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Expression and co-location of prohibitin genes in salt-tolerance QTLs in rice (*Oryza sativa*)

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ABSTRACT

Prohibitin proteins are involved in diverse biological functions including cell cycle regulation, mitochondrial activity, apoptosis, hormonal signalling and so on. Prohibitin family genes are conserved across the life taxa in both eukaryotes and prokaryotes. An experiment was conducted at ICAR-National Institute for Plant Biotechnology, New Delhi (2018-19) in which we identified 19 prohibitin genes present on 10 different chromosomes of the 'Nipponbare' reference rice (*Oryza sativa* L.) genome and analysed their possible role in salt tolerance, as they are known to regulate ion channels in plant and animal cells. A protein sequence based neighbour joining phylogentic tree clustered these 19 genes into three groups. Digital gene expression analysis revealed the highest level of expression of the prohibitin genes in anthers and radicle tips. Nine of these prohibitin genes were co-located in the salt-tolerance QTL regions of the rice genome, three of which (LOC_Os01g40560, LOC_Os03g60000 and LOC_Os08g15600) were significantly upregulated in response to salt stress in the tolerant genotype. These prohibitin genes are strong candidates for improving salt-tolerance in rice through marker-assited rice breeding after validation.

Key words: Gene expression analysis, Prohibitin (PHB), Quantitative Trait Loci (QTL)

Prohibitins (*PHB*s) family proteins are highly conserved proteins that contain a band-7 domain or SPFH (stomatin/prohibitin/flotillin/HflK/C) domain or *PHB* domain (Tamura *et al.* 2007).These proteins have been found in diverse taxa from prokaryotes like *Escherichia coli* to eukaryotes like *Homo sapiens.* These proteins are present throughout the plant kingdom ranging from chlorophyta, bryophyta to monocotyledonous and dicotyledonous species. Such a wide prevalence is also associated with diverse function of these proteins as *PHB*s are involved in regulating cell-cycle progression, apoptosis, maintaining crista morphology and different disease phenotypes in animals (Wang *et al.* 1999). In plants, *PHB*s are involved in cell division, cell expansion, growth and development processes like meristem development, senescence, NO signalling, circadian control, biotic and abiotic stresses, ABA signalling, lateral root formation by auxins and so on (Wang *et al.* 2010).

Van Aken *et al.* (2007) showed that over expressed *AtPHB3* or *AtPHB4* plants produce leaves with irregular shape and increased shoot branching. *NbPHB2* gene silencing leads to delay in plant growth, yellowing of leaves and cell death (Ahn *et al.* 2006). Xiang *et al.* (2015) showed that prohibitin family genes, *GmHIR1, GmHIR3* and *GmHIR4* are upregulated by *Phytophthora sojae* infection in soybean. Rice *OsHIR1* over expression in Arabidopsis led to enhanced resistance against *Pseudomonas syringae* pv. Rice (*Oryza sativa* L.) serves as major source of food for more than three billion people (Birla *et al.* 2017) and its productivity is badly affected by salinity stress in coastal as well as inland sodic and saline soils. However, the role of prohibitin in salt-stress response in rice has not yet been explored. Here, we present a genome wide analysis of *PHB* genes in rice and show their co-localization in the salt tolerance QTL regions of the genome. We also analysed the expression of five candidate *PHB* genes in response to salinity stress in rice.

MATERIALS AND METHODS

Plant material: For gene expression analysis a salt tolerant recombinant inbred line RIL41 derived from cross between a salt tolerant variety CSR 27 and salt-sensitive variety MI 48 was used. Seeds were germinated in a seed germinator at 28°C and three days after germination transferred to a hydroponic system in plastic trays at ICAR-National Institute for Plant Biotechnology, New Delhi (2018–19). Fifteen days after germination, 150mM NaCl solution was added to the Yoshida medium to impose salinity stress. Another set was kept as control, where only Yoshida nutrient medium was applied without NaCl. Hydroponics seedlings were grown in a controlled growth room in 28°C. Whole seedlings were collected 24 h after imposing the salt

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stress, snap frozen in liquid nitrogen and kept at -80ºC till RNA extraction.

Identification of Prohibitin (PHB) family genes: Hidden Markov model (HMM) profile of *PHB* superfamily (PF01145) was retrieved from Pfam database (https://pfam.xfam.org). The putative *PHB* protein sequences of maize were obtained from Phytozome database. BLAST search and HMM profile search (HMMER 3.0) were used to identify the *PHB* genes in the rice genome (IRGSP1.0 release 7). HMM profile search was performed taking hmm profile of *PHB* superfamily as query and rice protein sequences retrieved from Rice Genome Annotation Project (http://www.rice.plantbiology.msu.edu) as database. The putative protein sequences thus obtained from HMM search were reused as template and searched with BLASTP within rice protein database to find *PHB* genes with above 50% sequence identity and e-value $\leq 10^{-5}$. Maize *PHB* proteins reported by Wen *et al.* (2013) were also used as query to perform BLAST search against rice protein sequences. The presence of PHB domains in the predicted proteins was further confirmed with Pfam database search, CD database search and InterPro Scan search (https://www.ebi.ac.uk/interpro/interproscan.html). Only single isoform per gene out of several predicted isoforms of genes was used for further study.

Phylogenetic Analysis: The sequences of 19 rice prohibitin genes thus obtained were aligned using CLUSTALW in MEGA6 software (Tamura *et al.* 2013). Neighbor-Joining method was used to construct an unrooted phylogenetic tree with bootstrap reiterations of 1000. Replicates with ≤50% bootstrap value were reused to produce branches corresponding to partitions. Evolutionary distances were calculated using Poisson correction method.

Physiochemical property of the protein: Subcellular localization of rice prohibitin proteins were obtained using CELLO v2.5 (cello.life.nctu.edu.tw; Yu *et al.* 2006) and the isoelectric point was calculated using Protein isoelectric point calculator (www.isoelectric.org). TMHMM server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM) with standard parameter was used to predict the transmembrane helices in *PHB* proteins.

In silico *analysis of relative expression of PHB family genes*: *In silico* analysis of the relative expression of rice prohibitin genes was carried out in 12 different tissues using publicly available transcriptome data by GENEVESTIGATOR software (https://genevestigator.com). Percent expression potential (PEP=average expression of a gene across all the samples in a particular tissue / maximum expression value across samples) was used to denote *PHB* gene expression and was presented as heat map. In total 704 samples of expression in different tissues were taken for analysis by GENEVESTIGATOR.

RNA extraction and qRT-PCR analysis of differential gene expression: Total RNA from leaves was extracted by Trizol method (Logemann *et al.* 1987), cDNA was synthesized using Superscript III (Invitrogen, USA). For qRT-PCR primers were designed using web tool Primer 3.0 (Table 1). 10 µl master mix contained 1x KAPA SYBR®FAST qPCR Master Mix, 1µl (1:10 diluted) of cDNA and 0.4 µM each of forward and reverse primers. A two-step qRT-PCR protocol was carried out with initial denaturation of 95ºC for 3 min, followed by 40 cycles of denaturation and annealing /extension at 95ºC for 3 sec and 60ºC for 45 sec. Expression data were normalized against endogenous control gene ubiquitin (Table 1). Each data point represented average of three biological and three technical replicates. The expression was presented in the form of relative fold change (2^{-ddCT}) which is the relative change in expression of genes under stress conditions as compared to its expression under control condition.

RESULTS AND DISCUSSION

Prohibitin family genes in the rice genome: To identify all the prohibitin family genes in the rice genome, a HMM profile search was performed against rice protein database using PHB domain hmm profile (PF01145) as query. As the HMM profile search might have missed incomplete PHB domain containing proteins, a BLASTP search was also performed taking 16 maize *PHB* proteins and rice *PHB* proteins identified by hmm search as query. Together the HMM profile search and BLASTP search identified 19 non-redundant PHB genes in the rice protein database. After Pfam and SMART database search all these proteins were found to have PHB domain. These 19 protein sequences were taken for further analysis and all of them were found to be single domain protein carrying PHB domain. The phylogenetic tree (Fig 1) grouped 19 *PHB* protein sequences into three clusters. The largest cluster (I) has nine genes distributed on chromosome 1, 5, 6, 8, 9 and 10. All the 3 *PHB* genes of chromosome 8 (LOC_Os08g06200, LOC_Os08g15600

Table 1 List of primers used for differential expression of genes in response to salt stress

Genes	Forward primer	Reverse primer
LOC_Os01g40560	GACCAAGACCAAGGACAATGTG	GTGAGCCTGGATCTGTGACC
LOC_Os03g60000	AATTGAAGCCAAGCAGGGTGA	GGCAGCTTCAATCTGTCGGA
LOC_Os04g38900	CTGGCCCGTTCTCCTAATGT	CAAAGATACACCCCCGGCAG
LOC_Os06g04460	ACGTCTTTGATGTGGTCCGAG	CCTGAAGTACAGCTTTCGCC
LOC_Os07g15880	ACAAGCCTGAGCTTCGGAAA	TTTTAGCTTCACCCTGTGCCC
LOC_Os08g15600	ACGAACGCCTTCTTCTCCAG	ACGAACGCCTTCTTCTCCAG
Ubiquitin	CTCACCTACGTCTACAA	GTCAAGGTGTTCAGTTC

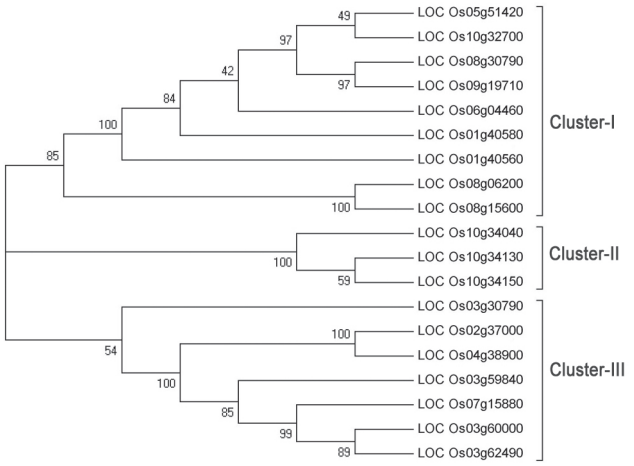

Fig 1 Neighbour-Joining unrooted phylogenetic tree of 19 prohibitin family genes present in the 'Nipponbare' reference rice genome. Percent bootstrap values from 1000 reiterations are shown at the branching points.

and LOC_Os08g30790) were grouped together in cluster-I. The smallest cluster (II) of three genes (LOC_Os10g34040, LOC_Os10g34130 and LOC_Os10g34150) were located very close to each other on the long arm of rice chromosome 10 and seem to have originated by tandem duplication. Cluster III has seven genes distributed on chromosome 2, 3, 4, and 7. All the four PHB genes of chromosome 3 (LOC_Os03g30790, LOC_Os03g59840, LOC_Os03g03600 and LOC_Os03g62490) were grouped together in Cluster III.

Structure and expression of prohibitin genes in different tissues of rice: In order to understand the structure of rice prohibitin genes we analysed the intron-exon organization of the 19 rice prohibitin genes using GSDS server. Number of intron in the rice prohibtin genes varies from 0 (LOC_Os02g37000) to 8 (LOC_Os03g30790, LOC_Os08g06200). Analysis of physiochemical properties revealed that these proteins have diverse properties though they belong to same family. Molecular weight of the rice prohibitin varied from 11.32KDa (LOC_Os08g15600) to

Table 2 Details of 19 prohibitin family genes present in the 'Nipponbare' reference gnome of rice (*Oryza sativa*)

MSU Locus Id.	Chromo. position (Mbp)	pI	Trans- membrane helices	Amino acid residues	Mr (Dalton)	Sub-cellular location	No. of introns	Co-located QTL (Ref.)
LOC_Os01g40560	22.90	5.562	No	288	30404	CT, MT	2	*qSNC1* (Gimhani *et al.* 2016)
LOC_Os01g40580	22.91	5.945	No	312	33396	CH	1	*qSNC1* (Gimhani *et al.* 2016)
LOC_Os02g37000	22.34	5.916	No	283	30251	CH, CT	0	-
LOC_Os03g30790	17.55	6.325	No	375	42669	CT, NL	8	*qSES10* (Alam *et al.* 2010)
LOC_Os03g59840	34.07	6.333	No	552	59191	NL	7	*qSES10* (Alam *et al.* 2010)
LOC_Os03g60000	34.12	5.500	193-215	421	46093	CT	4	*qSES10* (Alam *et al.* 2010)
LOC_Os03g62490	35.38	9.912	126-148	291	32190	CH, MT	4	-
LOC_Os04g38900	23.11	6.310	No	297	31624	CH	1	*qRFW4-1* (Bizimana *et al.* 2017
LOC_Os05g51420	29.48	6.120	No	306	33449	CT, MT	3	-
LOC_Os06g04460	1.90	5.329	No	289	32140	CT	4	*qSES6* (Bimpong *et al.* 2014)
LOC_Os07g15880	9.22	10.065	No	290	31954	MT, CH	4	*qPL7.4* (Hossain *et al.* 2015)
LOC_Os08g06200	3.42	7.151	No	378	41927	MT, NL	8	-
LOC_Os08g15600	9.48	5.912	No	106	11329	CT	1	*qSSISFH8.1* (Pandit *et al.* 2010)
LOC_Os08g30790	19.01	5.034	No	285	31379	CT	4	-
LOC_Os09g19710	11.79	5.023	No	288	31606	CT	4	-
LOC_Os10g32700	17.11	5.702	No	293	32125	CT	3	-
LOC_Os10g34040	18.16	5.273	No	486	52987	CT	1	-
LOC_Os10g34130	18.21	5.314	No	485	52283	CT	2	-
LOC_Os10g34150	18.22	6.131	No	500	54724	CT	2	-

CT-Cytosol, MT-Mitochondria, CH-Chloroplast, NL-Nucleus, - Not located in QTL

59.19KDa (LOC_Os03g59840). Though isoelectric point of most of the rice prohibitin was between 5 to 6 (Table 2), LOC_Os03g62490 and LOC_Os07g15880 were highly basic with isoelectric point near 10. It may be emphasized that these two proteins may have different function than other rice prohibitins. Subcellular localization of rice prohibitins was analysed by Cello web tool. It revealed that proteins coded by 9 of the 19 prohibitin genes (LOC_Os03g60000, LOC_Os06g04460, LOC_Os08g15600, LOC_Os08g30790, LOC_Os09g19710, LOC_Os10g32700, LOC_Os10g34040, O LOC_Os10g34130 and LOC_Os10g34150) were localized in cytosol, three (LOC_Os03g30790, LOC_Os03g59840 and LOC_Os08g06200) were localized in nucleus. Localization of LOC_Os01g40560, LOC_Os03g62490, LOC_Os05g51420, LOC_Os07g15880 and LOC_Os08g06200 in mitochondria indicates their role in energy metabolism. In case of human, it has been reported that prohibitin modulate mitochondrial stress response (Kathiria *et al.* 2012). Though similar function of prohibitin in plant cells is yet to be confirmed, but their localization in mitochondria indicates their putative role in mitochondrial function. Only two of the rice prohibitins (LOC_Os03g60000 and LOC_Os03g62490) were found to have transmembrane domain, as revealed by TMHMM prediction. Transmembrane domain of LOC_Os03g60000and LOC_Os03g62490 lies between 193rd aa to 215th aa and 126th aa to 148th aa respectively. Presence of TMHMM in prohibitin genes may be associated with their role in extracellular signal reception or signal transduction. Prohibitins have already been reported to mediate signalling in animal cells (Ande *et al.* 2017).

Expression of the 19 rice prohibitin genes was analysed in 12 different rice tissues using GENEVESTIGATOR software using 704 samples (Fig 2A). It was found that expression potential of LOC_Os10g34040 and LOC_Os10g34130 was very less across all the tissues (Fig 2A). LOC_Os10g34150 has higher expression potential in anther as compare to other tissues. In root all the genes were moderately expressed except LOC_Os03g59840, LOC_Os03g60000, LOC_Os10g34130 and LOC_Os10g34150. Interestingly, LOC_Os03g59840 - LOC_Os03g60000 and LOC_Os10g34130 - LOC_Os10g34150 are tandemly duplicated PHB gene pairs. All the four *PHB* genes present on chromosome 10 (LOC_Os10g32700, LOC_Os10g34040, LOC_Os10g34130 and LOC_Os10g34150) are poorly expressed in leaf. Five genes were highly expressed in anther (LOC_Os02g37000, LOC_Os03g59840, LOC_Os03g60000, LOC_Os08g06200 and LOC_Os10g34150). All PHB genes are moderately expressed in spikelet except LOC_Os01g40560, LOC_Os10g34040 and LOC_Os10g34150. Expression pattern of PHB genes are almost similar in radicle tip and root elongation zone. LOC_Os01g40560, LOC_Os01g40580, LOC_Os03g59840, LOC_Os03g60000, LOC_Os10g34040, LOC_Os10g34130 and LOC_Os10g34150 are poorly expressed in both radicle tip and root elongation zone. In root maturation zone, all genes are moderately expressed except three tandemly duplicated PHB genes on chromosome 10 (LOC_Os10g34040, LOC_Os10g34130 and LOC_Os10g34150).

Co-location of rice prohibitin genes in salt-tolerance QTLs and their expression under salt stress: The physical positions of *OsPHB* genes were obtained from Phytozome database using biomart data mining tool. The physical location of 19 prohibitin genes was distributed throughout the rice genome, except chromosomes 11 and 12. Each of the chromosomes 2, 4, 5, 6, 7 and 9 carries a single prohibitin gene, chromsome 1 carries two genes, chromosome 8 carries three genes, whereas chromosomes 3 and 10 carry four genes each (Table 2). There was tandem duplication of two prohibitin genes each on chromosomes 1 (LOC_Os01g40560, LOC_Os01g40580) and 3 (LOC_Os03g59840, LOC_Os03g60000) and three genes on chromosome 10 (LOC_Os10g34040, LOC_Os10g34130, LOC_Os10g34150). As membrane ion channels play

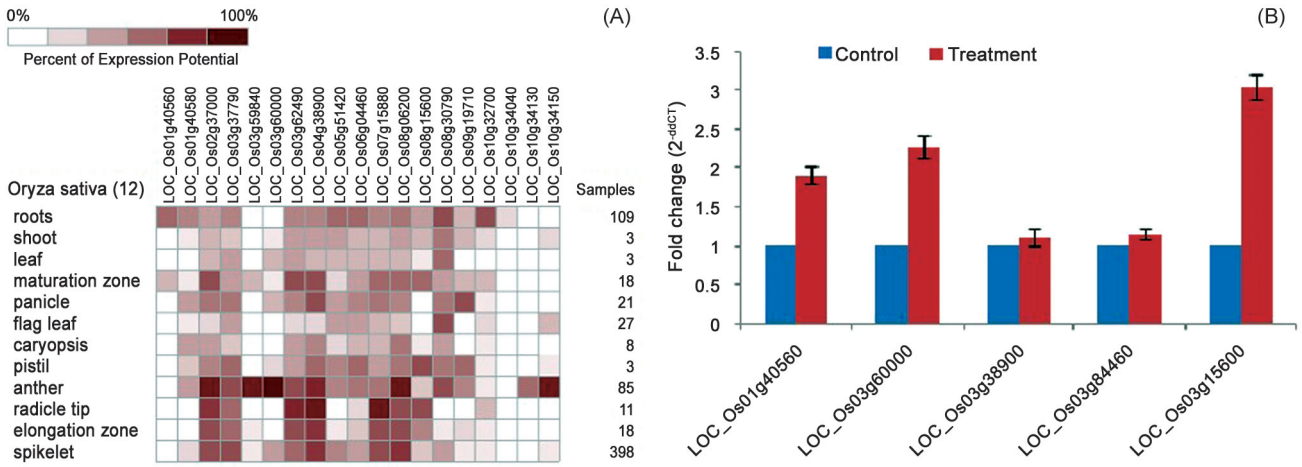

Fig 2A Heatmap of expression profiles of 19 prohibitin (OsPHB) genes in different tissues of rice based on the sequence read abundance in the available RNA sequence data analysed using GENEVESTIGATOR software; 2B Expression of five prohibitin genes co-located in the salt tolerance QTL regions of the rice genome (OsPHB genes) in response to salt stress. Expression of genes was studied using qRT-PCR.

important role in salt-tolerance and prohibitin being regulator of ion channel function we further investigated co-location of the prohibitin genes in salt tolerance QTL regions of the rice genome. We found that nine of the nineteen rice prohibitin genes are co-located in the salt-tolerance QTL regions on six different rice chromosomes mapped in earlier studies (Table 2). LOC_Os01g40560 and LOC_Os01g40580 are co-located in salt tolerance QTL *qSNC1* which contributes to shoot sodium concentration (Gimhani *et al.* 2016). LOC_Os03g59840, LOC_Os03g60000 and LOC_Os03g62490 are co-located in QTL *qSES3* responsible for salt injury score (Alam *et al.* 2012); LOC_Os04g38900 is co-located in QTL *qRFW4-1* responsible for root fresh weight (Bizimana *et al.* 2017), LOC_Os06g04460 is co-located in QTL *qSES6* responsible for trait salt injury score (Bimpong *et al.* 2014); LOC_Os07g15880 is co-located in QTL *qPL7.4* responsible for panicle length (Hossain *et al.* 2015); and LOC_Os08g15600 gene is co-located in reproductive stage salt tolerance QTL *qSSISFH8.1* responsible for salt-sensitivity index for spikelet fertility at high salt stress (Pandit *et al.* 2010).

We analysed the regulation of expression of five genes co-located in five different QTL in a salt-tolerant recombinant inbred line (RIL41) derived from CSR27/MI 48 cross. We have selected one PHB gene from each QTL region. However, LOC_Os07g15880 was not expressed in RIL41.We found that as compared to control plants the expression of LOC_Os01g40560, LOC_Os03g60000 and LOC_Os08g15600 was upregulated in the salt-stressed plants, but expression of LOC_Os04g38900 and LOC_Os06g04460 remain unchanged in the RIL 41 (Fig 2B). These results suggest possible role of prohibitin family genes in responsiveness to salt stress in rice. In mammals, they function as scaffolders and regulators of ion channels, in lipid raft micro-domains (Browman *et al.* 2007).

Prohibitin family genes are conserved across the wide range of life taxa including both eukaryotes and prokaryotes and are reported to play important role in stress tolerance. Here we have reported co-localization of nine PHB genes in salt tolerance related QTL regions of the rice genome and also validated their differential expression in response to salt stress. Upregulation of three *PHB* genes present in the QTL regions may indicate their potential role in salt tolerance in the corresponding genotype. Further validation studies are required to identify their exact physiological role and subsequent utilization in genetic improvement of rice.

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