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Genome-wide analysis of PHD finger gene family and identification of potential miRNA and their PHD finger gene specific targets in *Oryza sativa* indica

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ABSTRACT

Rice (*Oryza sativa* L.) is one of the most important cereal crops for one third of the world population. However, the grain quality as well as yield of rice is severely affected by various abiotic stresses. Environmental stresses affect the expression of various microRNAs (miRNAs) which in turn negatively regulate gene expression at the post-transcriptional level either by degrading the target mRNA genes or suppressing translation in plants. Plant homeo-domain (PHD) finger proteins are known to be involved in the plant response to salinity stress. In the present study, we identified 44 putative *OsPHD* finger genes in *Oryza sativa* Indica, using Ensembl Plants Database. Using computational approach, potential miRNAs that target *OsPHD* finger genes were identified. Out of the 44 *OsPHD* finger genes only three *OsPHD* finger genes i.e., *OsPHD2, OsPHD35* and *OsPHD11*, were found to be targeted by five newly identified putative miRNAs i.e., ath-miRf10010-akr, ath-miRf10110-akr, osa-miR1857–3p, osa-miRf10863-akr, and osa-miRf11806-akr. This is the first report of these five identified miRNAs on targeting PHD finger in *Oryza sativa* Indica. Further, expression analysis of 44 PHD finger genes under salinity was also performed using quantitative Real-Time PCR. The expression profile of 8 genes were found to be differentially regulated, among them two genes were significantly up regulated i.e., *OsPHD6* and *OsPHD12*. *In silico* protein-protein interaction analysis using STRING database showed interaction of the OsPHD finger proteins with other protein partners that are directly or indirectly involved in development and abiotic stress tolerance.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops and serves as the staple food for over one-third of the world's population [1]. However, the productivity of rice is greatly affected due to soil salinity which is the second most widespread soil problem next to drought in rice growing areas of the world [2,3].

Plant homeo-domain (PHD) finger proteins have been known to be involved in the plant response to various abiotic stress. The PHD finger is a chromatin interacting domain localized in the nucleus of eukaryotic organisms and involved in transcriptional regulation [4,5]. A typical PHD usually consist of 50–80 amino acid residues, stabilized by two zinc ions as metal binding RING domain in a "cross-brace" topology and possess a unique characteristic Cys4-His-Cys3 pattern [6–8]. It is conserved and has been found in more than 400 proteins in eukaryotes including the yeast [9–11]. The PHD finger protein plays diverse role in plants including development and plant growth [12–17]. In recent studies, it has been reported that PHD finger genes were also responsive under different abiotic stress treatment in *P. trichocarpa* [18], maize [5], carrot [19], tomato [20], *Oryza sativa* Japonica [21] and moso bamboo [22].

In plants, many studies have revealed that microRNAs (miRNAs) play a vital post-transcriptional regulatory role in gene expression by targeting mRNA cleavage or translational inhibition [23]. In plants, mature miRNAs are generated from the long stem-loop precursor (pre-miRNAs) by a DICER-like RNA endonuclease and then the RNA-Induced Silencing Complex (RISC) guided by ARGONAUTE 1 (AGO1) protein directs the miRNA to the complementary target mRNA sequence [23–26]. Plant miRNAs are reported to possess important functions in several metabolic and biological pathways such as tissue

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development and differentiation, biotic and abiotic stress responses, phytohormones signaling, and secondary metabolite production [27,28]. Nonetheless, the evolutionary highly conserved nature of an extensive number of miRNAs simplified the process of characterization of novel miRNA orthologs in new plant species through homologs identification [29]. Several abiotic stress-sensitive miRNAs have been reported over a period of time in various studies, e.g. In *Arabidopsis thaliana*, miRNA398 is involved in oxidative stress tolerance [30], and gene expression of 21 miRNAs are up-regulated in response to UV-B exposure [31].

Knowing the importance of miRNA and their roles in gene regulation, in the present investigation, experiments have been designed for *in silico* identification of miRNAs and their potential PHD targets in rice through computational approach. Further, to study the response of PHD genes under salinity stress, we performed gene expression analysis using qRT-PCR. Our results provide better framework to understand the role of PHD gene family in Indica rice and their potential role in salinity stress response.

2. Materials and methods

2.1. Identification and domain analysis of PHD family genes in rice

We identified candidate PHD family genes by using Pfam ID "PF00628" as a keyword against the rice genome database in the Ensembl Plant (http://plants.ensembl.org/Oryza_indica/Info/Index). The amino acids, as well as the cDNA sequence of all the selected PHD proteins, were then retrieved from the Ensembl Plant database for further analysis. The amino acid sequences obtained were used for domain analysis using Pfam [32] and HMMER database [33]. Multiple sequence alignment was performed in MEGA X program using MUSCLE algorithm using default parameters and visualized by Jalview software [34–36]. The aligned PHD finger protein sequences were used for construction of phylogenetic trees using default parameters in MEGA X program using Neighbor Joining (NJ) algorithm. The default parameters of MEGA X used during alignment are as follows - gap penalty was set at gap opening penalty at -2.90, extension at 0 and hydro-phobicity multiplier at 1.2. The maximum memory usage was set at 2048 MB and the Maximum iterations performed were 16. The cluster method used for iteration was UPGMA (Unweighted Pair Group Method with Arithmetic mean) as a simple agglomerative (bottom-up) hierarchical clustering method. UPGMA with a lambda value of 24 (Minimum diagonal Length) was used. While phylogeny reconstruction, neighbor joining (NJ) was used as a bottom-up (agglomerative) clustering method for the creation of phylogenetic trees. The Poisson model was used as a substitution model in this case. Substitution rates among sites were considered to be uniform and the pattern along lineages were also taken as homogeneous. The gaps and missing data were treated as pair wise deletion in each case.

2.2. Identification of potential miRNAs and their target PHD gene

Workflow of the identification and characterization of potential miRNAs and their target genes is depicted in Fig. 1. A total of 10,898 mature miRNA sequences were retrieved from PMRD: Plant micro RNA Database (http://bioinformatics.cau.edu.cn/PMRD/) [37]. With identity value 90, CD-HIT-v4.5.4 was used to remove the redundancy in miRNA sequences [38]. In order to identify miRNA-targeted PHD genes of Indica rice, local BLAST was performed using Blast2GO version 5.2 [39]. BLASTx analysis (E-value $\leq$ 1e-10) was performed in order to remove protein-coding sequences from precursor sequences.

2.3. Prediction of the secondary structure of pre-miRNAs

Prediction of the secondary structure was done by using the software MFOLD 3.1 available at (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) [40]. The following criteria were used for screening the candidates of potential miRNAs: minimum length of the pre-miRNA to be 60 nt; pre-miRNA should be folded into appropriate stem-loop hairpin secondary structure; mature miRNA sequence should be placed in one arm of the hairpin structure; not >6 nt mismatches in miRNA/miRNA duplex; No loops or breaks between the miRNA/miRNA duplex; A + U content within 30–70%; Predicted secondary structure should have higher minimal folding free energy index (MFEI) and negative minimal folding free energy (MFE) values [41]. The MFE or ΔG (-kcal/mol) values generated from the MFOLD web server of the stem-loop structures were used for calculating the MFE index values using the following formula:

$$\text{MFEI} = [(\text{MFE} \div \text{Length of precursor miRNA sequence}) \times 100] \div (\text{G} + \text{C content})\ \%$$

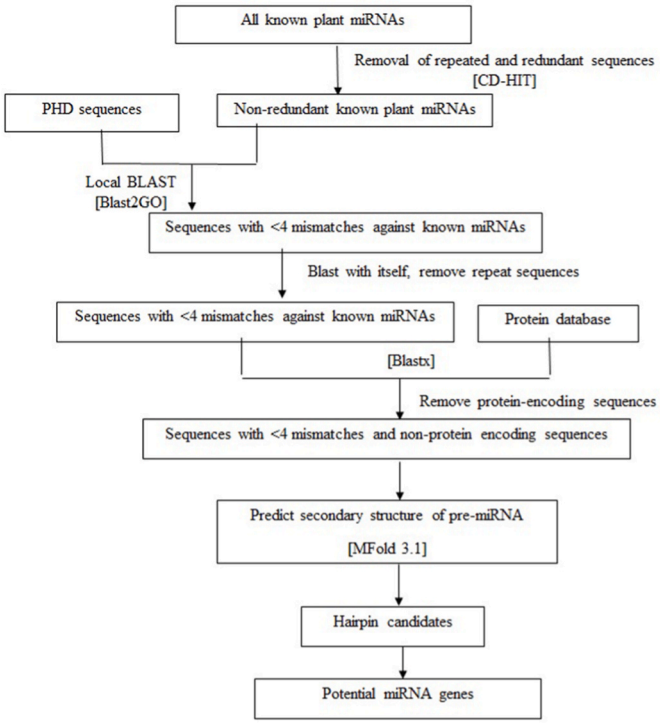

Fig. 1. Workflow of the identification and characterization of potential miRNAs and their target PHD finger genes in *Oryza sativa* Indica Group.

2.4. Plant material, growth condition and stress treatment

Seeds of *Oryza sativa* Indica variety Pusa Basmati 1 were surface sterilized with 70% ethanol for 1 min followed with 0.1% Mercuric chloride ($HgCl_2$) for 5 min and then 0.2% Bavastin for 10 min. All seeds were placed in the dark for 2 days then allowed to germinate for 15 days under control condition maintained at 26 ± 2 °C with 16/8 h light/dark photoperiods cycle. For salinity stress treatment, 15 day old seedlings were treated with 200 mM NaCl for 24 h using hydroponic system, whereas seedlings maintained in distilled H_2O were used as control. After 24 h of treatment, seedlings were harvested and immediately frozen into liquid nitrogen and stored at −80 °C.

2.5. RNA extraction and qRT-PCR analysis

Total RNA was isolated from 200 mM NaCl treated as well as untreated seedlings and was used as a template for the cDNA synthesis following the manufacturer's protocol (Thermo Scientific, EU). Gene

specific primers for all 44 *OsPHD* genes were designed manually using exon-exon junction region (Supplementary Table 1) to amplify 110-243bp (PCR amplicons) and their specificity were examined through Primer Blast of NCBI. The rice actin 1 gene was used as an internal control to normalize the gene expression level. The qRT-PCR was performed on an ABI 7900HT real time system (Applied Biosystems). The total reaction volume was 10 μl containing 5 μl of 2X KAPA SYBR FAST qPCR Master Mix Universal, 200 nM gene specific primers and 0.5 μl of cDNA in a total volume of 10 μl. The thermal cycle reaction conditions were 95 °C for 3min, followed by 40 cycles of 95 °C at 10 s and then 57 °C for 30 s. A melting curve was generated at the end of 40 cycles for analyzing the specificity of each gene. The experiment was conducted with 3 independent biological replicates and technical triplicates for each sample. The relative gene expression of individual gene was calculated via 2ˆ-ΔΔCT method [42].

2.6. Protein-protein interactions network analysis

For the construction of protein interaction network (PIN) associated with OsPHDs i.e., BGIOSGA001450 (OsPHD2), BGIOSGA004932 (OsPHD6), BGIOSGA009559 (OsPHD11), BGIOSGA009918 (OsPHD12) and BGIOSGA026108 (OsPHD35), the Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING v11) available at (http://string-db.org) was used [43]. The prediction methods selected for interaction analysis included data derived from high-throughput lab experiments, gene fusion, co-occurrence, co-expression, database and text-mining with default medium level of confidence (score $\geq$ 0.40).

2.7. Statistical analysis

All the experimental data are means of triplicates and represented as mean ± standard deviation (SD). The significance was tested using SPSS (Statistical Package for the Social Sciences) software (version 21 for Windows; IBM Ltd., Japan) for calculating Student's t-test at significance level $p \leq 0.05$.

3. Result

3.1. Identification and phylogenetic analysis of PHD family genes in rice

In the present investigation, we have identified 44 putative PHD family genes in the Indica rice genome. Domain analysis using Pfam database showed the presence of PHD-finger domain (PF00628) in all the identified 44 putative PHD proteins and were named OsPHD1 to OsPHD44 on the basis of their chromosomal location (Table 1). The length of OsPHD proteins was between 175 and 2275 amino acid. The

Table 1
List of the PHD family proteins from Indica rice and their chromosome localization.

S. No.	Gene Name	Gene ID	Chromosome	Location	CDS (bp)	Protein size (aa)	UniProt ID
1	*OsPHD1*	BGIOSGA000361	1	42190672–42195433	819	272	A2WXR5
2	*OsPHD2*	BGIOSGA001450	1	22715796–22731195	3681	1226	B8A9Z8
3	*OsPHD3*	BGIOSGA002118	1	6990785–6992,874	1218	405	B8AAL1
4	*OsPHD4*	BGIOSGA002243	1	4881590–4883,528	1821	606	A2WLG2
5	*OsPHD5*	BGIOSGA004914	1	41684692–41687747	2151	716	A2WXJ6
6	*OsPHD6*	BGIOSGA004932	1	41975605–41976132	528	175	A2WXN6
7	*OsPHD7*	BGIOSGA005241	1	46620372–46624865	1014	337	B8A9M3
8	*OsPHD8*	BGIOSGA007416	2	1286895–1288,511	1086	361	A2X0A9
9	*OsPHD9*	BGIOSGA008446	2	23087773–23090676	804	267	B8ADZ3
10	*OsPHD10*	BGIOSGA008941	2	31792835–31795557	2064	687	A2X907
11	*OsPHD11*	BGIOSGA009559	3	38692918–38696046	738	245	[a]
12	*OsPHD12*	BGIOSGA009918	3	33222181–33225186	2094	697	A2XLE3
13	*OsPHD13*	BGIOSGA011620	3	481,087–488,147	2805	934	B8AL16
14	*OsPHD14*	BGIOSGA012447	3	11725122–11728330	2379	792	A2XFQ9
15	*OsPHD15*	BGIOSGA012455	3	11834096–11841108	4239	1412	A2XFS2
16	*OsPHD16*	BGIOSGA013552	3	34960990–34968765	3918	1305	B8AJV6
17	*OsPHD17*	BGIOSGA013740	3	37620265–37624741	657	218	B8ALC1
18	*OsPHD18*	BGIOSGA014033	4	34552908–34559128	1554	517	B8AS61
19	*OsPHD19*	BGIOSGA014376	4	29761536–29780655	3741	1246	B8ATX2
20	*OsPHD20*	BGIOSGA016418	4	18846334–18853763	1413	470	B8ATR2
21	*OsPHD21*	BGIOSGA016480	4	20387442–20389,738	771	256	A2XTW9
22	*OsPHD22*	BGIOSGA018756	5	3895575–3899,686	777	258	A2Y0Q2
23	*OsPHD23*	BGIOSGA019093	5	1517426–1530,119	2775	924	B8AXJ3
24	*OsPHD24*	BGIOSGA019887	5	21735524–21739813	777	258	A2Y4R8
25	*OsPHD25*	BGIOSGA020494	6	:32893971-32906441	5100	1699	B8B2Y1
26	*OsPHD26*	BGIOSGA020496	6	32875895–32882513	1698	565	A2YH89
27	*OsPHD27*	BGIOSGA021368	6	12558762–12564437	1614	537	B8B125
28	*OsPHD28*	BGIOSGA022583	6	7754235–7763,674	2493	830	B8B494
29	*OsPHD29*	BGIOSGA022703	6	10978693–10988,365	3672	1223	B8B0L9
30	*OsPHD30*	BGIOSGA023675	7	27471873–27474528	1368	455	B8B6A0
31	*OsPHD31*	BGIOSGA023767	7	25898224–25906086	5091	1696	A2YPL6
32	*OsPHD32*	BGIOSGA024271	7	16826681–16838749	6828	2275	B8B6D8
33	*OsPHD33*	BGIOSGA024567	7	7483687–7486,750	735	244	B8B8I3
34	*OsPHD34*	BGIOSGA025290	7	4497651–4505,008	657	218	B8B7X1
35	*OsPHD35*	BGIOSGA026108	7	23085061–23088910	834	277	B8B8C5
36	*OsPHD36*	BGIOSGA027795	8	301,849–304955	1692	563	B8BA67
37	*OsPHD37*	BGIOSGA028692	8	21592433–21595318	651	216	B8BAV1
38	*OsPHD38*	BGIOSGA030686	9	11700867–11703782	651	216	B8BF25
39	*OsPHD39*	BGIOSGA030698	9	12147163–12152592	1158	385	B8BF36
40	*OsPHD40*	BGIOSGA034217	11	6793962–6800,967	765	254	B8BJV8
41	*OsPHD41*	BGIOSGA034246	11	6271774–6275,088	2055	684	A2ZCW5
42	*OsPHD42*	BGIOSGA034498	11	1983790–1991,277	5667	1888	B8BJ40
43	*OsPHD43*	BGIOSGA036080	12	16547927–16553285	1878	625	B8BM84
44	*OsPHD44*	BGIOSGA036283	12	1215253–11220922	3918	1305	B8BPD7

[a] Currently not available.

average length of proteins was 698.61 amino acid. Multiple sequence alignment using amino acid sequences showed the presence of PHD domain consensus sequence ranges from 40 to 54 amino acids in length (Fig. 2). Domain analysis using HMMER database showed the presence of other associated domains which include Alfin, BAH, DDT, SET, Jas, PWWP, SAP, AAA-34, DDT, JmjC, C5HC2 zinc finger, jmjN, PWWP, SET, Ring finger, twin BRCT, ARID/BRIGHT DNA binding domain and homeodomain (Fig. 3).

Phylogenetic tree analysis revealed that OsPHD proteins might be divided into ten major groups (Group I to X). Group I forms the largest clade with 9 members followed by group X which has 8 members. These two groups jointly represent 38.63% of total rice PHD proteins. The other groups like group IV-VI, II-III and VII had 5, 4 and 3 members respectively. Rest three groups V, VIII and IX remained the smallest ones with only 2 member each (Fig. 4).

3.2. Identification of miRNAs and their PHD specific target genes in rice

For identification of potential rice miRNA, a total of 10,898 plant miRNAs were retrieved from PMRD (Plant micro RNA database). With identity value 90, CD-HIT-v4.5.4 was used to remove the redundancy in miRNA sequences. After removing redundant sequences, a set of 5025 miRNA sequences (reference set of miRNA sequences) were analysed for sequence similarity (Local BLAST by using Blast2GO-v5.2) with the *OsPHD* genes assembly (Fig. 1). BLASTx analysis (E-value ≤1e-10) showed that out of 8 miRNA identified, sequences of only 6 miRNA i.e., ath-miRf10010-akr, ath-miRf10110-akr, gma-miR4993, osa-miR1857–3p, osa-miRf10863-akr and osa-miRf11806-akr were found to be non-coding, while sequences of 2 miRNA i.e., osa-miRf11501-akr and ptc-miRf12266-akr were coding for protein hence are removed. Further, in order to identify miRNA-targeted *OsPHD* genes, local BLAST was performed using Blast2GO. The potential PHD specific targets of ath-miRf10110-akr and gma-miR4993 were BGIOSGA026108 (OsPHD35) and BGIOSGA002243 (OsPHD4), respectively. However, miRNA osa-miR1857–3p and osa-miRf11806-akr had common target gene i.e., BGIOSGA009559 (OsPHD11), whereas miRNA ath-miRf10010-akr and osa-miRf10863-akr showed BGIOSGA001450 (OsPHD2) as their target gene (Table 2).

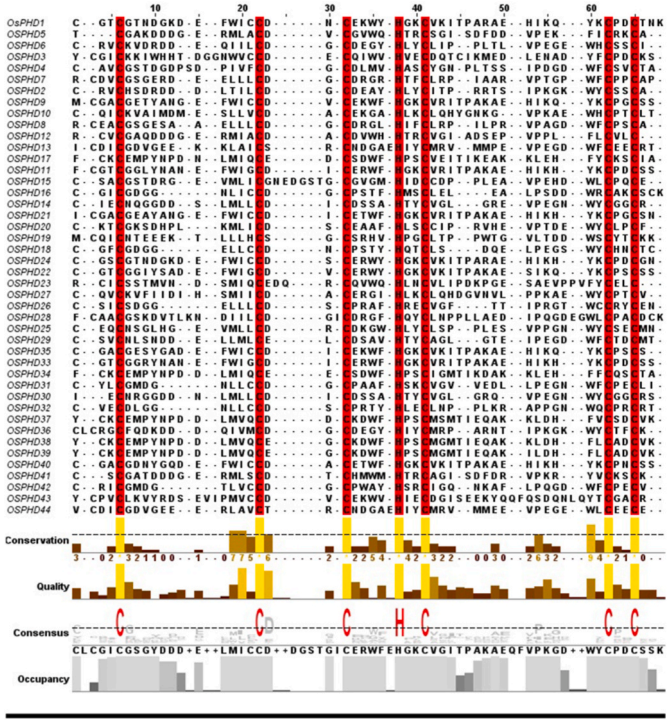

Fig. 2. Multiple sequence alignment of PHD finger protein of Indica rice showing characteristic domain feature in red color boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

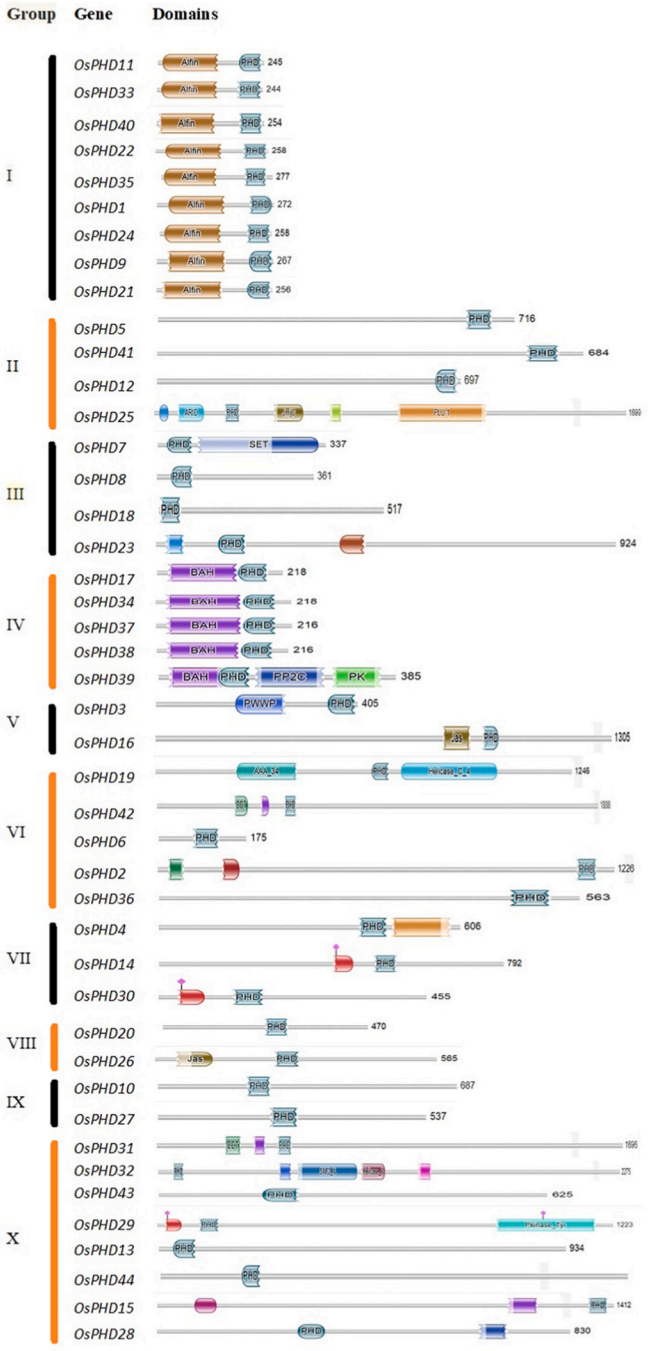

Fig. 3. Domain architecture of rice PHD finger protein and their distribution within different groups.

3.3. Prediction of the secondary structure of potential miRNAs

The six non-coding miRNA sequences i.e., ath-miRf10010-akr, ath-miRf10110-akr, gma-miR4993, osa-miR1857–3p, osa-miRf10863-akr and osa-miRf11806-akr, were further used for secondary structure analysis including hairpin stem-loop structure using MFOLD version 3.1 (Fig. 5A–F). The putative miRNAs obtained varied in their lengths ranging from 21 to 26 nucleotides. The negative MFE (-ΔG) of the

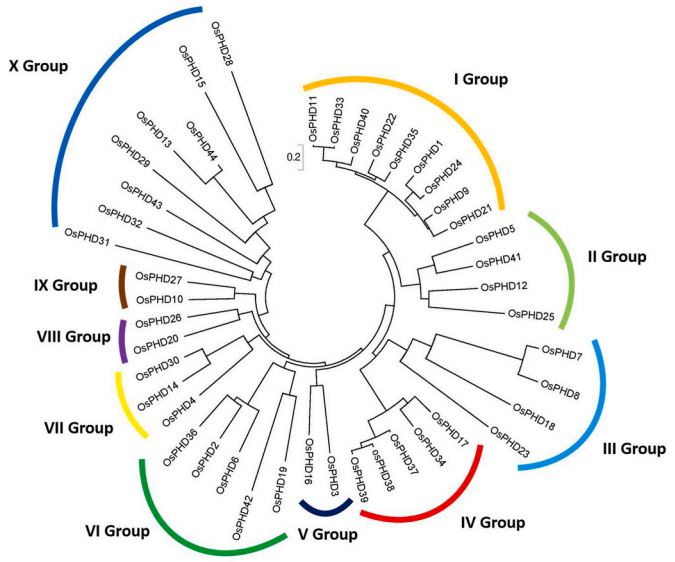

Fig. 4. Phylogenetic tree constructed using NJ method by MEGA X program. Amino acid sequences of all the 44 OsPHD finger proteins were used for tree construction.

Table 2
Identified potential miRNA and its target PHD finger specific genes in Indica rice.

S. No	miRNA	PHD gene	GO Term	Functions
1.	ath-miRf10010-akr	BGIOSGA001450 (*OsPHD2*)	GO:0046,872, GO:0003677	Metal ion binding, DNA binding
2.	osa-miRf10863-akr			
3.	osa-miRf11806-akr	BGIOSGA009559 (*OsPHD11*)	GO:0009058, GO:0006139	Biosynthetic process, Nucleic acid metabolic process
4.	osa-miR1857–3p			
5.	gma-miR4993	BGIOSGA002243 (*OsPHD4*)	GO:0046,872	Metal ion binding
6.	ath-miRf10110-akr	BGIOSGA026108 (*OsPHD35*)	GO:0006355, GO:0006325	Regulation of transcription, chromatin organization

miRNA precursors were also calculated to study the stability of the hairpin stem-loop structure (Table 3). In comparison to the length of miRNAs, the length of putative precursor miRNAs of rice also varied ranging from 70 to 130 nucleotides. The stability of the secondary hairpin structure of pre-miRNA was determined by MFE (-ΔG). The distribution of G, C, A, and U nucleotides in the pre-miRNA were found to be different, where it ranged from 14.29 to 33.85% for A, 12.75–34.13% for U, 17.69–35.71% for G and 17.46–36.27% for C, respectively (Table 4). In the present study, out of the six miRNAs, gma-miR4993 did not fulfil two criteria as it has >6 mismatches in miRNA/miRNA* duplex and there are loops or breaks between miRNA/miRNA* duplex, hence was not considered for further study (Fig. 5C) (see Fig. 6.

3.4. Quantitative real-time PCR analysis of OsPHDs expression in response to salt stress

In order to study the potential response of all *OsPHD* genes under salinity stress we further conducted quantitative real-time PCR. Out of 44 genes 8 genes were differentially expressed (Log2 fold change > 1)

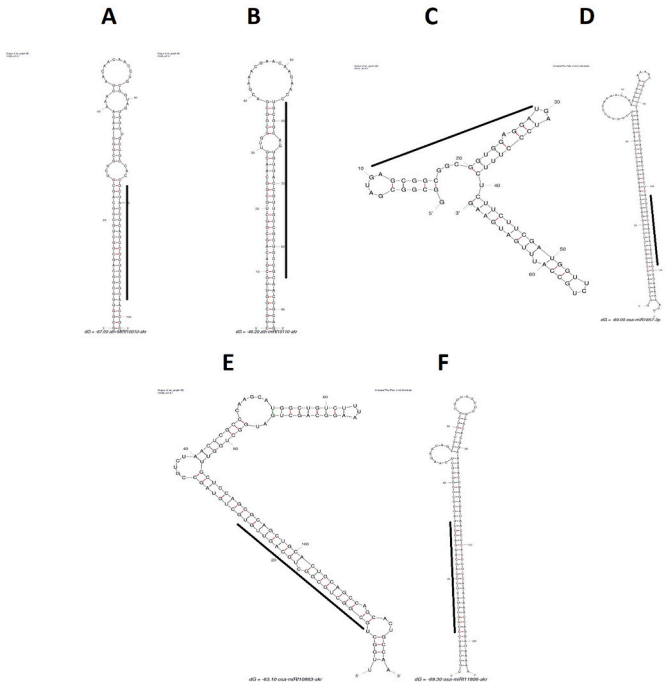

Fig. 5. A-F. Mature and precursor sequences and the predicted stem-loop structures of identified miRNAs in *Oryza sativa* Indica Group- (A) ath-miRf10010-akr, (B) ath-miRf10110-akr, (C) gma-miR4993, (D) osa-miR1857–3p, (E) osa-miRf10863-akr and (F) osa-miRf11806-akr. The mature miRNAs are indicated with bold black line.

under salt stress (Fig. 6). Two genes *OsPHD6* and *OsPHD12* were significantly up-regulated (>2 fold) from group I and VI, respectively. Other six genes i.e., *OsPHD* 5, 7, 8, 15, 19 and 43 corresponding to group II, III, VI and X showed the expression level between 1 and 2 fold change. The expression of remaining 36 *OsPHD* genes were slightly low (<1 fold change) under salinity stress (see Fig. 7).

3.5. Protein-protein interactions network analysis

Using the STRING database, protein-protein interaction of OsPHD6 protein was generated (Fig. 7A). The protein sequence of OsPHD6 showed similarity to uncharacterized PHD finger protein of *Oryza sativa* (OS01T0883400-00) with 373.2 bit score and 9e-104 E-value. OsPHD6 protein showed interaction with other proteins at confidence scores varied from 0.495 to 0.407 (Table 5A). Predicted functional partners of OsPHD6 includes Acetyltransferase (Os03g0747600), G-box-binding factor 1(OsJ_08237), EBS like protein (OsJ_27,353), small ubiquitin-like modifier (SUMO) E3 ligase (SIZ1 and SIZ2), PERSISTENT TAPETAL CELL 1 (Os09t0449000), Putative collagen protein (OS03T0716200) and uncharacterized protein (OS01T0367400). Among the parameters that describe the topology of the network, 11 nodes with a total of 20 edges, a 3.64 grade per node and a clustering coefficient of 0.851 were found. The totality of the network proteins is predicted computationally from its nucleic acid sequence and has not been demonstrated experimentally. Functional enhancement of the network was performed considering the information from the KEGG, PFAM, and INTERPRO databases, from which data on the biological routes and the predominant domains in the interacting proteins were collected. Two proteins i.e., SIZ1 and SIZ2, of the PPI network showed molecular function of SUMO ligase activity and involved in Ubi conjugation pathway.

Protein-protein interaction of OsPHD12 protein was generated using STRING database. The protein OsPHD12 sequence showed similarity to PHD-finger family protein of *Oryza sativa* (OS03T0716200-01). PPI consisted of 11 nodes and 14 edges with 2.55 average node degree and a

Table 3
Determination of minimal free folding energy (MFE) of the identified potential miRNA from Indica rice.

miRNAs	Mature miRNA sequence	Strand	Locus	Length of mature miRNA	Nucleotide mismatch	Length of precursor miRNA	(G + C)%	MFE (ΔG)	AMFE	MFEI
ath-miRf10010-akr	GCUACCGCAGCCGCUGCCGCA	+	3′	21	1	102	67.6	73.40	71.96	1.06
ath-miRf10110-akr	UGUUACCGUUGCCGCUGCCGC	+	3′	21	1	93	65.5	52.20	56.13	0.86
gma-miR4993	GAGCGGCGGCGGUGGAGGAUG	+	5′	21	1	70	57.1	18.6	26.57	0.47
osa-miR1857	UCAUGCUCCAAGAAAACCAGG	+	3′	21	1	130	35.3	75.30	57.92	1.64
osa-miRf10863-akr	GCGGCUGCGGCUGCAGUUGUGC	+	5′	22	1	120	60.8	69.50	57.92	0.95
osa-miRf11806-akr	CCUGGUUUUCUUGGAGCAUGAGGUU	+	5′	25	1	126	35.7	75	59.52	1.67

Table 4
The distribution of G, C, A, and U in the identified pre-miRNAs of Indica rice.

S.No.	miRNA	Mature miRNA sequence	miRNA Family	A%	U%	C%	G%	A/U ratio	G/C ratio	(A + U) %
1	ath-miRf10010-akr	GCUACCGCAGCCGCUGCCGCA	mir-935	19.61	12.75	36.27	31.37	1.54	0.86	32.35
2	ath-miRf10110-akr	UGUUACCGUUGCCGCUGCCGC	mir-935	20.43	13.98	32.26	33.33	1.46	1.03	34.41
3	gma-miR4993	GAGCGGCGGCGGUGGAGGAUG	miR4993	14.29	28.57	21.43	35.71	0.5	1.67	42.86
4	osa-miR1857	UCAUGCUCCAAGAAAACCAGG	miR1857	33.85	30.77	17.69	17.69	1.1	1	64.62
5	osa-miRf10863-akr	GCGGCUGCGGCUGCAGUUGUGC	miR8620	15.83	23.33	30	30.83	0.68	1.03	39.17
6	osa-miRf11806-akr	CCUGGUUUUCUUGGAGCAUGAGGUU	miR1857	30.16	34.13	17.46	18.25	0.88	1.05	64.29

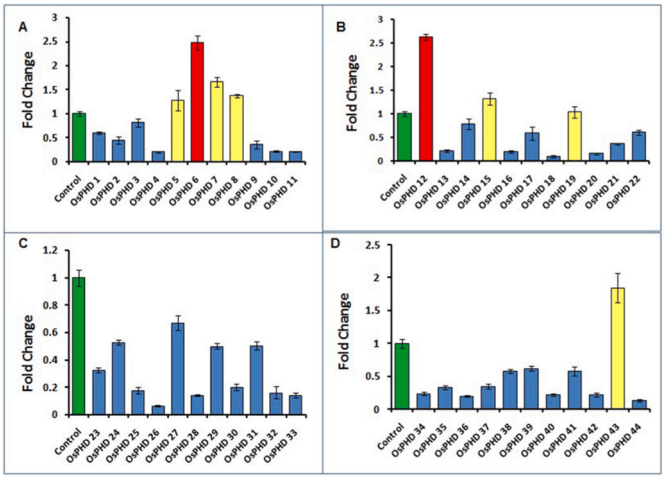

Fig. 6. A-D. Expression pattern of 44 OsPHD genes under salinity stress. The expression fold change analysis were examined by qRT-PCR and normalized by the expression of rice actin 1. The Y-axis represents fold change in gene expression. Bar represent standard error of the mean of three biological replicate.

clustering coefficient of 0.841 (Fig. 7B). OsPHD12 protein showed interaction with other proteins at confidence scores varied from 0.870 to 0.647 (Table 5B). Predicted functional partners of OsPHD12 includes Putative uncharacterized protein (OS05T0126300), Cytokinin dehydrogenase 2 (CKX2), Cytokinin dehydrogenase 4 (CKX4), E3 SUMO-protein ligase (SIZ1), Os07g0477500 protein, Os02g0689800 protein, Os07g0663500 protein, Probable monogalactosyldiacylglycerol synthase 3 (MGD3), Os05g0122400 protein and Os07g0405100 protein. Functional enhancement of the network proteins showed the involvement of two proteins (CKX2 and CKX4) in the Zeatin biosynthesis pathway. PPI network proteins were shown to have different biological process such as cytokinin metabolic process, organonitrogen compound metabolic process, cell communication and oxidation-reduction process. At molecular level, these proteins have cytokinin dehydrogenase activity (GO:0019,139), ion binding (GO:0043,167), oxidoreductase activity (GO:0016,614) and flavin adenine dinucleotide binding (GO:0050,660).

Similarly, PPI of OsPHD2, 11 and 35 proteins were also generated using STRING database. The sequence of these three protein OsPHD2, 11 and 35 showed similarity to *Oryza sativa* PHD-finger protein OS01T0547200-01, OsJ_13,123 and OsJ_25,070 (Fig. 7C–E). Interaction studies showed BGIOSGA001450 (OsPHD2) to interact with E3 SUMO-protein ligase (SIZ2), PERSISTENT TAPETAL CELL2 (PTC2) and Putative zinc-finger motif protein (OsJ_29,217). BGIOSGA009559 (OsPHD11) interacts with WD-repeat (WDR) proteins (OsJ_24,948), Retinoblastoma-binding protein (OsJ_09856) and Decapping protein 2-like (OS02T0805900-01). BGIOSGA026108 (OsPHD35) shown interaction with Auxin response factors (ARFs), SET domain containing protein (OS12T0613200-02) and YDG/SRA domain containing protein (OS11T0602200-01) (Table 5C).(Table 5D) (Table 5E)

4. Discussion

PHD finger transcription factor family have involved in numerous biochemical and physiochemical processes like transcription, chromatin structure regulation and few reports on involvement under abiotic stress response like drought, cold, heavy metals, ABA etc in different plant species [12,21,44–46]. It is also involved in fruit development and lignin synthesis in Chinese pear [47]. However, in rice no report has been published yet on the role of PHD finger proteins under salt stress. Hence, in the present study comprehensive genome wide analysis of PHD finger genes has been performed in Indica rice and studied their expression under salinity stress.

A total of 44 members of PHD finger family have been identified and were divided into 10 groups on the basis of phylogenetic tree analysis. From previous studies, it has been evident that the PHD finger proteins are evolutionary divergent [5,18,19]. It has been reported that PHD finger is a common structural motif present in all eukaryotic organisms and it shows characteristic typical conserved motif i.e., Cys4-His-Cys3 [48]. The PHD finger domain has been explained in *Daucus carota* L. (carrot) as C-X (1–2)-C-X (8–14)-C-X (2–8)-C-X (4–6)-H-X2-C-X

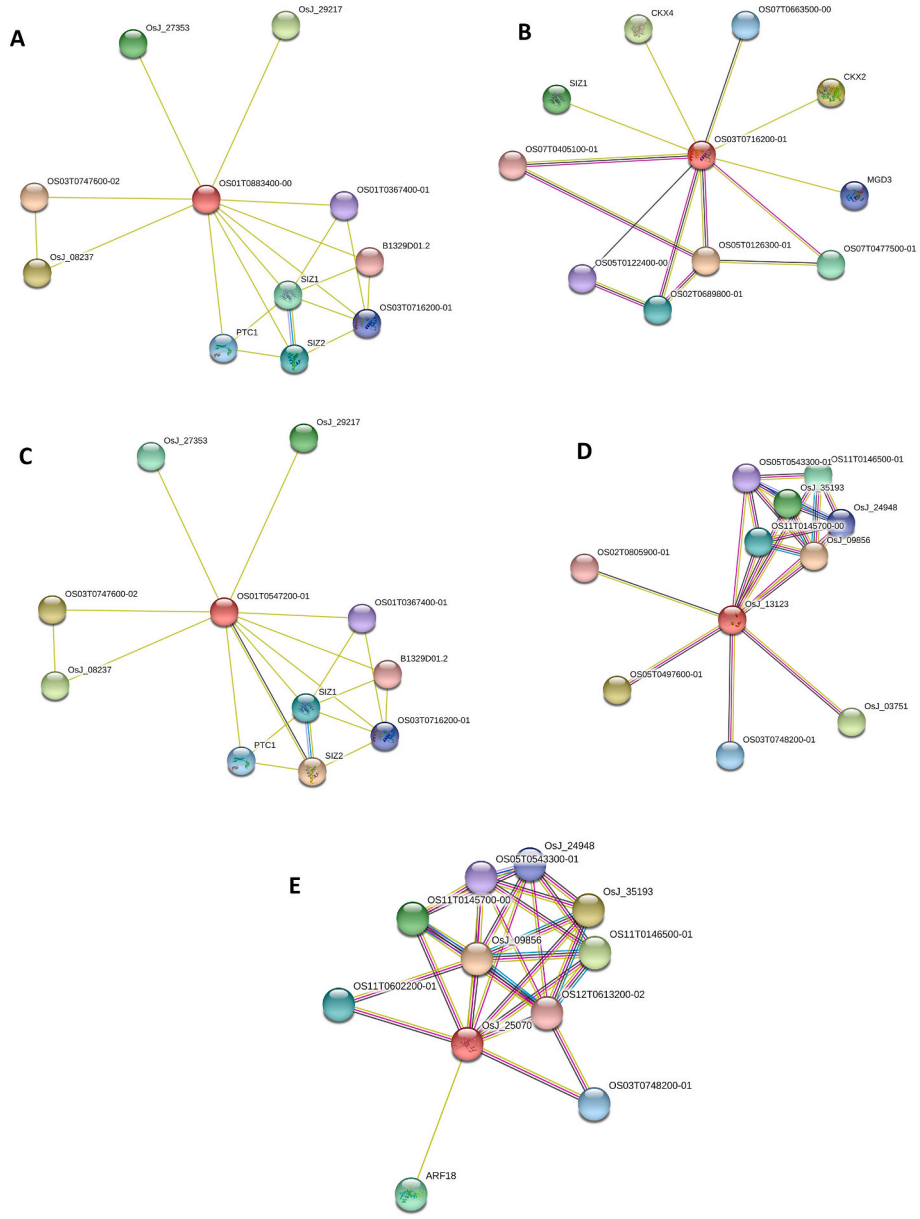

Fig. 7. A-E. Protein-protein interaction network of OsPHDs generated using STRING. Protein interactors as nodes and interactions as edges. Coloured lines between the proteins indicate the various types of interaction evidence. (A) OsPHD6 (B) OsPHD12 (C) OsPHD2 (D) OsPHD11 and (E) OsPHD35.

Table 5A
List of predicted OsPHD6 interacting proteins according to STRING.

Protein ID	Predicted functional	Confidence Score
OS03T0747600-02	Os03g0747600 protein (765 aa)	0.495
OsJ_08237	Os02g0728001 protein (169 aa)	0.495
OsJ_29,217	Os09g0386500 protein; Putative zinc-finger motif (216 aa)	0.457
OsJ_27,353	Os08g0421900 protein; Receptor like protein (216 aa)	0.457
SIZ1	E3 SUMO-protein ligase SIZ1 (875 aa)	0.45
SIZ2	E3 SUMO-protein ligase SIZ2 (813 aa)	0.45
PTC1	PHD finger protein PERSISTENT TAPETAL CELL 1 (109 aa)	0.407
OS03T0716200-01	PHD-finger family protein, expressed; Putative collagen (697 aa)	0.407
OS01T0367400-01	Os01g0367400 protein; Putative uncharacterized protein (288 aa)	0.407

(11–34)-C-X2-C and in *Populus* as C-X (1–2)-C-X (8–28)-C-X (2–8)-C-X (4–6)–H-X2-C-X (12–18)-C-X2-C [18,19]. However, in this study it showed as C-X (1–2)-C-X (8–14)-C-X (2–8)-C-X (4–5)-H-X2-C-X (12–20)-C-X2-C (X = any amino acid). This suggests that during evolution of different plant species the PHD finger proteins got significantly changed. The further analysis of PHD finger protein reveals that the length of PHD finger genes and their encoded proteins varied greatly from each other. In this study, we found that the length of PHD protein sequence varied from 175 (OsPHD6) to 2275 aa (OsPHD32); similar finding has also been observed with Japonica rice variety where it varied from 175 to 2192 aa and in maize it was between 72 and 2379 aa [5,21].

During the evolution of PHD finger genes few characteristic features were relatively conserved. For example, the presence of some common domains within particular groups, like the Alfin domain was exclusively present on group I and BAH on group IV. Furthermore, their corresponding size (amino acid) within the groups was also similar except for OsPHD39 from group IV. In another study, BAH was found only in group X in maize, and in Japonica rice group A and F were dominated by Alfin

Table 5B
List of predicted OsPHD12 interacting proteins according to STRING.

Protein ID	Predicted functional	Confidence Score
OS05T0126300-01	Putative uncharacterized protein (418 aa)	0.87
CKX2	Cytokinin dehydrogenase 2 (565 aa)	0.84
CKX4	Cytokinin dehydrogenase 4 (529 aa)	0.775
SIZ1	E3 SUMO-protein ligase (875 aa)	0.744
OS07T0477500-01	Os07g0477500 protein (794 aa)	0.718
OS02T0689800-01	Os02g0689800 protein (1066 aa)	0.71
OS07T0663500-00	Os07g0663500 protein (316 aa)	0.694
MGD3	Probable monogalactosyldiacylglycerol synthase 3 (406 aa)	0.691
OS05T0122400-00	Os05g0122400 protein (1334 aa)	0.679
OS07T0405100-01	Os07g0405100 protein (555 aa)	0.647

Table 5C
List of predicted OsPHD2 interacting proteins according to STRING.

Protein ID	**Predicted functional**	**Confidence Score**
SIZ2	E3 SUMO-protein ligase (813 aa)	0.498
OS03T0747600-02	Os03g0747600 protein (765 aa)	0.495
OsJ_08237	Os02g0728001 protein (169 aa)	0.495
OsJ_29,217	Putative zinc-finger motif protein (216 aa)	0.457
OsJ_27,353	Receptor like protein (216 aa)	0.457
SIZ1	E3 SUMO-protein ligase SIZ1 (875 aa)	0.45
PTC1	PHD finger protein PERSISTENT TAPETAL CELL 1 (109 aa)	0.407
OS03T0716200-01	Putative collagen (697 aa)	0.407
OS01T0367400-01	Putative uncharacterized protein (288 aa)	0.407
B1329D01.2	Putative uncharacterized protein (340 aa)	0.407

Table 5D
List of predicted OsPHD11 interacting proteins according to STRING.

Protein ID	**Predicted functional**	**Confidence Score**
OsJ_09856	Retinoblastoma-binding protein (570 aa)	0.823
OS05T0497600-01	Putative zinc finger protein (488 aa)	0.707
OsJ_03751	Putative ring finger protein 1 (489 aa)	0.707
OsJ_35,193	SPRY domain containing protein (414 aa)	0.701
OS11T0146500-01	SPRY domain containing protein (420 aa)	0.701
OS11T0145700-00	Os11g0145500 protein (351 aa)	0.701
OS03T0748200-01	Os03g0748200 protein (253 aa)	0.64
OsJ_24,948	Putative WD repeat protein (322 aa)	0.623
OS05T0543300-01	Putative uncharacterized protein (320 aa)	0.623
OS02T0805900-01	Decapping protein 2-like (323 aa)	0.609

Table 5E
List of predicted OsPHD35 interacting proteins according to STRING.

Protein ID	**Predicted functional**	**Confidence Score**
OsJ_09856	Putative retinoblastoma-binding protein (570 aa)	0.823
OsJ_35,193	SPRY domain containing protein (414 aa)	0.701
OS11T0146500-01	SPRY domain containing protein (420 aa)	0.701
OS11T0145700-00	Os11g0145500 protein (351 aa)	0.701
ARF18	Auxin response factor 18 (700 aa)	0.662
OS11T0602200-01	YDG/SRA domain containing protein (813 aa)	0.651
OS03T0748200-01	Os03g0748200 protein (253 aa)	0.642
OsJ_24,948	Putative WD repeat protein (322 aa)	0.623
OS05T0543300-01	Putative uncharacterized protein (320 aa)	0.623
OS12T0613200-02	SET domain containing protein (1212 aa)	0.607

and BAH respectively [5,21]. Also, DDT and BAH were the most common additional domain found within plant PHD-finger proteins. The DDT domain is a DNA binding domain in different transcription and chromosome remodeling factors [49,50], however, the BAH domain acts as a protein-protein interaction module specialized in gene silencing and plays an important role by linking DNA methylation, replication and transcriptional regulation.

Several studies have shown the involvement of rice miRNAs under various abiotic stress like drought [51–53], salinity [54–56], high temperature [57,58], low temperature [59], heavy metal [60–62], etc. In the present study, we identified miRNAs and their PHD-finger genes specific targets in *Oryza sativa* Indica using bioinformatics approaches. Using the Plant micro RNA Database, we obtained five newly identified putative miRNAs targeting three *OsPHD* genes i.e., *OsPHD2, OsPHD35 and OsPHD11*. Gene expression is tightly regulated by miRNAs at the post-transcriptional level by translational repression or target degradation and gene silencing [63–65]. Therefore, to study the expression pattern of all the 44 *OsPHD* genes under salinity stress, we performed qRT-PCR. We found that out of 44 genes, 8 genes were differentially expressed under salinity stress after 24 hours of treatment i.e., *OsPHD* 5, 6, 7, 8, 12, 15, 19 and 43. Among these 8 genes, two i.e., *OsPHD* 6 and 12, were significantly up-regulated in response to salinity stress treatment.

In the present study, the expression of *OsPHD* 6 and 12 were significantly up-regulated under salinity stress treatment. Protein-protein interaction studies using STRING database showed several functional partners of OsPHD6 and OsPHD12. Interacting partners of OsPHD6 such as Acetyltransferase, G-box-binding factor 1, SUMO E3 ligase (SIZ1 and SIZ2), and Persistent Tapetal cell 1 are reported to provide abiotic stress tolerance in plants. Acetyltransferase is involved in transcriptional regulation of nitrogen-associated metabolism and growth [66]. In *Arabidopsis*, it has been reported to be involved in the synthesis of secondary cell wall [67]. Another partner, G-box-binding factor 1 is reported to be involved in transcriptional regulation of environmentally inducible genes in plants [68]. In maize, GBF 1 is known to regulate maize Adh1 gene under hypoxia condition [69]. Many self-fertilising crops are particularly sensitive to abiotic stress at the reproductive stage. In rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.), for example, abiotic stress during meiosis and the young microspore stage indicates that the tapetum is highly vulnerable and that the developmental program appears to be compromised. One of the partners of OsPHD6 such as Persistent tapetal cell 1 reported to control the development and degeneration of tapetum as well as pollen development in rice [70]. In plants, the post-translational addition of SUMO is an essential protein modification that provides protection against numerous environmental challenges.

In the present study, we have identified SUMO E3 ligase (SIZ1 and SIZ2) as a partner of both the proteins OsPHD6 and OsPHD12. Role of SUMO E3 Ligase SlSIZ1 in reported to facilitate heat tolerance in several plants [71,72]. Overexpression of the rice gene *OsSIZ1* improved drought-, heat-, and salt-tolerance in *Arabidopsis* simultaneously [73]. Another functional partners of OsPHD12 such as chloroplastic

monogalactosyldiacylglycerol and cytokinin dehydrogenase (CKX2 and CKX4) are shown to provide tolerance to heat and drought in plant [74, 75]. Overexpression of cytokinin dehydrogenase gene in barley has shown to provide greater tolerance to drought stress [75]. Its overexpression has been reported to modify antioxidant defense against heat, drought and their combination in *Nicotiana tabacum* [76].

Expression of BGIOSGA001450 (*OsPHD2)*, BGIOSGA009559 (*OsPHD11)* and BGIOSGA026108 (*OsPHD35)* genes showed low expression (<0.5 fold change) under salinity stress treatment indicating the higher expression level of miRNA ath-miRf10010-akr, osa-miRf10863-akr, osa-miR1857–3p, osa-miRf11806-akr and ath-miRf10110-akr, respectively. The expression pattern of miRNAs and their target genes was reported to be inverse in response to different abiotic stress treatment [77]. Interaction studies showed BGIOSGA001450 (*OsPHD2)* to interact with PERSISTENT TAPETAL CELL2 (PTC2) which is required for normal tapetal programmed cell death and pollen wall patterning [78]. BGIOSGA009559 (*OsPHD11)* interacts with WD-repeat (WDR) proteins which is involved in signal transduction, cytoskeletal dynamics, protein trafficking, nuclear export, and RNA processing, and are especially prevalent in chromatin modification and transcriptional mechanisms [79]. BGIOSGA026108 (OsPHD35) shown interaction with auxin response factors (ARFs) which bind to the auxin response elements and regulates the expression of genes in auxin responses and plant development [80].

5. Conclusion

In the present investigation, we identified 44 putative *OsPHD* finger genes in *Oryza sativa* Indica and five potential miRNA targeting specific *OsPHD* genes. This is the first report of miRNAs namely ath-miRf10010-akr, ath-miRf10110-akr, osa-miR1857–3p, osa-miRf10863-akr and osa-miRf11806-akr, on targeting PHD finger gene in *Oryza sativa* Indica. Expression analysis of 44 *OsPHD* genes demonstrated that two genes *OsPHD* 6 and 12 showed up-regulation under salinity stress. Protein-protein interaction analysis showed several interacting protein partners that are directly or indirectly involved in development and abiotic stress tolerance. Thus, using genetic engineering tools the identified PHD finger genes or miRNAs can be manipulated for increasing the tolerance against abiotic stress and crop yield.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2020.10.002.

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