898)	0.293	*C/ ER*
AT2G15790		Chr 2: 6877774-6880898	AtCYP40	PPID	40.6/5.6	361	(0.093; 0.109)	0.120	*C*
AT4G33060		Chr 4: 15948490-15952223	AtCYP57	CWC27	56.5/7.7	504	(0.050; 0.076)	0.095	*N*
AT1G53720		Chr 1: 20056533-20059638	AtCYP59	PPIL4	58.8/6.1	506	(0.081; 0.358)	0.285	*N*
AT3G63400	AT3G63400.1	Chr 3: 23412024-23415659	AtCYP63a	PPID	63.5/11.5	570	(0.078; 0.139)	0.102	*N*
	AT3G63400.2		AtCYP63b	PPID	42.2/10.8	387	(0.078; 0.139)	0.102	*N*
	AT3G63400.3		AtCYP63c[#]	PPID	63.5/11.5	570	(0.078 0.139)	0.102	*N*
AT5G67530		Chr 5: 26941182-26944146	AtCYP65	PPIL2	65.0/8.3	595	(0.156; 0.101)	0.116	*N*
AT1G53780	AT1G53780.1	Chr 1: 20073996-20078228	AtCYP67-1a[#]	PSMC5	66.8/4.5	598	(0.082; 0.126)	0.126	*C*
	AT1G53780.2		AtCYP67-1b[#]	PSMC5	69.1/4.5	620	(0.066; 0.078)	0.104	*C*
	AT1G53780.3		AtCYP67-1c[#]	PSMC5	66.9/4.5	599	(0.082; 0.126)	0.126	*C*
AT3G44600		Chr 3: 16164935-16169262	AtCYP71	PPWD1	70.9/6.6	631	(0.025; 0.218)	0.104	*C*
AT4G32420	AT4G32420.1	Chr 4: 15647346-15652756	AtCYP95a	PPIG	94.6/12.4	837	(0.077; 0.104)	0.096	*N*
	AT4G32420.2		AtCYP95b	PPIG	82.6/12.6	727	(0.343; 0.010)	0.097	*N*

Table 1 (continued)

TAIR ID	Splice variants	Genome location	Nomenclature	KOBAS	MW/PI	AA length	Target P[a] (mTP; SP)	SignalP	pSORT[b]/ Predotar
	AT4G32420.3		AtCYP95c[#]	PPIG	94.6/12.4	837	(0.077; 0.104)	0.096	*N*
	AT4G32420.4		AtCYP95d[#]	PPID	92.8/12.4	820	(0.077; 0.104)	0.096	*N*

Alternatively spliced products have been designated as "a" or "b" after numerical. The new members of the family not reported earlier have been marked as "#"

[a] *SP* secretory proteins, *C* cytosol, *M* mitochondria

[b] *CS* cytoskeleton, *C* cytosol, *E* extra cellular, *Ch* chloroplast, *V* vacuole, *N* nucleus, *ER* endoplasmic reticulum, *P* plastid

treated (half-strength Hoagland medium +150 mM NaCl) leaf discs after 5 days of stress.

Total RNA isolation and cDNA synthesis

Total RNA was isolated using TRI reagent (Sigma, USA) according to the manufacturer's instructions. The concentration of RNA was determined using spectrophotometer and its integrity was checked on agarose gel. cDNA synthesis was carried out according to the manufacturer's protocol (Thermo Scientific, UK) using 5 μg of total RNA.

Real-time PCR

Primers for real-time PCR analysis of the OsCyp2-P gene members were designed using Primer Express 3.0 software (PE Applied Biosystems, USA). The sequences for these primers OsCYPRT FP: TTCGCCAGTATCAGTCGTCTTG, OsCYPRT RP: CGACACGAACTCATAAAAAGACT were designed from within the gene and 3′UTR region of the gene which gave a 104 bp amplicon. Real-time PCR was performed employing ABI Prism 7500 Sequence Detection System and software (PE Applied Biosystems, USA) using standard RT-PCR protocol. The specificity of amplification was tested by dissociation curve analysis. The expression of each gene in different RNA samples was normalized with the expression of internal control gene, elongation factor 1α for rice samples, and actin-specific primers for tobacco. The mRNA levels for each candidate gene in different tissue samples were calculated relative to its expression in control seedlings using ΔΔCT method of SDS 1.4 software (Applied Biosystems). Three technical as well as biological replicates were analyzed for each sample (n=3).

Protein extraction

One gram of sample tissue was taken and homogenized in liquid nitrogen to a fine powder form. The powder obtained was suspended in extraction buffer (Zivy et al. 1983). PVPP (50 mg/g fresh weight of the tissue) was added while homogenizing the samples to get rid of the phenolics. The crude homogenate was centrifuged twice at 12,000 rpm for 15 min at 4 °C and the total protein present in the supernatant were precipitated overnight at −20 °C using 8 volumes of prechilled acetone containing 10 mM 2-mercaptoethanol. The precipitated proteins were then solubilized in Laemmli buffer (Laemmli 1970), followed by heating at 100 °C for 5 min. The solubilized proteins were recovered by centrifugation at 15,000 rpm for 15 min and an aliquot of supernatant was used for protein quantification by Bradford assay (Bradford 1976).

Construction of binary vector and tobacco transformation

Full-length OsCyp2-P (519 bp) was cloned into plant expression vector pCAMBIA1304 under the control of 35SCaMV promoter by using the primer set (FP: 5′-GAAGATCTCATG TCGAACACGAGGGTGTTC-3′, RP: 5′-GGACTAGTGC TAGGAGAGCTGGCCGCAG-3′), at the *Bgl*II and *Spe*I site. Transformation of tobacco was done using *Agrobacterium*-mediated leaf disc method (Kumar et al. 2012). Leaf discs of 1-cm diameter were excised from 1-month-old tobacco leaves and incubated with *Agrobacterium* containing pCAMBIAOsCyp2-P (OD_{600}=0.8 was further diluted with MS media upto OD=0.3 to avoid overgrowth of *Agrobacterium*) for 15 min with intermittent shaking for co-infection. The co-infected leaf discs were co-cultivated on MS agar media in dark at 26 °C for 48 h. After 48 h, the co-cultivated leaf discs were further transferred to MS medium, containing 10 mM BAP, 1 mM NAA, 250 mg/l augmentin or cefotaxime (for combating *Agrobacterium* overgrowth sticking on surface of leaf tissue), and 25 mg/l hygromycin (for plant selection), for regeneration.

PCR for screening putative transgenic plants

Discs of about 0.5 to 0.7 cm from leaf tissue were punched into a 2-ml microcentrifuge tube using a standard one-hole paper punch. Then, 100 μl of extraction solution was added to the tube and vortexed briefly to mix properly, according to the manufacturer's protocol (Red Extract-N-Amp, Sigma, USA). The mix was incubated at 95 °C for 10 min. Furthermore, 100 μl of dilution solution was added and vortexed. The leaf

Table 2 List of putative cyclophilin gene members in rice along with their nomenclature based on molecular weight, isoelectric point, alternative spliced forms, KOBAS subtype, AA length, and predication localization (*MW* molecular weight, *PI* isoelectric point, and *AA* amino acid)

MSU ID	RAP (Os ID)	Splice variants	Genome location	Nomenclature	KOBAS	MW/PI	AA length	Target P[a] (mTP; SP)	SignalP	pSORT[b]/ Predotar
LOC_Os02g02090.1	Os02g0111200		Chr 2: 613113-613532	OsCYP17	PPIA	16.2/10.8	139	(0.106; 0.051)	0.097	*C*
LOC_Os06g04000.1	Os06g0130500		Chr 6: 1635098-1635912	OsCYP18-1	PPIL2	17.5/8.4	160	(0.109; 0.351)	0.124	*CS*
LOC_Os08g44520	Os08g0559400	LOC_Os08g44520.1	Chr 8: 27999844-28002323	OsCYP18-2a	PPIL1	19.7/9.8	178	SP (0.135; 0.446)	0.164	*C*
		LOC_Os08g44520.2		OsCYP18-2b	PPIL1	19.4/8.6	176	(0.069; 0.098)	0.119	*CS*
		LOC_Os08g44520.3		OsCYP18-2c	PPIL1	18.0/8.6	164	(0.083; 0.084)	0.119	*C*
LOC_Os10g06630.1	Os10g0154700		Chr 10: 3438474-3439397	OsCYP18-3	PPIA	18.9/9.3	181	(0.181; 0.114)	0.139	*C*
LOC_Os02g02890.1	Os02g0121300		Chr 2: 1116066-1116952	OsCYP19-1	PPIA	18.3/8.4	172	(0.371; 0.079)	0.132	*C*
LOC_Os09g39780	Os09g0571400	LOC_Os09g39780.1	Chr 9: 22809907-22810986	OsCYP19-2a	PPIA	19.1/7.8	179	(0.227; 0.108)	0.166	*C/ M*
		LOC_Os09g39780.2		OsCYP19-2b	PPIA	19.1/7.8	179	(0.227; 0.108)	0.166	*C/ M*
LOC_Os06g49470.1	Os06g0708400		Chr 6: 29971111-29972973	OsCYP19-3	PPIB	22.1/9.4	208	SP (0.039; 0.927)	0.930	*V/ ER*
LOC_Os06g49480	Os06g0708500	LOC_Os06g49480.1	Chr 6: 29973898-29976322	OsCYP20-1a	PPIF	24.0/10.6	225	SP (0.270; 0.702)	0.395	*M/ M*
		LOC_Os06g49480.2		OsCYP20-1b	PPIF	23.4/10.6	220	(0.283; 0.559)	0.734	*M/ M*
LOC_Os05g01270.1	Os05g0103200		Chr 5: 185654-188484	OsCYP20-2	PPIB	26.5/9.8	250	M (0.761; 0.070)	0.295	*Ch/ P*
LOC_Os01g18210.1	Os01g0284700		Chr 1: 10193658-10196611	OsCYP20-3	PPIB	25.3/7.9	238	M (0.885; 0.020)	0.255	*Ch/ P*
LOC_Os09g36670.1	Os09g0537600		Chr 9: 21151781-21154358	OsCYP21-1	PPIF	23.4/8.6	215	SP (0.237; 0.830)	0.899	*C/ ER*
LOC_Os07g29390.1	Os07g0476500	LOC_Os07g29390.1	Chr 7: 17254973-17260556	OsCYP21-2a	PPID	26.4/8.9	235	M (0.381; 0.313)	0.087	*M/ M*
		LOC_Os07g29390.2		OsCYP21-2b	PPID	26.4/8.9	235	M (0.381; 0.313)	0.087	*M/ M*
		LOC_Os07g29390.3		OsCYP21-2c	PPID	26.4/8.9	235	M (0.381; 0.313)	0.087	*M/ M*
LOC_Os03g59700.1	Os03g0811600		Chr 3: 33995633-33998631	OsCYP22	PPIH	21.7/8.2	204	Ch (0.387; 0.053)	0.128	*Ch/ P*
LOC_Os11g38990.1	Os11g0602900	LOC_Os11g38990.1	Chr 11: 23212475-23217465	OsCYP23a	PPWD1	25.8/7.2	235	SP (0.121; 0.934)	0.885	*Ch/ ER*
		LOC_Os11g38990.2		OsCYP23b	PPWD1	25.8/7.2	235	SP (0.121; 0.934)	0.885	*Ch/ ER*
		LOC_Os11g38990.3		OsCYP23c	PPWD1	25.8/7.2	235	SP (0.121; 0.934)	0.885	*Ch/ ER*
LOC_Os01g02080.1	Os01g0111100		Chr 1: 586445-587696	OsCYP26-1	PPIB	31.4/9.4	304	Ch (0.240; 0.151)	0.134	*Ch*
LOC_Os08g19610.1	Os08g0292600	LOC_Os08g19610.1	Chr 8: 11728223-11733106	OsCYP28a	PPIB	35.8/7.5	327	M (0.730; 0.126)	0.116	*Ch*
		LOC_Os08g19610.2		OsCYP28b	PPIB	35.8/7.5	327	M (0.730; 0.126)	0.116	*Ch*
		LOC_Os08g19610.3		OsCYP28c	PPIB	35.8/7.5	327	M (0.730; 0.126)	0.116	*Ch*
		LOC_Os08g19610.4		OsCYP28d	PPIA	28.1/7.0	258	M (0.730; 0.126)	0.116	*Ch*
LOC_Os03g01090.1	Os03g0100300		Chr 3: 63385-66118	OsCYP35[#]	PPIL2	35.4/8.1	331	SP (0.333; 0.476)	0.227	*Ch*
LOC_Os07g37830.1	Os07g0565600	LOC_Os07g37830.1	Chr 7: 22688900-22693282	OsCYP37a	PPIL1	49.7/5.5	465	M (0.402; 0.275)	0.606	*Ch/ ER*
		LOC_Os07g37830.2		OsCYP37b	PPIL1	46.0/5.0	432	M (0.478; 0.282)	0.601	*Ch/ ER*
LOC_Os08g29370.1	Os08g0382400	LOC_Os08g29370.1	Chr 8: 18020081-18023911	OsCYP38a	PPIL1	46.6/4.5	427	M (0.880; 0.042)	0.218	*M/ P*
		LOC_Os08g29370.2		OsCYP38b	PPIL1	46.4/4.5	426	M (0.886; 0.030)	0.217	*M/ P*
LOC_Os06g11320.1	Os06g0216800	LOC_Os06g11320.1	Chr 6: 5933850-5939505	OsCYP40-1a	PPID	43.2/5.7	396	(0.068; 0.153)	0.108	*C*
		LOC_Os06g11320.2		OsCYP40-1b	PPID	43.2/5.7	396	(0.068; 0.153)	0.108	*C*
LOC_Os02g52360.1	Os02g0761100		Chr 2: 32047766-32052300	OsCYP40-2	PPID	43.9/5.2	403	(0.070; 0.141)	0.109	*C*

Table 2 (continued)

MSU ID	RAP (Os ID)	Splice variants	Genome location	Nomenclature	KOBAS	MW/PI	AA length	Target P[a] (mTP; SP)	SignalP	pSORT[b]/ Predotar
LOC_Os01g40050.1	Os01g0582400	LOC_Os01g40050.1	Chr 1: 22591434-22596164	OsCYP57a	CWC27	56.8/6.1	499	(0.040; 0.131)	0.097	*N*
		LOC_Os01g40050.2		OsCYP57b	CWC27	56.8/6.1	499	(0.040; 0.131)	0.097	*N*
LOC_Os06g45900.1	Os06g0670400		Chr 6: 27782932-27791010	OsCYP59-1	PPIL4	63.1/6.3	548	N (0.026; 0.454)	0.180	*N*
LOC_Os06g45910.1	Os06g0670500		Chr 6: 27792515-27798510	OsCYP59-2	PPIL4	64.9/5.7	564	N (0.084; 0.242)	0.185	*N*
LOC_Os07g08190.1	Os07g0179200		Chr 7: 4175432-4181649	OsCYP63	PPID	69.9/11.5	632	N (0.116; 0.102)	0.139	*N*
LOC_Os03g10400.2	Os03g0201100		Chr 3: 5297334-5301353	OsCYP65	PPIL2	50.6/8.6	462	N (0.123; 0.045)	0.122	*C*
LOC_Os08g44330.1	Os08g0557500	LOC_Os08g44330.1	Chr 8: 27893370-27898831	OsCYP71a	PPWD1	72.9/6.9	651	C (0.073;0.082)	0.096	*C*
		LOC_Os08g44330.2		OsCYP71b	PPWD1	58.3/7.3	513	(0.035; 0.448)	0.161	*C*
LOC_Os02g10970	Os02g0204201	LOC_Os02g10970.1	Chr 2: 5826295-5834386	OsCYP95a	PPID	49.0/10.9	436	N (0.057; 0.135)	0.102	*N*
	Os02g0204300	LOC_Os02g10970.3		OsCYP95b	PPID	49.0/10.9	436	(0.057; 0.135)	0.102	*N*
LOC_Os10g06640.1	Os10g0154900		Chr 10: 3446108-3446808	OsCYP124[#]	PPIA	124/8.5	1089	(0.380; 0.122)	0.106	*N*

Alternatively spliced products have been designated as "a" or "b" after numerical. The new members of the family not reported earlier have been marked as "#"

[a] *C* cytosol, *SP* secretion, *M* mitochondria, *Ch* chloroplast, *N* nucleus

[b] *C* cytosol, *CS* cytoskeleton, *V* vacuole, *M* mitochondria, *Ch* chloroplast, *N* nucleus, *ER* endoplasmic reticulum, *P* plastid

extract was then used as template for PCR reaction. The PCR reaction was prepared using 10 μl of the supplied Red Extract-N-Amp PCR ReadyMix, 4 μl of the leaf extract, and 0.4 μM of each primer in a 20 μl of total reaction volume. Transgenic plants were screened using gene-specific forward and vector-specific reverse primer set (FP: 5′-CATGTCGAACACGAGG GTGTTC-3′, RP: 5′-CAAGAATTGGGACAACTCCAG-3′). The plasmid pCAMBIA1304OsCyp2-P was taken as the positive control template for the PCR reaction transformation.

Southern blotting

For Southern hybridization, 10 μg of digested genomic DNA (*Bgl*II and *Spe*I) was electrophoresed on a 0.8 % agarose gel and transferred to nylon membrane using the capillary transfer method as described in Sambrook and Russel (2001). The membrane was prehybridized for 3 h at 65 °C in buffer containing 5X SSC, 5X Denhardt reagent (0.5 % Ficoll, 0.5 % PVP, 0.5 % BSA), 0.1 % SDS, 100 μg/ml denatured salmon sperm DNA, and 10 % dextran sulphate. Probes were prepared by labeling the PCR-amplified samples of cDNA clones of the desired gene with α^{32}P-dATP (BRIT, Hyderabad, India) using HexaLabel DNA labeling kit (Thermo Scientific, UK) and purified using PCR purification kit (QIAGEN, USA). Thereafter, radiolabeled probe (pre-denatured by keeping on boiling water for 5 min) was added to the prehybridization solution. After incubation at 65 °C for 16–18 h in the hybridization solution, membrane was washed twice with 2X SSC for 5 min, 2X SSC +0.1 % SDS for 10 min, and 0.1 % SDS for 5 min. Washed membrane was exposed to phosphorimager plate (Amersham Pharmacia Biotech, England) for 15 h and scanned.

Raising of polyclonal antisera against OsCYP2-P

For expressing the recombinant protein OsCYP2, the coding region of 519 bp was cloned in the bacterial expression vector pET28a (Novagen, USA) at the multiple cloning site using the enzymes *Eco*RI and *Xho*I. The confirmed construct of pET28aOsCYP2 was transformed into BL21 (DE3) and induction of the protein OsCYP2 was carried out at 37 °C using 0.5 mM IPTG. Maximum induction of the OsCYP2 protein of the expected size of 18.6 kDa was observed at 37 °C at 4 h which was also confirmed using anti-HIS antibody Western blotting. The induced band was excised from the 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed again by peptide fingerprinting using MALDI-TOF-TOF. The spectrum gave 15 peaks with 36.6 % coverage, revealing high homology with *O. sativa* cyclophilin 2.

Polyclonal antisera against gel-purified OsCyp2-P was raised in rabbits as per the standard procedure (Harlow and Lane 1988) Three New Zealand White rabbits (3 months old)

were selected for raising antibodies at the Animal House, JNU. Pre-immune serum was obtained before immunization. Primary immunization was carried out with 100 μg protein antigen dissolved in normal saline (0.9 % NaCl). Before immunization, the protein antigen was emulsified with the complete Freund's adjuvant (CFA, Sigma, USA). Immunization with immunogens was carried out subcutaneously as well as intramuscularly. When immunized subcutaneously, the antigen was injected at multiple sites. For intramuscular immunization, single injection was given. Fifteen days after priming the animal, first booster shot was given. Purified antigen (50 μg protein) was emulsified with incomplete Freund's adjuvant (IFA) before injection. Subsequent booster shots were given weekly with 50 μg protein. After five booster shots, serum was collected separately from each animal and tested individually for cross reaction by performing Western analysis. High titer anti-OsCyp2 antisera were collected after five booster shots.

Western blotting using polyclonal antisera against OsCyp2-P

Thirty micrograms of protein separated on SDS-PAGE were transferred to Hybond-C^+, membrane (GE Healthcare, India) using Mini Transblot Electrophoretic cell (Bio-Rad, USA). Electroblotting buffer consisted of 150 mM glycine, 20 mM Tris, and 20 % methanol (pH 8.0). Prestained molecular weight markers (Sigma, USA) were employed to check the efficiency of protein transfer. The blot was rinsed briefly in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4 (pH 7.3)) containing 0.1 % Tween-20 and then incubated in blocking solution (5 % non-fat dried milk dissolved in PBS) for 45 min. Blot was washed thrice with PBS containing 0.1 % Tween-20 at 5-min interval and thereafter, incubated with primary antibodies at 1:1000 dilution with blocking buffer for 1 h. Blot was again washed thrice with PBS containing 0.1 % Tween-20 at 10-min interval followed by incubation in alkaline-phosphatase conjugated secondary antibodies for 1 h. The blot was washed thrice with PBS containing 0.3 % Tween-20 for 5 min. Thereafter, it was washed thrice with PBS containing 0.1 % Tween-20 for 5 min. 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 $MgCl_2$ containing 150 μg/ml of NBT and 75 μg/ml of BCIP). The reaction was terminated by rinsing the blot in water.

Subcellular localization of OsCYP2-P in rice

For the extraction of subcellular protein fractions, 10 g of sample tissue was used according to the manufacturer's instruction (Celllytic PN isolation kit, Sigma USA). Aliquots of 20 μg of protein of total, cytosolic, and nuclear protein was separated on 12.5 % SDS-PAGE and electroblotted onto Hybond-C membrane (Amersham, UK). Purity of the extracted cytosolic and nuclei proteins was checked by Western blots using primary antibodies raised against cytosolic marker enzyme—aldolase (CST, USA) and nuclear marker protein—histone 3 (CST, USA). Western analysis of OsCYP2-P was carried out using anti-CYP antibodies raised in our laboratory. All the three antibodies were treated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (Sigma, USA) and developed via standard enhanced chemiluminescence method.

Leaf disc senescence assay

Leaf discs of 1-cm diameter excised from healthy and fully expanded leaves of tobacco plants maintained under greenhouse conditions (28±1 °C; 12 h day/night) were kept in half-strength Hoagland media containing 150 mM NaCl. Leaf discs kept in half-strength Hoagland were taken as control.

Chlorophyll estimation

Estimation of chlorophyll from leaf discs or seedlings were done following Arnon's method (Arnon 1949): 100 mg of tissue was homogenized thoroughly in 1 ml of 80 % acetone and centrifuged at 3000 rpm for 2–3 min. The supernatant was retained and absorbance was recorded using spectrophotometer at 663 and 645 nm. The experiment was repeated thrice with transgenic line L5 (n=3).

Total chlorophyll

$$= [(20.2 \times A645 + 8.02 \times A663)/(1000 \times W)]XV$$

Seed germination and measurement of growth under stress conditions

T_2 generation seeds of OsCyp2-P ectopically expressing transgenic tobacco lines along with WT were subjected to various abiotic stresses. Half MS agar plates containing 150 mM NaCl (for high salt stress), 5 mM H_2O_2 (sublethal dosage of oxidative stress), 200 mM mannitol (high osmotic stress), or 5 % PEG (drought stress) were prepared. Seeds of transgenic lines and WT were sterilized by washing with 70 % ethanol for 1 min and subsequently washed with double-distilled autoclaved water to remove any trace of residual ethanol. The sterilized seeds were sown on the freshly prepared half MS agar plates (containing various stress inducing agents). After 21 days, images were taken and various physiological parameters, e.g., % seed germination, root length, shoot length, and chlorophyll content, were measured. Data from one representative line L5 is shown here (n=3).

K^+/Na^+ estimation

For determination of endogenous K^+ and Na^+ contents, 100 mg of tissue (control or salinity stressed) was taken and digested in 0.1 % HNO_3 (Singla-Pareek et al. 2003). Ions were extracted in distilled H_2O by boiling for 30 min twice. The filtered extract thus obtained was used to measure specific ions with flame photometer. Experiment was repeated thrice and standard error was calculated (n=3).

The assessment of membrane damage through lipid hydroperoxide assay

Lipid hydroperoxide accumulation was detected as per manufacturer's protocol (LPO assay kit, Cayman Chemical, USA). Tissue homogenate from unstressed and stressed seedlings were added into 500 μl of Extract R saturated methanol and vortexed followed by addition of 1 ml of chloroform. This mixture was centrifuged at 15,000 rpm for 5 min at 0 °C followed by collection of bottom layer. Then, 500 μl of bottom chloroform layer was mixed with 450 μl of chloroform methanol solvent mixture and thereafter 50 μl of freshly prepared chromagen was added. Tubes were kept at room temperature for 5 min and absorbance was measured at 500 nm. Final amount of lipid hydroperoxide in the sample was calculated as per the manufacturer's protocol. Experiment was repeated thrice and standard error was calculated (n=3).

Electrolyte leakage

For measuring electrolyte leakage, 100-mg tissue was taken from salt-stressed seedlings (along with the unstressed controls). They were washed immediately with distilled water and electrolyte leakage was measured as described earlier (Kumar et al. 2009). The tissue was dipped in 20 ml deionized water and incubated at 32 °C. The electrical conductivity (E1) of the solution was measured after 2 h using a conductivity meter (ELEINS Inc., India). The total conductivity (E2) was determined further by autoclaving the same immersion solution for 15 min at 121 °C, and the conductivity was measured after cooling it down to room temperature. Relative electrical conductivity was calculated as (EL=E1/E2×100). Experiment was repeated thrice and standard error was calculated (n=3).

In planta histochemical estimation of H_2O_2

Accumulation of H_2O_2 was examined based on histochemical staining by 3, 3′-diaminobenzidine (DAB) as described earlier (Kumar et al. 2009). Leaves from 21-day-old seedlings WT and transgenic plants subjected to various stresses (as described above) were vacuum infiltrated into 1 mg/ml fresh DAB solution (pH 3.8) prepared in 10 mM phosphate buffer (pH 7.8) and placed in a plastic box under high humidity and light until brown spots were observed (5 to 6 h). The stained leaves were then fixed with a solution of 3:1:1 ethanol: lactic acid: glycerol and photographed. Experiment was repeated thrice and standard error was calculated (n=3).

Peptidyl-prolyl-*cis*-trans activity assay

The fine chemicals and reagents used in this study were purchased from Sigma Chemical Co, St. Louis, USA. Peptidyl-prolyl-*cis*-trans isomerase activity was assayed in a coupled reaction with chymotrypsin, as described earlier (Kumari et al. 2009a). The assays were performed at 15 °C for 360 s. The 1 ml assay mixture contained 80 micro M *N*-succinyl-ala-ala-prophe- *p*-nitroanilidine as test peptide, assay buffer (50 mM HEPES (pH 8.0), 150 mM NaCl, 0.05 % Triton X-100) along with varying concentration of the plant protein from either WT or transgenic line. The reaction was initiated by the addition of chymotrypsin (300 mg/ml), and the change in absorbance at 390 nm was monitored using a spectrophotometer (Perkin-Elmer Mol Biotechnol Lambda Bio20) equipped with Peltier temperature control system. For calculating the PPIase activity, the difference between the catalyzed and uncatalyzed 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

Multi-stress responsive CYP gene family has expanded in rice as compared to *Arabidopsis*

In the present study, we have employed genome-wide analysis of *Arabidopsis* and rice in order to identify their respective cyclophilin gene family members. Detailed features of these cyclophilin gene family members in *Arabidopsis* and rice have been shown in Tables 1 and 2, respectively. We identified 31 distinct cyclophilin genes in *Arabidopsis* including two additional genes to that of 29 identified by Romano et al. (2004) encoding 41 putative proteins. In silico analysis of the *O. sativa* genome retrieved 29 CYPs genes encoding 46 putative proteins instead of 27 (Ahn et al. 2010) and 28 (Trivedi et al. 2012) gene members reported previously. Rice cyclophilin gene members show comparatively higher incidence of alternative splicing as compared to *Arabidopsis*. Chromosomal localization analysis showed that cyclophilin gene family members of *Arabidopsis* and rice were dispersed throughout their respective genomes. In *Arabidopsis*, the family of 31 cyclophilin genes was distributed randomly on all the 5 chromosomes while in *Oryza*, a family of 29 cyclophilin genes was distributed on 10 of the 12 chromosomes (Supplementary Fig. Ia, b).

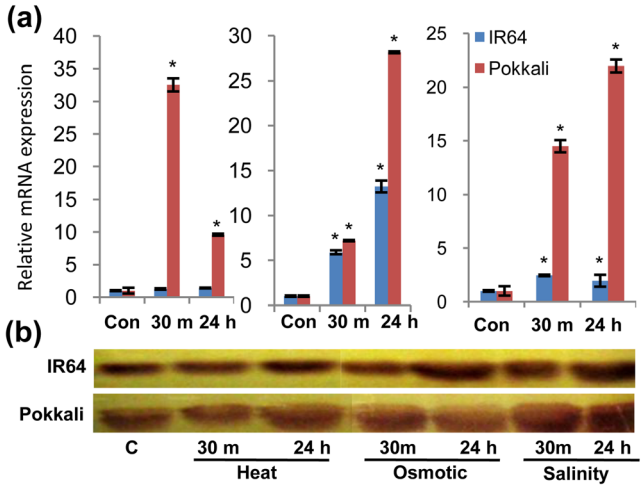

Fig. 1 qRT-PCR and Western blot analysis of OsCyp2-P gene in contrasting cultivars of rice. **a** Real-time PCR analysis of OsCyp2-P expression in shoots of 14-day-old rice seedlings under various abiotic stresses such as heat (42 °C), salinity (200 mM NaCl), and drought (5 % PEG) for various durations, i.e., 30 min and 24 h. The expression of each gene in different RNA samples was normalized with the expression of internal control gene elongation factor 1α. Data are means±SE. Each data set represents an average of minimum three separate experiments. *Asterisk* mark shows values significantly different from respective cultivar under control conditions at $P<0.05$, by Student's *t* test. **b** Western blot using anti-OsCYP2-P antisera showing OsCyp2-P protein accumulation in response to various stresses in contrasting rice genotypes i.e. IR64 and Pokkali

In order to classify and to investigate their evolutionary relationships, derived protein sequences of *Arabidopsis* and rice cyclophilins were subjected to phylogenetic analyses. The detailed phylogenetic tree depicting evolutionary relationship among cyclophilin members of rice and *Arabidopsis* is shown in Supplementary Fig. II, inner circle. The classification of cyclophilin members of rice and *Arabidopsis* into various subtypes was performed using KOBAS 2.0 database as shown by middle circle (middle circle). Cyclophilin members falling in same molecular weight category and subtype were grouped together in the phylogenetic tree indicating their co-evolution in rice and *Arabidopsis*. The outermost circle depicts the domain architecture of cyclophilin members of rice and *Arabidopsis*. The membrane-bound cyclophilin members (having a characteristic transmembrane domain) of rice and *Arabidopsis* and those localized in nucleus (possessing nuclear localized signals (NLS)) (Tables 1 and 2) identified using WOLF PSORT (http://wolfpsort.org/) and Nucpred (http://www.sbc.su.se/) have also been shown in the outer circle. The TPR containing cyclophilin members include AtCYP40, OsCYP40b, and OsCYP40a (Supplementary Fig. II, outer circle).

We have used the publicly available 17 and 20 bp MPSS libraries of *Arabidopsis* and rice for carrying out expression analysis of cyclophilin genes identified in this study. Expression analyses of *Arabidopsis* cyclophilin genes revealed that *AtCyp19*-1 transcript is most abundant in various tissues and stress treatments (Supplementary Fig. IIIa), thereby indicating its functional significance at all the developmental stages and stress treatments. Expression analysis of rice cyclophilin genes using MPSS database revealed that *OsCyp19-3* and *OsCyp40-2* were the only cyclophilin genes that were highly expressed in mature pollen and developing seeds, respectively (Supplementary Fig. IIIb). One of the cytosolic cyclophilin gene member from rice, *OsCyp19-1*, showed highly abundant transcript accumulation at various developmental stages and tissues under control and both biotic and abiotic stresses, as evident in the MPSS data.

Microarray data analysis of cyclophilin genes of rice and *Arabidopsis* depicts differential yet specific stress-inducible nature of these genes. Heat maps based on expression pattern of these genes under various stresses show distinct classes of these proteins, as shown by the hierarchical clustering (Supplementary Fig. IVa, b). *AtCyp19*-2, ortholog of rice gene *OsCyp2* (*OsCyp19*-1), was found to be highly induced by both high- and low-temperature stress, besides showing moderate to high expression under salt and osmotic stress. Microarray analysis of rice cyclophilin genes in response to cold, salinity, and drought revealed only few members to be perturbed by low-temperature stress whereas a large number of genes were altered under salinity and drought stress (Supplementary Fig. IVb). This analysis clearly indicated towards uniquely orchestrated action of various members of the cyclophilin family under various abiotic stresses and developmental stages. Microarray data analysis of cyclophilin genes in *Arabidopsis* and rice supports the MPSS data for their stress- and tissue-specific expression patterns (Supplementary data I).

Expression of OsCyp2-P gene is induced by multiple stresses in rice, both at transcript and protein level

As per MPSS and microarray data, cytosolic single-domain cyclophilin, OsCyp19-1, shows stress-dependent and developmental stage-specific transcript induction. In our previous study, we had obtained a single-domain cyclophilin in salinity-specific subtractive library (referred as *OsCyp2* in Kumari et al. (2009a, b) and now being referred as OsCyp2-P). On closer inspection, based on sequence similarity, it was found that OsCyp19-1 and OsCyp2-P show 100 % sequence similarity. To revalidate if OsCyp2-P is regulated by multiple abiotic stresses, qRT-PCR

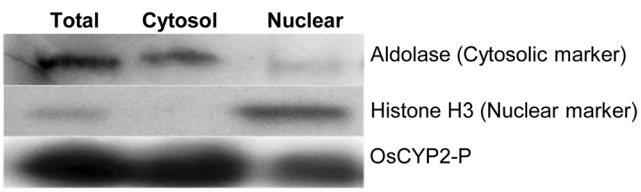

Fig. 2 Localization of OsCYP2-P protein by Western blots of subcellular protein fractions in rice leaves. Presence of OsCYP2-P could be seen clearly in the fractions corresponding to the cytosol and nucleus of the cell. Purity of the cytosolic and nuclear fractions was checked by the marker proteins—aldolase and histone H3, respectively

was carried out with cDNA prepared from shoots of IR-64 (salt sensitive) and pokkali (salt tolerant) rice seedlings (14 days old) subjected to heat, osmotic, and salinity stress. Analysis of transcript abundance of OsCyp2 in shoot tissue of these two contrasting cultivars of rice at seedling stage revealed its differential stress-inducible expression (Fig. 1a). This analysis indicated that *OsCyp2* is upregulated rapidly (after 30 min) under salinity stress in both IR-64 and Pokkali. However, the induction in Pokkali is much higher (7–8-fold) than IR-64. Similar observations were made when the transcript abundance for OsCyp2-P was analyzed in seedlings in response to high temperature or osmotic stress.

To corroborate these findings further at protein level, Western analysis was carried out with the crude protein extracts isolated from the above experimental setup. Polyclonal antibodies were raised against full-length OsCyp2-P protein and used for studying the protein accumulation in crude plant protein extract isolated from 4-day-old seedlings. A single specific band corresponding to 18.6 kDa of OsCyp2-P protein was obtained in the blots. Differential accumulation of OsCyp2-P protein was observed for the two genotypes under study. The tolerant cultivar showed higher level under both control and stress conditions (Fig. 1b), thus implying the role of OsCyp2-P in multi-stress response in rice.

OsCyp2-P is localized both in cytosol and nucleus in rice

OsCyp2-P protein lacks any nuclear signal (NLS) and is predicted to be cytosolic. To confirm it, we carried out Western analysis of the subcellular protein fractions in rice (Fig. 2). The cytosolic marker enzyme aldolase and nuclear marker protein histone H3 showed predominance in cytosolic and nuclear fractions, respectively, establishing the purity of these fractions. However, both the cytosolic and nuclear fractions showed the presence of OsCYP2-P protein suggesting the predominance of this protein in both cytosol and nucleus. The presence of this protein in the cytosolic fraction is much higher as compared to the nuclear, suggesting its major involvement in the cytosolic events.

Transgenic tobacco plants ectopically expressing OsCyp2-P showed constitutive higher Cyp transcript and protein accumulation as well as higher PPIase activity

Since OsCyp2 was highly represented in a subtractive library and its overexpression conferred tolerance to *Escherichia coli* and *Saccharomyces cerevisiae* (Kumari et al. 2009b), further characterization of this gene OsCyp2-P (isolated from a salinity-tolerant landrace, i.e., Pokkali) and its ectopic expression in tobacco was carried out to assess its effect as a candidate gene conferring multi-stress tolerance to plants. The putative transgenic plants which formed proper roots on hygromycin containing MS media were screened using tissue PCR (see "Materials and methods" section) with a combination of gene-specific and vector-specific primer. Genomic DNA from WT (non-transgenic) tobacco cv. Petit Havana and pCAMBIA1304OsCyp2-P plasmid (Supplementary Fig. Va) were taken as negative and positive controls, respectively. Among ten lines screened, five lines were found to be positive (L1, L2, L4, L5, L6) as they showed the desired band amplified from the genomic DNA of the putative transgenic plants (Supplementary Fig. Vb).

Genomic Southern blot analysis of PCR positive plants (four lines L1, L2, L4, and L5) was carried out to check the stable integration of the transgene in the tobacco genome. The genomic DNA from each of the transgenic lines and WT tobacco plants was digested with *Bgl*II and *Spe*I which released the OsCyp2-P fragment of 519 bp. The digested DNA was fractionated on agarose gel (Supplementary Fig. Vc) and transferred to the nitrocellulose membrane and probed with full-length OsCyp2-P specific α-P^{32}-radiolabeled probe (see "Materials and methods" section). Only a single specific cross hybridizing band, which exactly matched with the size of the positive control, was visible for all four samples and none in the WT (Supplementary Fig. Vd). These results confirmed the transgene (OsCyp2-P) insertion in the tobacco genome. However, based on visual analysis of thickness of band, it appears that lines L1 and L2 may be multicopy transgenic lines.

To confirm the expression of OsCyp2-P at the transcript level, semiquantitative PCR and qRT-PCR analysis were performed in transgenic tobacco ectopically expressing OsCyp2-P lines L1, L2, L4, and L5. Accumulation of OsCyp2-P transcripts in three lines L1, L4, and L5 could be clearly detected on gel (Fig. 3a). Though line L2 was found to be positive for the presence of transgene by PCR and Southern hybridization, it did not show any expression of the transgene. The results of qRT-PCR again confirmed these observations (Fig. 3b). It is possible that the transgene was silenced in the line L2, hence no expression of the transcript could be found. To examine whether increase in transcript expression in transgenic lines was also reflected in the protein accumulation, Western blot analysis was carried out for transgenic lines using anti-OsCyp2-P antibodies. High level of OsCyp2-P protein was detected in lines L1, L4, and L5, whereas wild-type tobacco plants and line L2 did not show accumulation of OsCyp2 protein (Fig. 3c, d). Though cyclophilin proteins are highly conserved in nature, the absence of an immunological homolog of OsCyp2-P in tobacco (non-transgenic) indicates toward the high quality of the antisera raised against OsCyp2-P. Among the four lines tested here, tobacco line L5 showed highest transcript as well as its corresponding protein accumulation as revealed by qRT-PCR and Western blot analysis, respectively. To test if the increased OsCyp2-P protein is also enzymatically active, we performed PPIase assay in WT and L5 transgenic lines using standard protocol as done earlier (Kumari et al. 2009b). The transgenic line L5 was found to

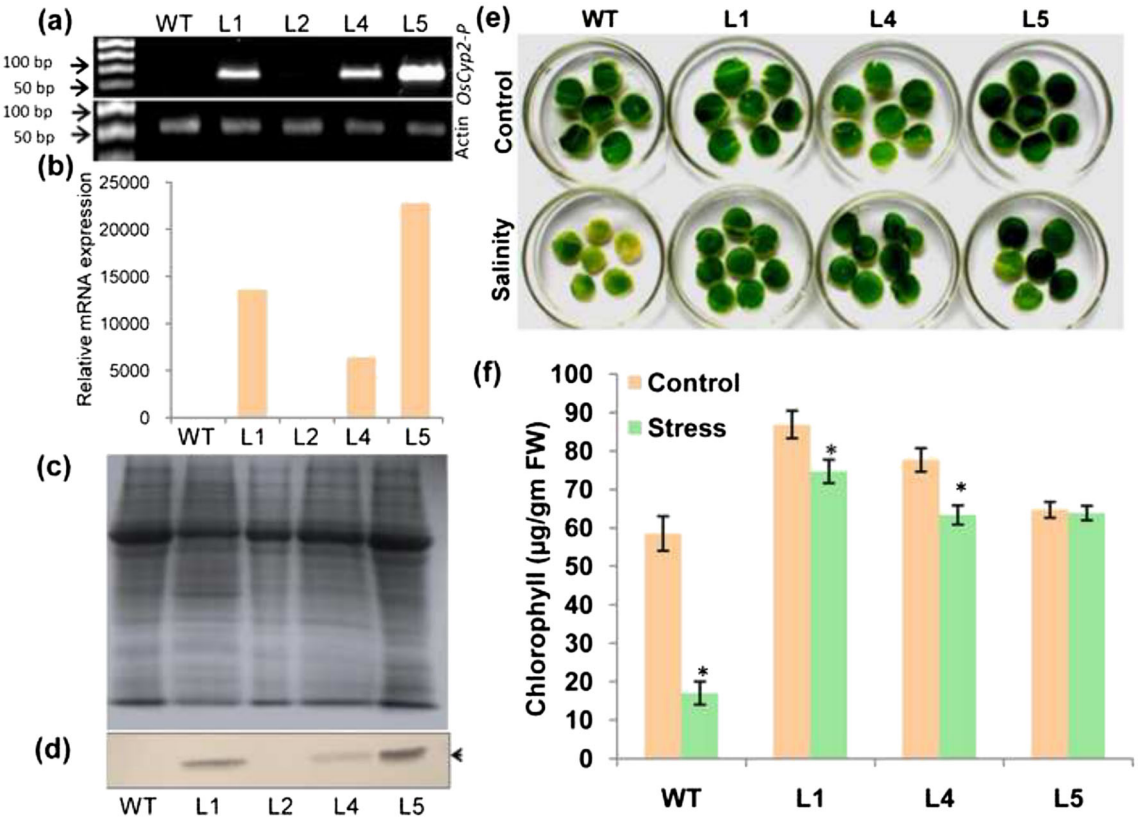

Fig. 3 Confirmation of OsCyp2-P ectopically expressing transgenic tobacco plants and assessment of their tolerance towards salinity stress. **a** Ethidium bromide stained agarose gels showing semiquantitative RT-PCR amplicons of actin gene (*lower panel*) and OsCyp2-P gene (*upper panel*) in wild-type and transgenic lines. Note that lines L1, L4, and L5 are positive and line L2 is negative for the expression of OsCyp2-P. **b** Bar diagram showing relative expression of OsCyp2-P in wild type and OsCyp2-P ectopically expressing transgenic lines analyzed through qRT-PCR, again confirming the expression pattern for OsCyp2-P as observed in RT-PCR shown above. **c** Commassie stained SDS-PAGE showing total protein isolated from wild-type tobacco plants and OsCyp2-P ectopically expressing lines and **d** corresponding Western blot analysis for the presence of recombinant protein using anti-OsCYP2-P antisera. Note that lines L1, L4, and L5 are positive and line L2 is negative for the expression of OsCyp2-P protein as revealed by the immunoblot. **e** Leaf disc assay of wild-type tobacco plants and OsCyp2-P lines (L1, L4, and L5) to obtain a comparative estimate of chlorophyll retention capability under salinity stress (150 mM NaCl). Leaf discs floated in Hoagland medium served as the experimental controls. **f** Bar diagram depicting chlorophyll content retained in leaf discs after 48 h in OsCyp2-P lines along with the WT. The data represent mean±SE of three biological replicates (n=3). *Asterisk* shows values significantly different from respective control under same conditions at $P<0.05$, by Student's t test

have ~sixfold PPIase activity as compared to WT (Supplementary Fig. VI) which confirmed that the orthologous OsCyp2-P protein is also enzymatically active.

All the transgenic plants overexpressing OsCyp2-P matured normally under control conditions. The seed setting of these transgenic plants was similar to that of the untransformed WT plants thereby suggesting that overexpression of the transgene did not hamper the yield potential. Furthermore, the basis of stress tolerance in OsCyp2-P ectopically expressing plants was studied by examining various physiological and biochemical parameters.

OsCyp2-P plants show better capability to retain chlorophyll under salinity stress

To study whether ectopic expression of OsCyp2-P in tobacco plants can alter their sensitivity towards salinity, leaf disc chlorophyll retention assay was performed. Fully expanded healthy leaves of WT tobacco plants and transgenic T_2 lines were used for this assay. Leaf discs of 1-cm diameter were cut from each transgenic line and were kept in stress (i.e., half-strength Hoagland media supplemented with 150 mM NaCl) or control (i.e., half-strength Hoagland media). Significant differences in the "green-ness" of the leaf discs were observed among WT and transgenic plants (line no. L1, L4, L5) treated with 150 mM NaCl within 48 h (Fig. 3e). However, line no. L5 showed significant tolerance towards salinity as observed by its chlorophyll retention under stress condition (Fig. 3f). Under salinity stress, L5 had almost same levels of chlorophyll as under control (non-stressed conditions) as revealed by t test.

OsCyp2-P transgenic plants show enhanced tolerance to multiple abiotic stresses

In our study, OsCyp2-P (both mRNA and protein) has been found to be induced in rice seedlings in response to various

abiotic stresses such as salinity, high temperature and drought. Previous studies have also documented similar induction of *OsCyp2* under various stresses (Ruan et al. 2011). Based on these findings, we hypothesize that OsCyp2-P may be playing crucial role in providing tolerance to plants for multiple abiotic stresses. To validate this hypothesis, we have performed various physiological experiments with the tobacco plants ectopically expressing OsCyp2-P which is presented below.

Comparative analysis of germination and growth of T_2 seeds of tobacco plants ectopically expressing OsCyp2-P and wild-type tobacco was carried out under multiple stress conditions. Results for one of the representative line, i.e., L5 has been presented here, since in the leaf disc assay (Fig. 3f), this line exhibited highest chlorophyll retention when exposed to salt stress. Equal number of seeds of line L5 and WT tobacco plants were inoculated on MS media supplemented with various stressors (see "Materials and methods" section). Differential growth response of WT and transgenic line towards various stresses was observed (Fig. 4a–f for salinity and oxidative stress response and Supplementary Fig VIIa–e for drought and osmotic stress response). Under these saline conditions, only 76 % of WT seeds could germinate. In contrast, 96 % seed germination was observed for OsCyp2-P transgenic plants under similar conditions. Similarly, germination percentage was found to be 86 and 98 % in L5 tobacco plants under drought and osmotic stress, respectively, while it was 80 and 94 % in WT plants under same conditions (Fig. 4e and Supplementary Fig VIIc). However, under oxidative stress, almost all of the WT as well as transgenic seeds could germinate.

Interestingly, in terms of growth under stress conditions, the root and shoot growth of the L5 transgenic plants showed significant difference in comparison to the respective WT under similar conditions. Imposition of different stress conditions resulted in significant decrease in shoot growth of both WT and OsCyp2-P L5, but the reduction in shoot length of L5 was significantly lower than WT (Fig. 4c and Supplementary Fig VIId). L5 transgenic line also showed decrease in root length under mannitol and salt stress, though as compared to control, it was significantly lower (Fig. 4d and Supplementary Fig VIIe); however, it showed significantly high root growth under drought stress (PEG), whereas the wild-type roots were severely retarded. It thus appears that the presence of OsCyp2-P prevented the decrease in root as well as shoot growth under drought stress. Using mutant rice lines, it has been demonstrated that OsCyp2 plays a critical role in degradation of auxin-responsive proteins and thus plays a crucial role in its lateral root initiation (Kang et al. 2013). Oxidative stress (5 mM H_2O_2) severely affected the root and shoot growth in both WT and transgenic plants. However, the decrease in shoot growth of L5 was significantly less than the WT under oxidative stress. On the contrary, root growth was adversely affected to the same extent in both wild-type and transgenic lines (Fig. 4d).

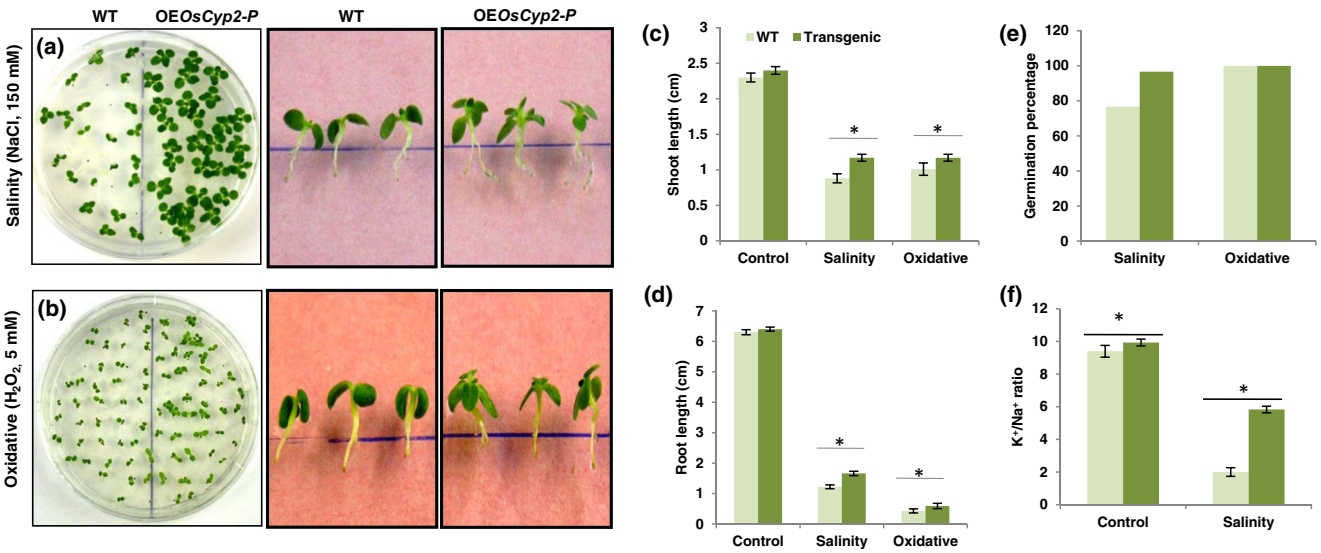

Fig. 4 Germination assay of OsCyp2-P ectopically expressing transgenic tobacco seeds on MS media supplemented with various stressors. For this purpose, medium was supplemented with either **a** NaCl (150 mM) for salinity stress or **b** H_2O_2 (5 mM) for oxidative stress. Bar diagram depicting **c** shoot length and **d** root length of wild-type and transgenic seedlings under control and stress conditions as indicated below the bars. **e** Bar diagram showing germination percentage of wild-type and transgenic seedlings (L5) under various stress conditions. **f** Bar diagram depicting K^+/Na^+ ratio in WT and transgenic lines grown under control and saline (NaCl, 150 mM) conditions. The data represent mean±SE of three biological replicates (n=3). *Asterisk* shows values significantly different from respective WT under same conditions at $P<0.05$, by Student's t test

OsCyp2-P ectopically expressing plants show increased tolerance towards multiple abiotic stresses in terms of their K^+/Na^+ ratio, electrolyte leakage, and chlorophyll contents

We measured the Na^+ and K^+ concentration in the whole seedlings of WT plant and OsCyp2-P transgenic lines exposed to salinity stress (150 mM NaCl). It was observed that the OsCyp2-P ectopically expressing tobacco seedlings were able to maintain K^+/Na^+ homeostasis better than the WT plants under salinity stress (Fig. 4f). Another report by Ruan et al. (2011) showed that the transgenic rice plants overexpressing *OsCyp2* maintain reduced K^+/Na^+ ratio than WT even under non-stress conditions. However, in our study, OsCyp2-P tobacco seedlings showed significant advantage over WT in terms of maintaining K^+/Na^+ ratio even under non-stress (control) conditions (Fig. 4f). The reasons for these observed differences needs to be explored further.

Chlorophyll content measured in WT and different OsCyp2-P transgenic seedlings grown under greenhouse conditions revealed a clear advantage of OsCyp2-P ectopic expression under various stress conditions (Fig. 5a). This is in conformity with the observation related to chlorophyll retention leaf disc assay (Fig. 3e). Electrolyte leakage is correlated with cell membrane stability and integrity and is of primary concern in plants grown under abiotic stress conditions (Wang et al. 2010a). Compared to WT, the OsCyp2-P ectopically expressing transgenic lines showed significantly lower electrolyte leakage under all the stress conditions tested (Fig. 5b). For all these studies, the silenced line L2 exhibited the response similar to that of WT.

OsCyp2-P transgenics show reduced lipid peroxidation and reduced H_2O_2 accumulation under various abiotic stress conditions

To validate the protective effects of OsCyp2-P on the photosynthesis machinery (hub for ROS generation) in tobacco

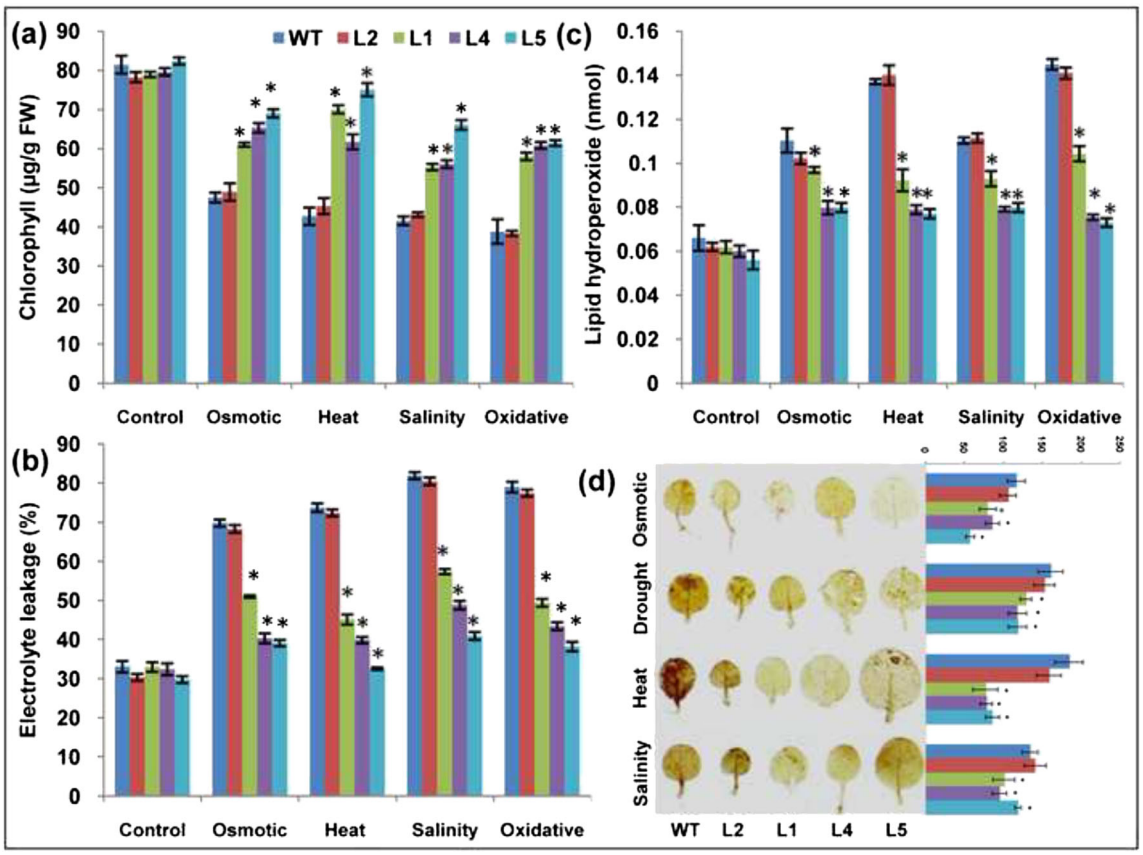

Fig. 5 Assessment of various physiological parameters of WT and OsCyp2-P transgenic tobacco lines grown under control or stress conditions (i.e., mannitol, 200 mM; heat, 42 °C; NaCl, 150 mM and H_2O_2, 5 mM). Bar diagram depicting **a** chlorophyll retention; **b** electrolyte leakage (%). **c** Lipid hydroperoxide accumulation in WT and OsCyp2-P ectopically expressing lines grown under control and stress conditions. **d** Assessment of relative H_2O_2 levels in tobacco plants under stress conditions. Stress-induced levels of H_2O_2 in leaves of WT as well as OsCyp2-P ectopically expressing lines (L2, L1, L4, L5) were visualized by DAB staining. *Bar diagram* depicting relative quantity of H_2O_2 in these leaves in terms of their color intensity. Transgenic line L2 represents a silenced line taken as a control. The data represents the means of three biological replicates ($n=3$). *Asterisk* shows values significantly different from respective WT under same conditions at $P<0.05$, by Student's *t* test

leaves, we compared salt stress-induced changes in the lipid peroxidation and reactive oxygen species (ROS) scavenging in OsCyp2-P transgenic and wild-type seedlings. The level of lipid hydroperoxide in plant tissues was used as an indicator of lipid peroxidation. Under control conditions, the lipid hydroperoxide levels were comparable in OsCyp2-P transgenic seedlings and wild-type seedlings (Fig. 5c). However, under stress conditions (osmotic, heat, salinity, H_2O_2), the lipid hydroperoxide levels were significantly lower in all OsCyp2-P transgenic lines (except the line L2 where the gene appears to be silenced), as compared to WT (Fig. 5c), indicating that OsCyp2-P ectopic expression decreased the lipid peroxidation levels. Diaminobenzidine tetrahydrochloride (DAB) staining was used to visualize the accumulation of H_2O_2 in stressed WT and OsCyp2-P ectopically expressing lines. Under stress conditions, OsCyp2-P transgenic tobacco seedlings contain substantially lower H_2O_2 levels than wild-type seedlings (Fig. 5d).

Discussion

Genome-wide analysis of cyclophilin members in *Arabidopsis* and rice revealed this family to have expanded in the latter via alternative splicing events. Similar observations were made when we compared the cases of alternative splicing of two-component system family (TCS) between *Arabidopsis* and rice (Pareek et al. 2006). Alternative splicing is a molecular mechanism utilized by broad range of eukaryotes to extend the repertoire of normal functions associated with a single gene (Haas 2008). It has been suggested that alternate splicing of many genes in rice is affected by stress conditions, suggesting that manipulation of alternate splicing may be an effective strategy of rice to adapt to abiotic stresses (Zhiguo et al. 2013). Expansion of Cyp family in rice may indicate towards involvement and varied functions of the members in handling diverse stresses. Random distribution of salinity-related ESTs on different chromosomes in rice has also been reported by our group recently (Kumari et al. 2009a). In contrast, we have also reported clustering of few metallothionein genes in rice which may be representing a kind of "regulon" (Kumar et al. 2012). This kind of genome-wide analysis allows studying the gene-specific molecular evolution of species possibly correlating with their adaptive responses towards abiotic stresses. Analysis of cyclophilin family in rice and *Arabidopsis* showed their random distribution on chromosomes and also showed that some of the members were duplicated on certain regions of the chromosome (for details, see the supplementary data).

Stress-dependent accumulation of cyclophilins has been documented in several plant species including bean, maize, sorghum, pigeon pea, *Arabidopsis*, and tomato (Marivet et al. 1994, 1995; Scholze et al. 1999; Godoy et al. 2000; Sharma and Singh 2003; Sekhar et al. 2010). These reports suggest a pivotal role of cyclophilins in plants under adverse conditions either through their direct role in chaperoning or transcriptional activation of other stress-regulated genes. Recently, OsCyp2 has also been shown to participate in auxin signal transduction by acting as molecular chaperone and interacting with the co-chaperone OsSGT1 (Kang et al. 2013), which further strengthens the possibility of its role as molecular chaperone. Mammalian *CypA* was predicted earlier as a cytosolic *Cyp* but it was found to be distributed in both the nucleus and cytosol of mouse macrophages and interact with a zinc-finger transcription factor (Krummrei et al. 1995). It has also been hypothesized that yeast cyclophilin A (which also lacks NLS) passes through nuclear pores passively due to its small size. Similarly, OsCyp2-P is also a small protein of 18.6 kDa and thus it can physically pass into the nucleus by diffusion. This leads us to hypothesize that OsCyp2-P is delivered into the nucleus through diffusion, similar to the yeast cyclophilin A, and interact with specific nuclear proteins thereby regulating the transcriptional activation of downstream genes. Furthermore, in contrast to mammalian multiprotein NADPH oxidase complex (Cross and Segal 2004), plant NADPH oxidases are single cytosolic proteins (Davis et al. 1998). AtCyp19 has been reported to be localized in cytoplasm and hypothesized to interact with cytosolic ROS-producing proteins, particularly with NADPH oxidase or act its upstream regulator to produce ROS (Pogorelko et al. 2014). These reports confirm our study and serve as an indication towards the key regulatory role played by OsCyp2-P as a modulator of the gene function and protein expression during stress responses.

Differential accumulation pattern of OsCyp2-P transcripts under various stresses indicate its possible role in multiple stress responses. This is in agreement with our previous findings where we found differential accumulation pattern of OsCyp2 transcripts at various developmental stages indicating its developmental and stress responsive regulation (Kumari et al. 2009b). The high salinity tolerance in L5 line was in strong correlation with the high abundance of transcript, protein, and activity of OsCyp2-P. In order to survive under higher concentrations of NaCl, plants have to either sequester or extrude excess Na^+ ions and thereby maintain higher K^+ ions required for various cellular processes (Zhu 2001). Maintenance of K^+/Na^+ ratio under stress condition is considered as a vital determinant of stress tolerance (Glenn et al. 1999). The OsCyp2-P transgenic plants could maintain higher K^+/Na^+ ratio and better membrane integrity than WT plants under salinity stress, thereby implying that the ectopically expressing lines showed unequivocal adaptive advantage in terms of ion homeostasis and membrane integrity. Thus, OsCyp2-P may be playing a role in protecting cellular membranes via its chaperoning activity as proposed earlier (Lippuner et al. 1994; Ruan et al. 2011).

Diverse stresses have been shown to ultimately lead to oxidative stress due to the disturbance in reactive oxygen species (ROS) production and scavenging, leading to generation of ROS such as H_2O_2 (Mittler et al. 2004; Huang et al. 2010; Ouyang et al. 2010). ROS are highly detrimental to biological systems. They partially reduce or activate derivatives of oxygen and thus damage DNA, proteins, and carbohydrates, resulting in cell death (Jambunathan et al. 2010). They have also been implicated in lipid peroxidation and cell membrane damage, and their levels can reflect the degree of damage to cellular components (Huang et al. 2010). Stress-induced H_2O_2 accumulation can result in increase in lipid peroxidation (Jambunathan et al. 2010). It has been proposed earlier that ROS act as primary signaling molecules during abiotic stresses, activating mitogen-activated protein kinase (MAPK) pathways (Karali et al. 2012). ROS are reported to be produced by cell wall, mitochondria, plastids, endoplasmic reticulum, peroxisomes, NADPH oxidase metabolisms, and due to the consequence of cell membrane electron transporting (Sharma et al. 2012). The study by Ruan et al. (2011) has also demonstrated the role of OsCyp2-P towards providing tolerance to salinity stress in rice seedlings via ROS scavenging. They have observed that OsCyp2-P transgenic rice seedlings had lower MDA content and H_2O_2, which is well correlated with higher antioxidant enzyme activities (such as SOD, CAT, and APX) than WT seedlings. It has been suggested that OsCyp2 up-regulates the activities of several important enzymes such as SOD, CAT, and APX at post-transcriptional level (Kong et al. 2001). This in turn protects photosynthetic components of leaves against oxidative stress. Taken together, our findings also indicate the role of OsCyp2-P as a key regulator controlling ROS levels.

The proliferated growth of OsCYP2-P transgenic plants under stress conditions (this study) are in confirmation with the earlier studies that reported improved germination and growth in the transgenic plants that overexpressed single-domain cyclophilin (*Gjcyp-1*) in red algae (Cho and Kim 2008) and *ThCyp1* in tobacco (Chen et al. 2007). Overexpression of a pigeonpea cyclophilin gene, CcCyp in *Arabidopsis*, also resulted in improved germination and biomass accumulation in transgenic plants (Sekhar et al. 2010). Our results clearly show that OsCyp2-P plays a crucial role in preventing oxidative damage in cells thus protecting the photosynthetic machinery. Since generation of ROS is central to various abiotic stresses studied here and OsCyp2-P expressing plants exhibit considerable tolerance to these varied abiotic stresses, we confirm the role of OsCyp2-P as a key regulator controlling the levels of ROS in plants.

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Conflict of interest The authors declare that there are no conflicts of interest.

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