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Comprehensive expression analysis of rice phospholipase D gene family during abiotic stresses and development

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Phospholipase D is one of the crucial enzymes involved in lipid mediated signaling, triggered during various developmental and physiological processes. Different members of PLD gene family have been known to be induced under different abiotic stresses and during developmental processes in various plant species. In this report, we are presenting a detailed microarray based expression analysis and expression profiles of entire set of PLD genes in rice genome, under three abiotic stresses (salt, cold and drought) and different developmental stages (3-vegetative stages and 11-reproductive stages). Seven and nine PLD genes were identified, which were expressed differentially under abiotic stresses and during reproductive developmental stages, respectively. PLD genes, which were expressed significantly under abiotic stresses exhibited an overlapping expression pattern and were also differentially expressed during developmental stages. Moreover, expression pattern for a set of stress induced genes was validated by real time PCR and it supported the microarray expression data. These findings emphasize the role of PLDs in abiotic stress signaling and development in rice. In addition, expression profiling for duplicated PLD genes revealed a functional divergence between the duplicated genes and signify the role of gene duplication in the evolution of this gene family in rice. This expressional study will provide an important platform in future for the functional characterization of PLDs in crop plants.

Introduction

Various abiotic stresses impose a set of adverse conditions on plants in a given environment, which hamper their growth and development, longevity and productivity. The major abiotic stresses include high salinity, drought and temperature fluctuations i.e., cold and heat. To combat these adverse growth conditions plants have devised some adaptive mechanisms, which include the triggering of a number of signaling networks.[1-4] Lipid mediated signaling has emerged as one of the major signaling pathways and recently well recognized to be triggered in response to various stresses in plants.[5-8] Various environmental cues trigger the hydrolysis of membrane phospholipids leading to the generation of different classes of lipid and lipid-derived signal messengers such as phosphatidic acid (PA), diacylglycerol (DAG), DAG-pyrophosphate (DGPP), lysophospholipids, free fatty acids (FFAs), phosphoinositides, and inositol polyphosphates.[9-11] Phospholipase D (PLD) group of enzymes hold a central place in the catalysis of membrane lipid hydrolysis in plants. PLD cleaves the terminal phosphodiesteric bond of phospholipids to release phosphatidic acid (PtdOH) and water-soluble free head group.[12] PLD represents a major family of phospholipases in plants and comprised of multiple isoforms. Arabidopsis genome encodes 12 PLD members while 17 members have been reported in the genome of crop plant rice.[13,14] In Arabidopsis, PLDs have been grouped into five classes namely; α, β, γ, δ and ζ based on their gene structure, domain organization, sequence similarity and biochemical properties.[13] In rice genome; α, β, δ and ζ PLDs have been identified as orthologs of Arabidopsis PLDs, however PLDs of γ class are not encoded.[14] In addition, two new classes of PLDs have been predicted in rice and designated as PLDκ and PLDφ, comprised of one member each. Strikingly, PLDφ class is structurally different from any other plant PLDs, as it harbours a signal peptide at N-terminal instead of a C2 domain (found in C2-PLDs) or PX/PH domain (found in PX/PH-PLDs) and therefore belongs to a unique structural class of PLDs known as SP-PLD. This PLD was identified in rice genome for the first time[14] and recently also has been reported in grapes and poplar.[15] Different PLD isoforms have been associated with abiotic stress (salt, drought and cold) triggered lipid signaling and associated responses in a number of plant species.[16-21] Similarly, PLDs have also been implicated in the various stages of plant development.[6,22,23] Although, functional role of PLDs have been well dissected in stress and development related processes in various plant species, knowledge about their involvement in similar pathways in crop plant rice has not been investigated adequately.

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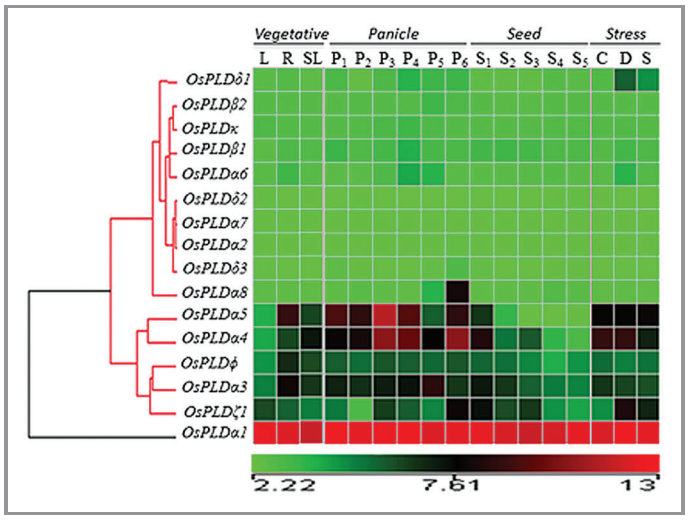

Figure 1. Expression of rice PLD gene family has been represented by a heat map. Developmental stages comprising three vegetative stages (L-leaf, R-root and SL-7-d-old seedling), six stages of panicle [P1 (0–3 cm), P2 (3–5 cm), P3 (5–10 cm), P4 (10–15 cm), P5 (15–22 cm), and P6 (22–30 cm)] and five stages of seed [S1 (0–2 DAP), S2 (3–4 DAP), S3 (4–10 DAP), S4 (11–20 DAP) and S5 (21–29 DAP)]. Clustering of the expression profile was done with log transformed average values taking mature leaf as base line. Three stress conditions are denoted as C, cold stress; D, drought stress; S, salt stress and SL, control, seven day old unstressed seedling. The scale at the bottom of each heat map is given in $\log_2$ intensity value.

To comprehend the underlying functional mechanism of a gene, expression profiling is a very handy tool as it provides clue to its functional relevance. At the same time, this also leads to development of preliminary platform from where the detailed functional role of respective genes can be unearthed by adopting comprehensive molecular, biochemical and genetic tools. With this view in mind, in this report we are providing a detailed genome-wide expression (transcriptomic) analysis of rice PLD gene family using gene chip microarray under abiotic stress conditions (salt, drought and cold) and during various stages of plant development (vegetative and reproductive). We have identified a set of differentially expressing PLD genes under these conditions and stages, and validated the expression profile for a few selected candidates under abiotic stresses using quantitative real time PCR. Moreover, we have marked the duplicated PLD members and generated an expression profile to show their functional relatedness.

Results

Expression profile of rice PLDs under abiotic stresses. Genome-wide expression profile of rice PLDs was generated using Affymetrix rice genome arrays data for 7 day old rice seedling treated for three abiotic stress conditions (salt, cold and drought). In comparison to control (untreated 7 day old seedling), a total of seven PLD members expressed differentially (either up or down-regulated) under three abiotic stress conditions with several fold change in expression and significant p value (**Fig. 1 and Table 1**). Out of the seven genes, six genes; *OsPLDα1*, *OsPLDα5*, *OsPLDα4*, *OsPLDα6*, *OsPLDδ1* and *OsPLDζ1* were found to be significantly upregulated whereas only *OsPLDφ* was down-regulated (gene names are adopted from Li et al. 2007[14]). Analysis for the specific or overlapping expression of genes under stress conditions revealed that *OsPLDα5* was commonly expressed under all three stresses. Similarly, *OsPLDδ1* and *OsPLDζ1* were commonly upregulated under both salt and drought stress

Table 1. Microarray expression data for entire set of rice PLD genes under abiotic stresses

			DROUGHT			SALT			COLD		
Probe Set ID	RGAP Locus	Gene	Fold	Regulation	p value	Fold	Regulation	p value	Fold	Regulation	p value
Os.155.1.S1_a_at	LOC_Os01 g07760	*OsPLDα1*	1.76	U	0.0014	1.45	U	0.0063	1.19	U	0.1379
OsAffx.4272.1.S1_at	LOC_Os05 g07880	*OsPLDα2*	1	D	0.644	1.01	D	0.543	1	U	0.778
Os.9878.1.S1_at	LOC_Os06 g40190	*OsPLDα3*	1.22	D	0.3375	1.39	D	0.1648	1.05	U	0.9337
Os.50455.1.S1_at	LOC_Os06 g40170	*OsPLDα4*	3.09	U	0.0031	1.2	D	0.55	3.16	U	0.0599
Os.50104.1.S1_at	LOC_Os06 g40180	*OsPLDα5*	2.71	U	0.0029	3.22	U	0.0297	2.93	U	0.0724
Os.54571.2.A1_at	LOC_Os03 g27370	*OsPLDα6*	1.99	U	0.0352	1.08	U	0.4458	1	U	0.9794
OsAffx.29446.1.S1_at	LOC_Os08 g31060	*OsPLDα7*	1	U	0.644	1	D	1	1	D	1
Os.50273.1.S1_at	LOC_Os09 g25390	*OsPLDα8*	1	D	1	1	D	0.5798	1	U	1
Os.9389.2.S1_s_at	LOC_Os10 g38060	*OsPLDβ1*	1.3	U	0.0135	1.07	U	0.0754	1.06	D	0.8352
Os.34954.1.S1_at	LOC_Os03 g02740	*OsPLDβ2*	1.02	U	0.6604	1	U	0.9118	1.01	U	0.8858
Os.11408.1.S1_at	LOC_Os09 g37100	*OsPLDδ1*	8.52	U	8.24E-04	4.12	U	0.0781	1.23	U	0.7385
Os.52881.1.S1_at	LOC_Os03 g62410	*OsPLDδ2*	1.01	D	0.644	1.02	D	0.4164	1	U	1
Os.5744.1.S1_at	LOC_Os07 g15680	*OsPLDδ3*	1	D	0.6778	1.01	D	0.2723	1	D	1
OsAffx.14869.1.S1_at	LOC_Os05 g29050	*OsPLDζ1*	9.93	U	0.0019	3.79	U	0.0193	1.27	D	0.8026
Os.6185.1.S1_at	LOC_Os06 g44060	*OsPLDφ*	2.22	D	0.0088	1.79	D	0.0094	1.65	D	0.0351
Os.56801.1.S1_at	LOC_Os02 g02790	*OsPLDκ*	1.05	U	0.027	1	D	0.9464	1	D	1

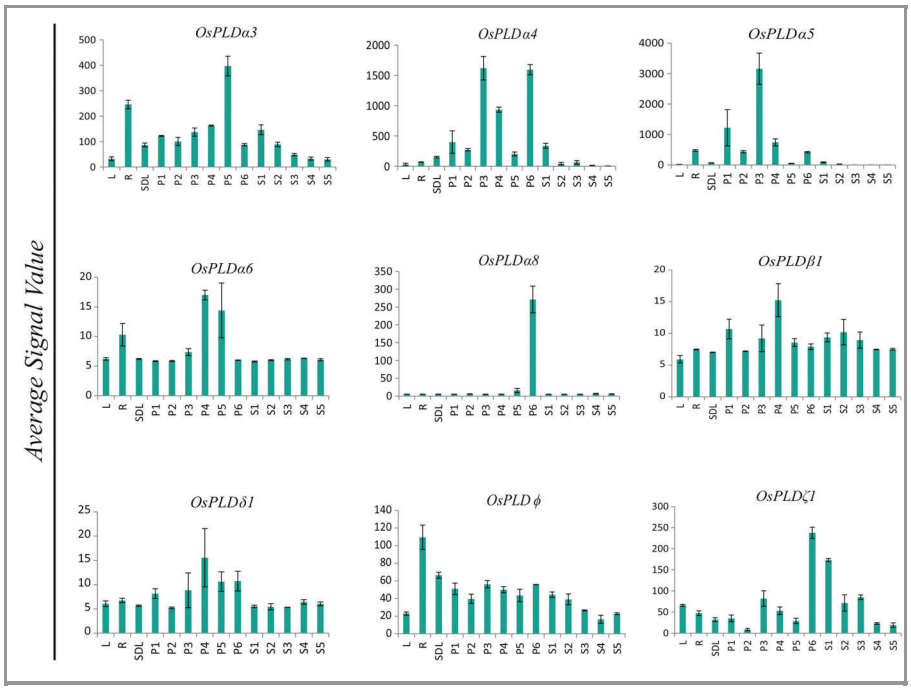

Figure 2. Expression profiles of differentially expressed PLD genes during developmental stages. Average signal intensity value of three replicates from microarray for all the developmental stages has been plotted to show the differential expression. Standard error bars have been shown. Y-axis represents signal values from microarray and X-axis shows different developmental stages.

conditions whereas *OsPLDα4* was upregulated under both cold and drought stresses. Two PLD members; *OsPLDα1* and *OsPLDα6* were specifically expressed and upregulated under drought condition. None of the PLD members was found to be expressed exclusively either under salt or cold stress conditions (**Table 1 and Fig. 3A**).

Expression profile of rice PLDs during development. Expression profile for rice developmental stages was generated from the gene chip microarray data. Eleven reproductive developmental stages were analyzed including, six panicle stages (P1-P6) and five seed developmental stages (S1–S5) along with three vegetative stages namely; mature leaf, root and seedling. A detailed analysis of the expression profile revealed that a total of nine PLD genes expressed differentially during reproductive developmental stages when compared with three vegetative developmental stages (**Fig. 2 and Table S1**). Out of the nine genes, eight PLDs; *OsPLDα3, OsPLDα4*, *OsPLDα5, OsPLDα6*, *OsPLDα8*, *OsPLDβ1, OsPLDδ1* and *OsPLDζ1* were upregulated

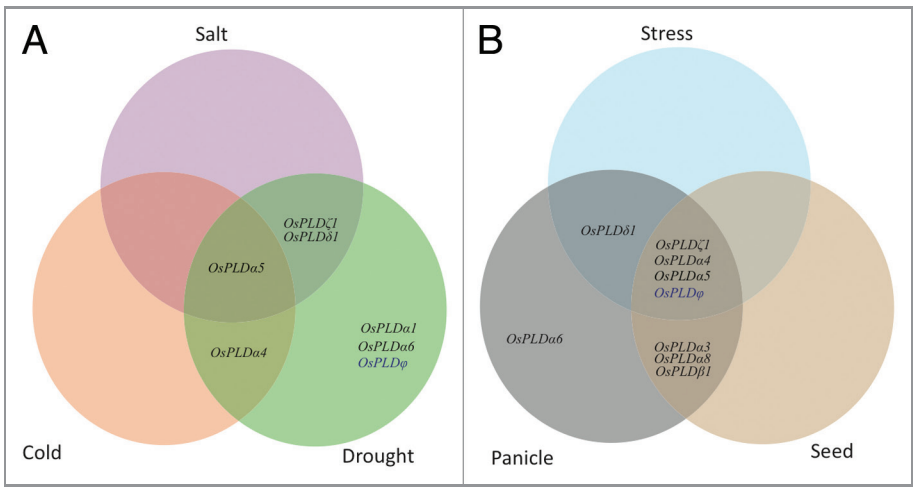

Figure 3. Venn diagram for differentially expressed PLDs. PLD genes upregulated and downregulated (A) under different abiotic stress conditions and (B) under stresses and developmental stages. Different compartments showing genes specific to either a particular stress/developmental stage or common to more than one stress and/or developmental stage.

and a single member *OsPLDφ* was downregulated. Six PLD members were commonly upregulated in both panicle and seed developmental stages. Two PLD members; *OsPLDα6* and *OsPLDδ1* were expressed exclusively in the panicle stages whereas none of the PLD gene was exclusively expressed in seed developmental stages (**Fig. 3B and Table S1**).

Overlapping expression under abiotic stresses and development. Keeping the fact in mind that abiotic stresses and reproductive development are interconnected processes in the plant life cycle, which has also been proven by overlapping expression of genes in these conditions in prior studies,[24-26] we attempted to investigate overlapping expression of PLDs involved in stress and development. Interestingly, all seven PLD genes, which were expressed differentially under abiotic stresses, were also found to be expressed significantly during reproductive developmental stages (**Fig. 3A and B**). In this subset of seven genes, three genes; *OsPLDα4*, *OsPLDα5* and *OsPLDζ1,* which were highly upregulated under abiotic stress (mainly drought and high salinity) conditions, also found to be upregulated in panicle and seed developmental stages. *OsPLDα4* and *OsPLDα5* were mainly upregulated during different stages of panicle development whereas *OsPLDζ1* was found to be highly upregulated in the later stages of panicle and early stages of seed development (**Fig. 2**). A single gene *OsPLDδ1* was commonly upregulated under stresses and later stages of panicle development and had no significant expression in seed stages. On the other hand, only *OsPLDφ* was found to be downregulated in all abiotic stress conditions and developmental stages. Surprisingly, not a single PLD member was found to be commonly expressed in stress and seed developmental stages.

Expression profile of duplicated PLDs. Analysis for the chromosomal duplication revealed that six PLD members (three pairs); *OsPLDα1* (chr. 1): *OsPLDα2* (chr. 5), *OsPLDβ1* (chr. 10): *OsPLDβ2* (chr. 3) and *OsPLDζ1* (chr. 5): *OsPLDζ2* (chr. 1), were present on the duplicated segments of chromosome and hence, exhibited segmental duplication. On the other hand, three genes; *OsPLDα3, OsPLDα4* and *OsPLDα5* exhibited tandem duplication and were located in a cluster on the chromosome 6 (**Table S2**). Expression profile was generated for the duplicated PLD genes using microarray data for the three abiotic stresses and entire spectrum of developmental stages. Corresponding probeset was not available for *OsPLDζ2* on the microarray gene chip, therefore expression profile could not be generated for the respective gene pair (*OsPLDζ1*:*OsPLDζ2*). Average signal values for abiotic stress conditions and developmental stages have been presented as an area-diagram (**Fig. 4**). Expression profile revealed that one of the segmentally duplicated pair *OsPLDα1*:*OsPLDα2* exhibited pseudo-functionalization as one of the paired partners did not have any expression in tested conditions or developmental stages. On the other hand, another segmentally duplicated pair *OsPLDβ1*:*OsPLDβ2* showed retention of expression as both the partners followed similar expression pattern. In the tandemly duplicated cluster of gene, while *OsPLDα3* had negligible expression, pair of *OsPLDα4*: *OsPLDα5* exhibited retention of expression in most of the conditions and stages. However, the magnitude of expression varied between the paired duplicated partners.

Validation of microarray expression under abiotic stress conditions. Expression pattern and profile of the seven PLD genes, which were expressing differentially and significantly either exclusively or overlapping under abiotic stress conditions were validated employing quantitative real time PCR. Interestingly, all the candidate genes exhibited anticipated expression pattern and clearly revealed the differential regulation of transcript level under different abiotic stresses (**Fig. 5**). For a few genes, the level of transcript was found to be much higher than estimated by microarray profile in conditions such as for *OsPLDα6* and

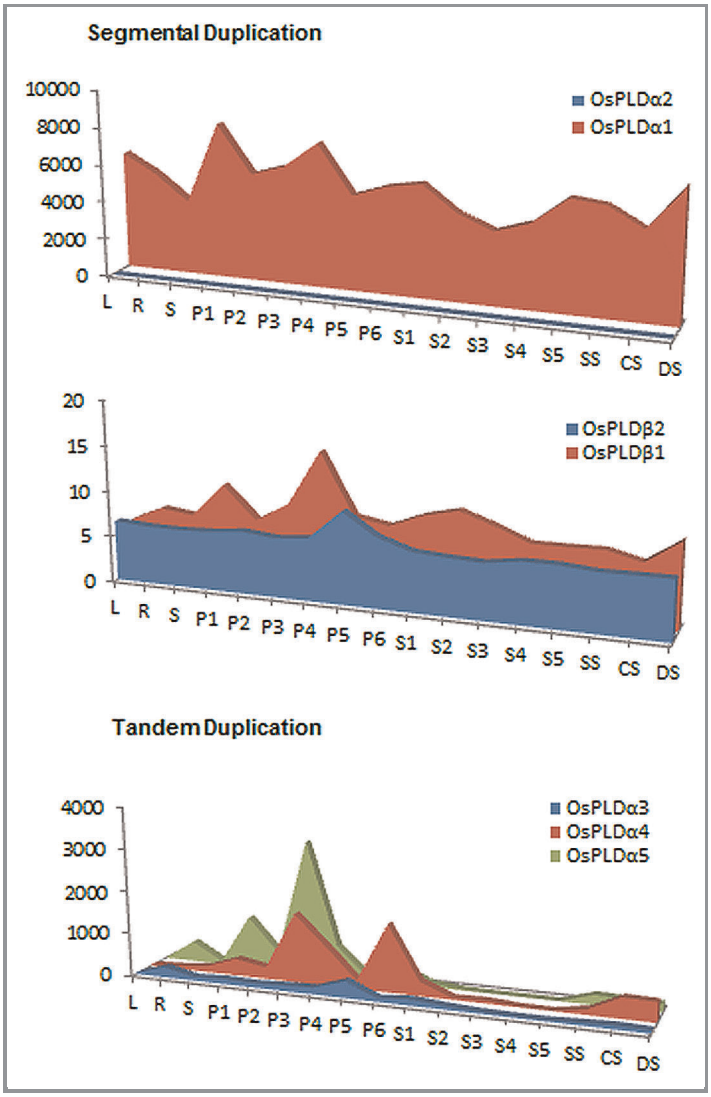

Figure 4. Expression profiles of duplicated PLD genes. Expression profiles of segmentally and tandemly duplicated gene pairs/clusters from microarray data were compared in various developmental stages including leaf (L), root (R) and seven day old seedling (S) tissue, in various stages of panicle development (P1–P6), seed development (S1–S5) and under cold stress (CS), dehydration stress (DS) and salt stress (SS). Each area graph represents compilation of the mean normalized signal intensity values.

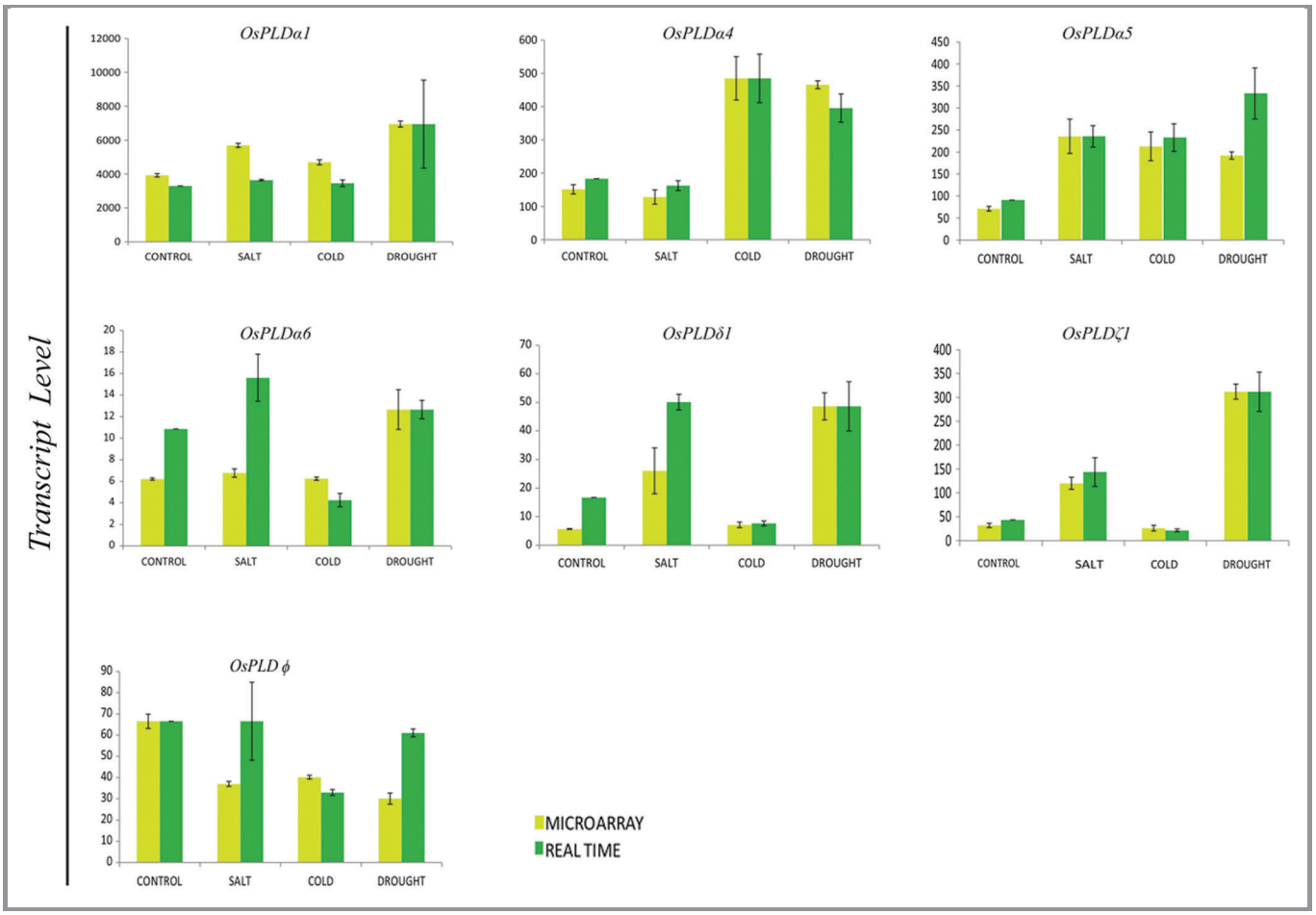

Figure 5. Validation of microarray expression profiles for differentially expressing PLD genes by quantitative RT-PCR. Two and three biological replicates were analyzed by real time PCR and microarray, respectively. Standard error bars have been shown for both microarray and real time PCR data. Y-axis represents raw expression values from microarray and normalized expression value from real time PCR and X-axis shows different experimental conditions.

OsPLDδ1 in salt stress and for *OsPLDφ* in salt and drought stresses.

Discussion

Abiotic stresses are the major factors, which hamper the normal plant growth and productivity. After extensive research, it has been appreciated that plants acquire tolerance or adaptability against these stresses by a plethora of signal transduction network. One of the important signaling networks is mediated by lipid molecules generated in response to multiple stimuli in plants. Therefore, it is imperative to understand the role of major lipid modifying enzymes such as phospholipase A, C and D. Phospholipase D (PLD) class has been implicated in multiple stress responses in Arabidopsis. In crop plant such as rice, not enough has been investigated for the role of PLD, which enticed us to look for the functional role of this family by focusing on gene expression analysis during different stages of development and abiotic stresses.

Expression profile of a gene provides an important clue regarding its functional role and molecular action; therefore, we performed a whole-genome transcript profiling for the rice PLD genes in three different abiotic stresses and during various developmental stages. Microarray data revealed that a subset of PLD genes expressed differentially and significantly under specific or multiple abiotic stress conditions (**Table 1**). In this subset of genes, out of six upregulated PLDs, four genes belong to α subfamily (*OsPLDα1*, *OsPLDα4*, *OsPLDα5* and *OsPLDα6*), whereas one member each from δ and ζ subfamilies. This observation suggests that α subfamily of PLDs, which have evolved together in due course of time in rice and Arabidopsis, exhibited functional conservation and is majorly responsive to abiotic stresses. A single member of φ subfamily (*OsPLD φ*) was found to be repressed in all the three abiotic stresses. This deviation in expression for *OsPLDφ* suggests that this might act as a negative regulator of stress signaling or it has functional role in some processes other than stress signaling, which might be attributed to its novel domain structure (N-terminal signal peptide), because C2 domain and PX/PH domains in other PLDs are known to mediate the targeting of these enzymes to plasma membrane where the lipid hydrolysis occur in response to various stresses.[27,28] These results were further supported by quantitative

transcript analysis by real time PCR, which showed that all the tested PLD members had expression pattern and transcript level similar to microarray expression pattern under different abiotic stress conditions (**Fig. 5**). However, in case of *OsPLDφ*, the transcript abundance was not as low in case of salt and drought stresses as revealed by the microarray data. This type of variation in expression level from these two techniques (microarray and real time PCR) has been observed in previous studies also.[24-26] Gene induction and transcript accumulation for PLDs in response to similar abiotic stresses has been observed previously, in different plant species such as Arabidopsis and tomato.[21,29] *AtPLDα1*, Arabidopsis ortholog of rice *PLDα1* has been studied most extensively and was found to regulate ABA and drought mediated responses.[30-33] Overexpression of *AtPLDα1* made plants highly sensitive to ABA, which lead to enhanced stomatal closure and resulted in reduced transpirational water loss and drought tolerance, whereas the knockout plants showed opposite response.[30,31] Similarly, *AtPLDα1* has also been implicated in salinity and freezing stress mediated signaling pathways.[21,34] Tobacco and tomato *PLDα1* were also reported to mediate drought and salt stress responses.[21,35] Another study showed that, *AtPLDα3* knockout mutant plants exhibited high sensitivity towards salinity, dehydration and ABA, while gain-of-function of *AtPLDα3* led to reduced sensitivity in transgenic plants.[5] These observations are supportive to our findings of involvement of various PLDα subfamily members in different abiotic stresses in rice. Other PLD member, *AtPLDδ* has also been found to be involved in salinity and freezing responses and tolerance.[17,21] With similar kind of expression profiles, sequence conservation and close evolutionary relationship,[14] a functional conservation might also be predicted and significant role can be corroborated in abiotic stress signaling for rice PLD gene family members. Involvement of PLDs in various stress responses has been further supported by the presence of various *cis*-regulatory elements in their promoter, including ABRE (abscissic acid responsive element), LTR (low temperature responsive), MBS (myb binding site), Skn-1, GCN4, RY-element and motif IIb (**Table S3**). These motifs have been previously known to regulate various stress responses and plant development.[36-40] Promoter of several PLD genes, which were significantly and differentially regulated in stress and developmental stages, and found to contain multiple *cis*-regulatory elements especially ABRE, MBS and Skn-1 known to control ABA, drought and development mediated induction of genes. Notably, *OsPLDα1, OsPLDα5, OsPLDβ1, OsPLDδ1* and *OsPLDζ1* were commonly induced in abiotic stresses and development, and found to contain most of these motifs. This observation indicated regulation of PLD genes by the *cis*-regulatory promoter elements and also accounted for their overlapping expression in abiotic stresses and development. Expression profiling for various developmental stages revealed that nine PLD genes showed significant variation in their expression level through a spectrum of vegetative and reproductive developmental stages (**Table S1**). Out of the nine PLDs, eight genes were upregulated in one or more panicle and seed development stage w.r.t three vegetative stages, whereas a single gene *OsPLDφ* was downregulated in the entire spectrum of reproductive development, similar to abiotic stresses. Among the upregulated genes, five PLD members belong to α subfamily and all of them were significantly expressed in different panicle stages and interestingly none of them is expressing during seed developmental stages (**Fig. 2**). Transcripts of *OsPLDα3* were accumulated at P5 stage (vacuolated pollen), *OsPLDα6* at P5-P6 and *OsPLDα8* specifically at P6 (mature pollen), whereas *OsPLDα5* expressed at P3 (meiosis) and *OsPLDα4* in almost all the panicle stages.This has suggested the significant role of this subfamily of PLDs in the floral organ development in rice. *OsPLDβ1* significantly expressed in panicle and seed developmental stages whereas *OsPLDζ1* had significant expression in the late panicle stage (P6) and early seed developmental stages S1-S3 (early globular embryo to embryo morphogenesis stages) (**Fig. 2**). Previously, *OsPLDβ1* expression was found to be upregulated in immature seeds and it was implicated in seed germination process in rice where it activated ABA signaling by SAPK leading to inhibition of seed germination.[14] Some other studies also supported our finding regarding PLD genes in various developmental stages. In soybeans, the expression of a PLD gene varied during seed developmental and seed germination stages.[22] Similarly, the high expression for the two Arabidopsis PLD members; *PLDζ1* and *PLDζ2* was observed in the roots and both of these genes were implicated in root elongation and patterning.[41] Phosphatidic acid (PA) generated by the activity of PLD has been found to regulate ABA mediated seed germination in Arabidopsis.[42] Apart from PLDs, the differential expression pattern has been observed for the members of phospholipase A (PLA) class under similar developmental stages in rice.[26] Analysis for overlapping expression under abiotic stresses and reproductive development provided interesting results and revealed that all the seven PLD members, which expressed significantly and differentially under abiotic stresses were also differentially expressed during reproductive developmental stages (**Fig. 3A and B**). This kind of overlapping expression has been observed earlier for various gene families in plants,[24-26,43,44] which further strengthen the fact that abiotic stresses and reproductive development are interconnected phenomena in plants, as dehydration conditions set in during the later stages of seed maturation and leads to seed dormancy.[43,45,46] The overlapping expression as discussed earlier, can be attributed to the presence of *cis*-regulatory element such as ABRE in the promoters (especially for the genes such as *OsPLDβ1*), which controls both stress response and development through regulation of phytohormone ABA responses.[25,49-51] Therefore, signaling pathways triggered by abiotic stresses and during development can be hypothesized to be commonly controlled by ABA involving PLDs. Additionally, expression profile of duplicated PLD genes revealed variable expression pattern for the paired partners. In the segmentally duplicated PLDs one pair (*OsPLDα1*: *OsPLDα2*) exhibited pseudo-functionalization while other pair (*OsPLDβ1*: *OsPLDβ2*) showed retention of expression (**Fig. 4 and Table S2**). This observation is consistent with previous reports, which mention that segmentally duplicated genes exhibit a functional divergence.[50] Among the three tandemly duplicated genes, one gene (*OsPLDα3*) had almost negligible expression exhibiting pseudo-functionalization while

other two had similar expression pattern hence showed retention of expression. However, the amplitude of expression was variable, which could be attributed to the variability in the *cis*-regulatory elements.[25,51] This kind of variable expression pattern among the duplicated genes could be due to the lack of intense selection pressure during the course of evolution.[50,52-54] Gene duplication, therefore, might have played a significant role in the functional diversification of this gene family in rice.

In conclusion, this study provides a comprehensive expression profile for the entire set of rice PLD genes under abiotic stress conditions and during developmental stages, which has not been performed until now. Subsets of PLD genes with differential expression profile during abiotic stresses and reproductive development have been identified, which signify the role of PLDs in abiotic stress signaling and during development in rice. Moreover, most of the differentially expressed PLD genes exhibited overlapping expression under stress conditions and during development, indicating the crosstalk of these pathways involving PLDs possibly through a common component such as ABA. Duplicated PLD genes showed variable expression pattern indicating the role of gene duplication in the functional diversification of PLD gene family in rice.

Materials and Methods

Plant material, growth conditions and abiotic stress treatment. The tissues samples were collected from field grown rice plants (*Oryza sativa* ssp. *Indica* var IR64), at different panicle and seed development stages. To avoid wounding, collected panicles were instantly frozen in liquid nitrogen. Stress treatment was given to IR64 rice seeds, which were first sterilized with 0.1% $HgCl_2$ and grown in culture room conditions at 28 ± 1°C with a daily photoperiodic cycle of 14 h light and 10 h dark. After seven days growth, seedlings were subjected to different stress treatments according to Ray et al. 2007.[51]

Microarray expression analysis. To perform the microarray based expression analysis, the total RNA was isolated from the three replicates of rice tissues representing different stages of plant development, which included three vegetative stages (mature leaf, 7 days old seedling and their roots), 11 reproductive stages (P1–P6 and S1–S5; representing panicle and seed developmental stages, respectively) and three abiotic stress conditions, i.e., cold, salt, and drought. Microarray experiments were then performed using 51 Affymetrix GeneChip Rice Genome Arrays (Gene Expression Omnibus, GEO, platform accession number GPL2025). The raw data (*.cel) files generated from all the chips were imported to Array Assist 5.0 software (Stratagene) for detailed analysis according to Arora et al. 2007.[24] The microarray expression data have been deposited in the gene expression omnibus (GEO) database at NCBI under the series accession numbers GSE6893 and GSE6901.

Quantitative expression analysis by real time PCR. Real time PCR was performed to verify the microarray data for a few selected genes, which showed significant differential expression pattern under abiotic stress conditions. The primers were designed for all the selected genes preferentially, from 3' end, using PRIMER EXPRESS (PE Applied Biosystems), with default parameters. Primers were further checked for their specificity, using BLAST tool of NCBI and dissociation curve analysis after the PCR reaction (**Table S4**). First strand cDNA was prepared from 4 µg of DNase treated total RNA, in 100 µl of reaction volume using high-capacity cDNA Archive kit (Applied Biosystems). SYBRGreen PCR Master Mix (Applied Biosystems) was used to determine the transcript levels in ABI Prism 7000 Sequence detection System (Applied Biosystems). Biological duplicates of each sample were taken for the analysis. The average Ct values were calculated by taking the average of three technical replicates for each sample. The cDNA variance among samples was normalized using *ACTIN* as the endogenous control. Relative expression values were calculated by ΔΔCt method and normalized the data against the maximum average expression value from microarray.

Gene duplication. The RGAP (rice genome annotation project) version 6.1 (http://rice.plantbiology.msu.edu/) segmental duplication database was explored to find out the segmentally duplicated genes. Genes separated by five or fewer genes were considered as tandemly duplicated and amino acid sequence homology of these gene products was computed using MegAlign software 5.07©.

Promoter analysis. To find out various *cis*-acting regulatory elements in the promoter of all the PLD genes, 1 kb upstream region from translation start site was extracted from RGAP ver 6.1. One kilo base pair upstream region was subsequently scanned in PlantCARE database[55] for the presence of various regulatory motifs and elements. Various motifs involved in stress responses and plant development were identified.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/psb/article/20385

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