

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/320067174>

RETRACTED ARTICLE: Molecular characterization of the 14-3-3 gene family in rice and its expression studies under abiotic stress

Article in *Planta* · September 2017

DOI: 10.1007/s00425-017-2779-4

CITATIONS

36

READS

662

4 authors, including:



Saurav Bhattacharya
Changshu Institute of Technology

14 PUBLICATIONS 243 CITATIONS

[SEE PROFILE](#)



ORIGINAL ARTICLE

Molecular characterization of the 14-3-3 gene family in rice and its expression studies under abiotic stress

Niti Yashvardhini¹ · Saurav Bhattacharya¹ · Shubho Chaudhuri² · Dibyendu Narayan Sengupta¹

Received: 30 June 2017 / Accepted: 16 September 2017
© Springer-Verlag GmbH Germany 2017

Abstract

Main Conclusion 14-3-3 isoforms were relatively less conserved at the C-terminal region across plant groups. Both *Os14-3-3f* and *Os14-3-3g* were inducible with differential gene expression levels under different abiotic stress and developmental stages in sensitive and tolerant indica rice cultivars as confirmed both at transcript and protein level.

Plant 14-3-3s has been well characterized to function in several signaling pathways, biotic as well as abiotic stress and nutrient metabolism. We attempted comprehensive analysis of 14-3-3 genes in different plant lineages such as green algae (*Chlamydomonas reinhardtii*), moss (*Physcomitrella patens*) and lycophyte (*Selaginella moellendorffii*), dicot *Arabidopsis thaliana* and monocot *Oryza sativa* subsp. japonica at the gene and protein level. Sequence alignment results revealed that 14-3-3 isoforms were evolutionarily conserved across all taxa with variable C-terminal end. Phylogenetic analysis indicated that the majority of 14-3-3 isoforms in rice belong to the non-epsilon group that clustered separately from the dicot group. Segmental duplication event played a significant role in the expansion of both,

Arabidopsis and rice, 14-3-3 isoforms as revealed by synteny studies. *In silico* gene expression using Massive Parallel Signature Sequencing and microarray analysis revealed that 14-3-3 isoforms have variable expression in different tissue types and under different abiotic stress regime in *Arabidopsis* and japonica rice. Both, semi-quantitative and qPCR results, confirmed that *Os14-3-3f* and *Os14-3-3g* were inducible under abiotic stress in lamina and roots of indica rice and relatively higher under salinity and cold stress in Nonabokra, under dehydration stress in N-22 and under exogenous ABA in IR-29 usually after 3–6 h of treatment. Both, 14-3-3f and 14-3-3g, were highly expressed in flag leaves, stems and panicles and mature roots. These results were further confirmed by immunoblot analysis of rice cultivars using *Os14-3-3f* antibody generated from recombinant *Os14-3-3f* protein. The results provide the first comprehensive report of 14-3-3 gene expression in indica rice cultivars which differ in tolerance to abiotic stress that might be useful for further research.

Keywords 14-3-3 gene · Abiotic stress · Gene expression · Indica rice · Microarray · MPSS · Phylogenetic tree · Sequence alignment · Synteny mapping

Abbreviations

DAP	Days after pollination
MPSS	Massive parallel signature sequences
TPM	Transcript per million

Introduction

Plants being sessile develop complex mechanism to adjust physiological response to environmental stress. All these modulations involves complex biological process that are

Electronic supplementary material The online version of this article (doi:10.1007/s00425-017-2779-4) contains supplementary material, which is available to authorized users.

✉ Dibyendu Narayan Sengupta
dibyendu@jcbose.ac.in; sengupta_dibyendu@rediffmail.com

¹ Division of Plant Biology, Bose Institute, Main Campus, 93/1, A.P.C. Road, Kolkata, West Bengal 700009, India

² Division of Plant Biology, Bose Institute, Centenary Campus, P1/12, C.I.T. Scheme VII(M), Kolkata, West Bengal 700054, India

controlled by signal transduction and metabolism regulation that have been known to occur via phosphorylation-mediated transition of protein states (Tian et al. 2015). 14-3-3 Proteins are a large family of small acidic proteins (~ 30kD), which are highly conserved even between kingdoms (Fu et al. 2000; Paul et al. 2009) and typically function as dimers. They are functionally characterized as phosphor-serine binding proteins (bind to phosphorylated motifs) and modulate cellular processes such as signal transduction, cell cycle, metabolism, membrane trafficking, stress responses and apoptosis in mammalian cells (Shin et al. 2011).

14-3-3 Proteins in plants comprise multiple isoforms with differential subcellular localization (Bihl et al. 1997; Ferl et al. 2002; Chen et al. 2006). Unlike animals which have seven 14-3-3 proteins, the majority of plants possess multiple 14-3-3 isoforms suggesting that their isoforms specifically function and are involved in multiple protein–protein interactions including response to environmental stress (Roberts et al. 2002; Yan et al. 2004; Xu and Shi 2006), metabolism or nutrient stress (Xu and Shi 2006; Shin et al. 2011; Xu et al. 2012), biotic/pathogenic stress (Roberts et al. 2002), membrane transport processes (Shin et al. 2011), regulation of floral transition process, and brassinosteroid (BR) and phytohormone signaling in plants (Purwestri et al. 2009; Jaspert et al. 2011; Taoka et al. 2011; Roberts 2017). To date, several 14-3-3 isoforms have been reported in plants such as *Arabidopsis* (15 isoforms), rice (8 isoforms), *Brachypodium distachyon* (7 isoforms), barley (5 isoforms), tobacco (17 isoforms), cotton (6 isoforms), soybean (18 isoforms) and maize (26 isoforms), but only few have been phylogenetically and functionally characterized (Denison et al. 2011).

Although the 14-3-3 proteins are found throughout the plant taxa, the majority of our knowledge on gene structure, organization and evolutionary history has been derived from studies in *Arabidopsis* owing to its large family of 14-3-3 isoforms (DeLille et al. 2001) and fully sequenced genome. Rice being a staple cereal crop has been a focus of diverse research in plant science aimed at crop improvement, both qualitatively and quantitatively. In general, rice shows differences in sensitivity towards excess salinity at various developmental stages during its life cycle. It is considered relatively tolerant to salinity and dehydration at the germination stage, the young seedling stage and early reproductive stages, i.e., panicle initiation and pollination are the most salinity-sensitive growth stages, directly affecting the crop yield (Zeng et al. 2001; Ganguly et al. 2012; Shankar et al. 2016). Several recent studies have revealed rice genes to be either differentially expressed at different growth stages and/or one or more genes differentially regulated in stress-sensitive as compared to stress-tolerant rice cultivars under stress conditions (Walia et al. 2005; Muthuramalingam et al. 2017). A large number of stress-tolerant rice cultivars/

landraces have been identified based on their phenotypical and physiological responses under various abiotic stress conditions (Degenkolbe et al. 2009). Rice exhibits genetic diversity in their sensitivity to salt stress. The high yielding indica rice varieties such as IR-64, IR-72 and IR-29 are mostly salt susceptible, while the low-yielding varieties such as Pokkali and Nonabokra are salt tolerant. Similarly, varieties such as Nagina-22 (N22), Vandana and Sahabhogi are low-yielding cultivars, but highly tolerant to extreme water stress condition. Such contrasting physiological differences are considerably useful and could be utilized for analysis and manipulation (introgress or overexpress) of stress-inducible genes/pathways from tolerant cultivars to high-yielding sensitive cultivars with an aim to increase crop productivity under extreme conditions (Reddy et al. 2017).

Plant 14-3-3 proteins have been widely reported to be involved in metabolism (carbon and nitrogen); however, several recent studies have shown their involvement in biotic and abiotic stress response (Denison et al. 2011). These studies have also indicated the dynamic expression of 14-3-3 proteins briefly regulated in response to diverse stress stimuli (Chen et al. 2006; Liu et al. 2016) and stress-activated kinases (Shin et al. 2007). Preliminary reports of 14-3-3 in rice identified four putatively expressed 14-3-3 isoforms among eight rice 14-3-3 genes which were differentially regulated by biotic as well as abiotic stimuli (Chen et al. 2006). Yao et al. (2007) reported that rice 14-3-3 genes were phylogenetically similar to *Arabidopsis* 14-3-3 isoforms with rice 14-3-3s exhibiting differential gene expression under different abiotic stresses and hormonal regime. To confirm these findings, further in-depth analysis of 14-3-3 isoforms among different plant taxa including *Arabidopsis* and rice based on gene structure and detection of shared synteny is necessary to understand the evolutionary aspects of 14-3-3 genes in monocots and dicots. As 14-3-3 interacts with diverse clients and is regulated by diverse stress stimuli and hormonal regime, a comprehensive gene expression study under various abiotic stress module utilizing stress-sensitive as well as -tolerant cultivars simultaneously will be beneficial to decipher its role in abiotic stress.

Indica rice cultivars with contrasting (both sensitive and tolerant) tolerance to abiotic stress provide a suitable system to study the gene expression pattern of 14-3-3 for further research. Keeping in view the importance of 14-3-3 proteins, we made a genome-wide *in silico* characterization of the 14-3-3 gene isoforms in different plant lineages including *Arabidopsis* and rice and presented a comprehensive phylogenetic analysis of the 14-3-3 gene/isoforms with their gene and protein structure. We have attempted to gain knowledge on the overall gene expression pattern under different environmental stress using the open access Microarray and Massive Parallel Signature Sequencing (MPSS) database-derived information emphasizing on the *Arabidopsis* and

Oryza genome. We further expanded our study to investigate comparative expression response of the 14-3-3 genes under different abiotic stress such as salt, drought, cold and exogenous ABA in indica rice varieties such as IR29, Nonabokra and N-22 with contrasting tolerance to abiotic stress.

Materials and methods

Sequence analysis and characterization of 14-3-3 isoforms in different plant lineages

The gene sequence, coding sequences (CDS) and protein sequences of different 14-3-3 isoforms were retrieved from *Arabidopsis thaliana* available in TAIR (<http://www.arabidopsis.org/>) and also from *Oryza sativa*, Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/analyses> search locus.shtml). Homologous 14-3-3 gene sequences in major plant lineages including green algae (*Chlamydomonas reinhardtii*), moss (*Physcomitrella patens*) and lycophyte (*Selaginella moellendorffii*) were obtained from Phytozome (<http://phytozome.jgi.doe.gov/pz/portal.html>) by performing homology-based tBLASTn searches using *Arabidopsis* and rice 14-3-3 protein sequences as queries and downloaded those sequences from the National Centre for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>). Multiple sequences alignment (MSA) of the 14-3-3 nucleotide and protein sequences from rice and *Arabidopsis* and three other plant species were performed with the integrated MUSCLE alignment program in MEGA 5.0 (Molecular Evolutionary Genetics Analysis) (Tamura et al. 2011), with default parameters. Phylogenetic analysis was conducted by the maximum-likelihood (ML) method using MEGA5 software and bootstrap tests replicated 1000 times. In addition, full length amino acid sequences from both rice and *Arabidopsis* were aligned with MEGA5 and used to construct a phylogenetic tree in the same way.

Gene structure, promoter, chromosomal location and synteny analysis

The exon–intron structures of the *Os14-3-3* and *At14-3-3* isoforms were determined from aligning their coding sequences (CDS) to their corresponding genomic sequences. An illustrated map of exon–intron structures of all the isoforms was obtained online using Gene Structure Display Server (GSDS: <http://gsds.cbi.pku.edu.cn>). The 1 kb upstream promoter sequence of each 14-3-3 isoforms of *Arabidopsis* and rice was downloaded from the plant promoter database 2.1 (<http://ppdb.agr.gifu-u.ac.jp/ppdb/cgi-bin/index.cgi>) using the GeneBank accessions as query. The 1 kb upstream promoter region of each gene sequence was searched for cis-acting regulatory elements for abiotic stress

in plant CARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) (data not shown). These sequences were used for p-distance calculations. Pairwise sequence divergence was estimated for the 1 kb upstream region and the coding region of each of the 14-3-3 genes from *Arabidopsis* and rice genome based on nucleotide substitution per site using p-distance in MEGA5. The chromosomal locations of the 14-3-3 genes were also determined using the *Oryza* and *Arabidopsis* genome browser (<http://www.phytozome.net/>). The chromosomal location of 14-3-3 genes were drawn and visualized using CIRCOS (Krywinski et al. 2009). The conserved domains were identified in rice and *Arabidopsis* protein sequence using SCANPROSITE (<http://prosite.expasy.org/scanprosite/>). For synteny analysis, synteny blocks within the *O. sativa* genome and between *O. sativa* and *A. thaliana* genomes were downloaded from the Plant Genome Duplication Database (PGDD, <http://chibba.agtec.uga.edu/duplication/>) and those containing rice 14-3-3 isoforms were identified and analyzed.

To calculate the synonymous substitution (Ka) and non-synonymous substitution (Ks), codon alignment of duplicated genes was done using MEGA ver. 5.0 with ClustalW codon alignment tool and Ka/Ks values were calculated using the Ka/Ks calculator (Zhang et al. 2006). The approximate date of duplication events was calculated using $T = Ks/2E$, where E represents a constant rate of synonymous substitutions for monocots of 6.5×10^{-9} substitutions per synonymous site per year (Gaut et al. 1996). The 3D structure of rice 14-3-3 proteins were predicted using Phyre² software at intensive mode (Kelley et al. 2015) and the structures were validated using Ramachandran plot analysis (Lovell et al. 2003).

To predict rice 14-3-3 targeted miRNAs, *Os14-3-3* coding region was searched against published miRNAs in PMRD (Plant microRNA Database) (Zhang et al. 2010).

Expression analysis of 14-3-3 genes: exploring the microarray and MPSS database

To gain information on the putative role of different 14-3-3 isoforms in different growth stages and in lamina and root tissue under different stress response, we analyzed the publicly available microarray database of *Arabidopsis* and rice. *Arabidopsis* microarray data for the 14-3-3 genes in response to different abiotic stresses along with different developmental and reproductive stages were retrieved from AtGenExpress (<http://jsp.weigelworld.org/expviz/expviz.jsp>) for different abiotic stress conditions such as salt, drought, cold and various developmental stages (Schmid et al. 2005). Microarray data were obtained for different stresses at different time points, viz., 0.5, 1, 3, 6, 12 and 24 h for both root and shoot tissues. Fold change at transcript level of different genes under stress was calculated

with respect to their controls. For the developmental stage data, Affymetrix values were log₁₀ transformed, heat maps generated and hierarchical clustering done using the MeV software package (Eisen et al. 1998). For microarray analysis of rice 14-3-3 genes, Affymetrix GeneChip rice genome arrays (<http://www.ncbi.nlm.nih.gov/geo/>; Gene Expression Omnibus platform accession nos. GSE6893) was used. The Affymetrix values were log₂ transformed and heat maps generated using TIGR MeV software (Eisen et al. 1998). Microarray dataset in rice is described as: roots of 7-day-old seedlings, mature leaf (collected before pollination, young leaf, 7-day-old seedlings, P(0–3 cm panicle), P2 (3–5 cm panicle), P3 (5–10 cm panicle), S1 (0–2 DAP), S2 (3–4 DAP), S3 (5–10 DAP), S4 (11–20 DAP), GC (growing callus), R1 (regenerating callus for 2 days), R2 (regenerating callus for 4 days), R3 (regenerating callus for 6 days) and R4 (regenerating callus for 8 days). MPSS database is an important tool to get information on expression profiles of genes across different tissues and treatment types (Brenner et al. 2000). The expression profiles were analyzed for the 14-3-3 genes in the Plant MPSS database (Nakano et al. 2006) for *Arabidopsis* (<http://mpss.udel.edu/at/mpssindex.php>) and rice (<http://mpss.udel.edu/Rice/mpssindex.php>) in various tissues, developmental stages and abiotic stress based on the 20-nucleotide signature sequences using the locus IDs given in the TIGR database. The signature was considered to be significant when it uniquely identified an individual gene and showed perfect match (100% identity over 100% length of the tag). The quantitative estimate of gene expression for a selected signature sequence in a given library was represented as normalized abundance and transcript per million (TPM). The TPM values obtained for different libraries for all 14-3-3 isoforms in rice and *Arabidopsis* were plotted and analyzed.

Expression studies of rice 14-3-3 gene at the mRNA level by semi-quantitative RT-PCR and qRT-PCR under abiotic stress and in different developmental stages

Among eight 14-3-3 isoforms in *O. sativa*, we selected two isoforms 14-3-3f (non-epsilon) and 14-3-3g (epsilon) for gene expression analysis in three different indica rice cultivars: salt sensitive but high-yielding IR29, salt tolerant but low-yielding Nonabokra and drought tolerant but low-yielding Nagina-22. Gene expression was studied under salinity (200 mM), dehydration (20% PEG 6000), cold (4 °C) and ABA (100 µM) treatment at 0, 3, 6, 12 and 24 h in 12-day-old seedlings, and also during different developmental stages such as dry seeds, 4-day-old germinating seedlings, 12-day-old seedlings and 90-day-old mature plant's panicles, flag leaf, stems and roots.

Full length 14-3-3f and 14-3-3g gene (promoter + ORF) from indica rice varieties (IR29, Nonabokra and N-22) was amplified from genomic DNA sequence as well as from cDNA using gene-specific primers (Supplementary Table S1) designed according to BLAST results from TIGR database, cloned in TA cloning vector and sequenced using M13Forward–M13 reverse primers. The sequence was matched with published sequence of 14-3-3f and 14-3-3g. For upstream analysis, 1 kb upstream was amplified from genomic DNA of all three rice varieties, cloned and sequenced. The upstream sequence was analyzed and compared for probable cis-regulatory elements for abiotic stress using PlantCARE and PlantPAN database. All the primers were prepared according to the sequence of the japonica rice database and used for amplification in indica rice cultivars.

For gene expression analysis, total RNA was isolated from seeds, lamina, roots, flag leaf, stems and panicles using RNA express reagent (Himedia, Mumbai, India). RNA samples were treated with RNase-free DNase I (NEB) and concentration and purity were measured with a spectrophotometer (NANODROP 2000; Thermo Scientific). First-strand cDNA synthesis was carried out with 5 µg of total RNA using Sensiscript RT-PCR Kit (Qiagen) and oligo(dT) primers. Semi-quantitative RT-PCR reactions were carried out with 1 µl of RT mix from different samples using *Os*14-3-3f and *Os*14-3-3g gene-specific primers.

For qRT-PCR, the reaction mixture contained 2 µl of 1:10 diluted cDNA, 10 µl of 2× SYBR Green Master Mix (GenetBio, Nonsan, South Korea) and 200 nM of each gene-specific primer in a final reaction volume of 20 µl in a 96-well optical reaction plate (Applied Biosystems). Applied Biosystems 7500 Fast machine was used to perform the qRT-PCR experiment. The PCR cycle was the same for all genes, i.e., 10 min at 95 °C and cycle of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. Curve analysis was done and graphs were prepared by normalizing the values with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression values. Relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. The expression ratio was represented relative to the control value observed for the gene and fold change was calculated by the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{gene}} - Ct_{\text{gapdh}})_{\text{treatment}} - (Ct_{\text{gene}} - Ct_{\text{gapdh}})_{\text{control}}$. The treatment referred to Ct value for samples grown under salinity, drought, cold and ABA along with the respective control set grown under similar environment without any stress treatment. Ct_{gene} values denoted both *Os*14-3-3f and *Os*14-3-3g derived from qPCR experiment done separately. Two cDNA replicates were used for each reaction.

Production of isoform-specific anti-14-3-3f antibody and Western-blot analysis

Primers specific to the C-terminal region of rice 14-3-3f (Supplementary Table S1) were used to amplify the C-terminal specific region and the generated fragment was cloned into pET28a+, bacterial expression vector (Clontech), and then transformed into BL21 DE3 (pLYs), (Clontech) bacterial strain. The protein was overexpressed using 1 mM IPTG for 5 h at 30 °C and the recombinant protein was purified using Ni-NTA column (Qiagen); the protein was then eluted using 250 mM imidazole and then dialyzed against the same buffer. This recombinant protein was used for immunization in rabbits (Abgenex, Bhubaneswar, Odisha, India). The antibodies were then purified on an affinity column using the recombinant protein.

For expression analysis, total protein was extracted from the lamina of salt-, drought-, cold- and ABA-treated 12-day-old seedlings of IR29, Nonabokra and N-22 after 3, 6, 12 and 24 h of treatment along with a control set. Total protein was also extracted from roots, flag leaf, stems and seeds for expression analysis of *Os14-3-3f*. Tissue samples were homogenized in liquid nitrogen using phosphate buffer (pH 8.0) along with 150 mM NaCl and 1 mM PMSF and the extract was centrifuged at 10,000g for 30 min at 4 °C. The protein was then precipitated with 80% ammonium sulfate and the resulting pellet was dissolved in phosphate buffer (pH 8.0) and dialyzed against the same buffer. Protein samples were quantified using Bradford assay. Equal concentration (5 µg) of protein was taken from each sample and separated on 15% SDS-PAGE and blotted onto nitrocellulose membrane (Hybond-P, Amersham). Blots were incubated overnight with anti-14-3-3f primary antibody (1:20,000 at 4 °C) and with H3 antibody (as constitutive protein), followed by incubation with alkaline phosphatase-labeled goat anti-rabbit IgG as secondary antibody (1:1000 dilution, 4 h at 4 °C) and incubation with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Himedia) in alkaline phosphatase buffer.

The bands obtained from the H3 protein blot and 14-3-3 protein blots were scanned using Quantity One Software (Biorad) and then the graph was prepared after normalization of the values.

Results

Characterization of 14-3-3 isoforms in major plant lineages

We compared the 14-3-3 genes among major plant groups with whole genome sequences available including green algae *Chlamydomonas reinhardtii*, moss *Physcomitrella*

patens, *Selaginella moellendorffii*, *Arabidopsis thaliana* and *Oryza sativa* sub sp. *japonica*. The nucleotide sequence length, number of exons, number of introns, predicted amino acid length, cellular localization, chromosomal location and gene annotation and accession numbers of each of the 14-3-3 isoforms in all plant lineages are represented in Supplementary Table S2. From our studies, all lower and higher plant groups possess 14-3-3 isoforms in varying number. The highest number of 14-3-3 isoforms was found in *A. thaliana* (15) followed by *P. patens* (12), *O. sativa* (8), *S. moellendorffii* (6) and *C. reinhardtii* (2). From alignment results using all isoforms in all taxa, the 14-3-3 proteins were found to be highly variable at the C-terminal amino acid residues. Protein alignments of 15 *Arabidopsis* 14-3-3's across all taxa show the highest similarity within the core region. The C-terminal end is the part of the 14-3-3s that differs most, both in sequence and length, and the length of *Arabidopsis* C termini varies from 8 to 31 amino acids, counting from the last conserved residue (Fig. 1).

Phylogenetic and gene structure analysis

To gain information on the phylogenetic and evolutionary relationships, we constructed a phylogenetic tree by maximum-likelihood (ML) method aligning full length protein sequences of 14-3-3 from all selected taxa including rice. The 14-3-3 proteins of all five plant species were grouped into two major groups (epsilon group and non-epsilon group (Fig. 2) based on their gene structure (Ferl 2004). The epsilon group has a gene structure of six to seven exons and four to six introns (Fig. 3) and comprises *EPSILON*, *OMICRON*, *MU*, *IOTA* and *PI*. The majority of the 14-3-3 isoforms from the lower plant groups including the green alga *C. reinhardtii*, the lycophyte *S. moellendorffii* and the moss *P. patens* were included in the non-epsilon group. The non-epsilon group of *Arabidopsis* has four exons and three introns and consists of *KAPPA*, *LAMBDA*, *PSI*, *NU*, *UPSILON*, *OMEGA*, *PHI* and *CHI*. This group is further divided into three sub-branches: the kappa group harbors *KAPPA* and *LAMBDA*; the psi group contains *PSI*, *NU* and *UPSILON*; the omega group is made up of *OMEGA*, *PHI* and *CHI*. While, in *Arabidopsis*, the epsilon group cluster included five isoforms, namely epsilon, mu, rho, omicron and pi, *P. patens* included six and *S. moellendorffii* included four isoforms (Supplementary Table S2). However in *O. sativa*, the majority of the 14-3-3 isoforms were included in the non-epsilon group, except *Os14-3-3g* and *Os14-3-3h* that clustered in the epsilon group.

◀Fig. 1 Multiple sequence alignment of all 14-3-3 proteins from different plant lineages. The conserved motif is shown in blue color. The different domain names are denoted above the sequences. 14-3-3 Proteins contain nine alpha helices, in which alpha 1 and 2 constitute Domain 1, while alpha 3 and 4 denote Domain 2 and alpha 9 has NES (nuclear export signal). The variable C-terminal end is shown as a red box

Chromosomal location and synteny analysis of the *Arabidopsis* and rice 14-3-3 isoforms

Gene duplication events play an important role in the amplification of gene families in the genome. All the 15 14-3-3 isoforms were distributed throughout the five chromosomes of *A. thaliana* and eight 14-3-3s in six chromosomes in *O. sativa* (Fig. 4). All 15 14-3-3 isoforms in *Arabidopsis* were located in the same synteny block (Fig. 5), with 8 isoforms exhibiting duplication events in the same chromosomes (Chr1 and Chr5) and 7 isoforms showing segmental duplication events between different chromosomes. Isoform paired in Chr1, At14-3-3.2 and At14-3-3.13 as well as At14-3-3.11, At14-3-3.4 and At14-3-3.14 were located close in chromosomal position and also clustered together in the phylogenetic tree and paralogous to At14-3-3.1 at Chr4. However, in *Oryza*, the synteny block (Fig. 5) with all eight isoforms showed segmental duplication events between different chromosomes, with the majority of duplications centered on *Os14-3-3c* at chromosome 8 that showed four duplicated pairs with Chr11, Chr1, Chr2 and Chr3, indicating that segmental duplication event played a significant role in the expansion of rice 14-3-3 isoforms. Both *Arabidopsis* and rice 14-3-3 isoforms were well studied and, to compare the origin and evolutionary relatedness between them, we did comparative synteny mapping between *Arabidopsis* and rice 14-3-3 orthologs. According to the synteny map, only two *Arabidopsis* 14-3-3 orthologs (At14-3-3.5 and At14-3-3.7) were found to have syntenic pairing (13%) with only three *O. sativa* 14-3-3 isoforms (14-3-3b, 14-3-3c, and 14-3-3e). These results were in congruence with earlier reports of 14-3-3 genes where segmental duplication events contributed predominantly to 14-3-3 gene expansion (Wu et al. 1997; Rosenquist et al. 2001; Cao et al. 2016).

Estimation of duplication event dates

We estimated the genetic divergence and gene duplication events assuming that synonymous silent substitutions per site occur at a constant rate over time. For this analysis, we calculated the Ks between the coding sequences of each paralogous pair of *Os14-3-3* genes and used the mean Ks to estimate the approximate date of duplication event, with an estimated rate of silent-site substitutions of 6.5×10^{-9} substitutions per synonymous site per year. Information about the mean Ks values for each duplication event and the

estimated dates are shown in Table 1. Furthermore, the ratio of non-synonymous (Ka) and synonymous (Ks) substitution rates among the duplicated genes was calculated. As standard, the Ka/Ks ratio equal to one indicates “neutral mutation or no selection” and less than one Ka/Ks indicates “negative or purifying selection”, whereas Ka/Ks greater than one shows “positive or Darwinian selection”. The Ka/Ks ratio was calculated to elucidate the evolutionary constraint and selection pressure acting on the 14-3-3 gene family in rice. The pairwise comparison results showed that all Ka/Ks ratios were smaller than 1, suggesting that the duplicated regions were subjected to purifying selection (Table 1).

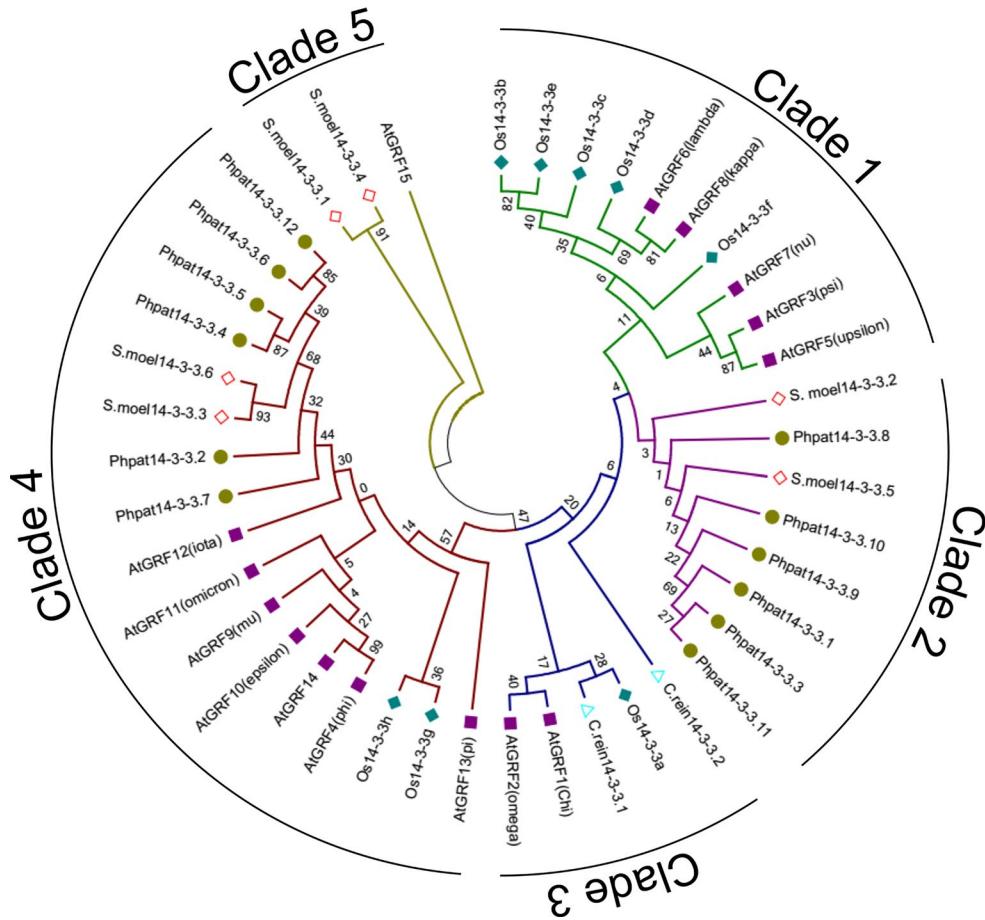
Homology modeling of the 14-3-3 proteins in rice

We constructed 3D models of eight *Os14-3-3* proteins of *Oryza sativa* subsp. *japonica* using protein homology/analogy recognition engine V 2.0, Phyre2 server (Kelley et al. 2015) with intensive mode modeling (Fig. 6). In modeling, the following templates were used to heuristically maximize the alignment coverage, percentage identity and confidence score for the submitted sequences: 2C1N:A and 2O02 templates for *Os14-3-3h*; 3E6Y:B, 2NPM:B and 1O9D:A templates for *Os14-3-3a*, *Os14-3-3b*, *Os14-3-3c*, *Os14-3-3g* and *Os14-3-3f*; 2O8P, 2NPM:B, 3E6Y:B and 1O9D:A for *Os14-3-3e* and *Os14-3-3d*. The models were constructed with an accuracy of > 90% confidence (73–86% of residues) in Phyre2 as well as model quality was verified by Ramachandran plot analysis with > 90% confidence of residues in the allowed region, indicating the reliability of the predicted models for further analyses. Secondary structures in almost all the 14-3-3 isoform constituted α -helices (72–82%) and predicted disordered regions (14–20%) primarily spanning the C-terminal domain of 14-3-3 proteins.

Analysis of the 14-3-3 expression profile based on Massive Parallel Signature Sequencing (MPSS) database

We have analyzed the expression profile of the 14-3-3 isoforms from *Arabidopsis* and rice MPSS database using 20 bp signature sequence from tissue-specific and abiotic stress-specific libraries (Fig. 7). Analysis of selected *Arabidopsis* tissue library revealed that a significant amount of gene expression (in terms of transcript abundance per million or TPM) was present only in ten isoforms from *AtGRF1* to *AtGRF10*. Distinctly high expression levels in all tissue types except in germinating seedlings (GSE) were shown by *AtGRF1*, *AtGRF3*, *AtGRF4*, *AtGRF9* and *AtGRF11*. Isoforms *AtGRF3* and *AtGRF6* showed a relatively higher expression in actively growing callus tissue (CAS). Isoforms *AtGRF1* to *AtGRF5* and *AtGRF9* to *AtGRF11* showed high expression in roots (ROS/ROF). Similarly, MPSS data

Fig. 2 Phylogenetic relationships among *Chlamydomonas*, *Physcomitrella*, *Selaginella*, *Arabidopsis* and rice 14-3-3 proteins. Multiple sequence alignment was done using MEGA5 software by the maximum-likelihood tree method with 1000 bootstrap replicates. 14-3-3 proteins were categorized into five different clades as shown according to the homology percentage. Each lineage protein is depicted by a different shape



indicated that *AtGRF4*, *AtGRF5* and *AtGRF6* were highly expressed in mature and immature inflorescences. Rice MPSS database revealed root-specific expressions of *Os14-3-3a* and *Os14-3-3b* isoforms as indicated by their consistent expression levels in young roots (NYR) and mature roots (NRA and NRB), and a significant increase in gene expression levels was observed under cold stress in young roots (NCR) in *Os14-3-3b* and under drought in *Os14-3-3a*. Similarly isoform *Os14-3-3f* was found to be relatively highly expressed in both young and mature leaves and germinating seedlings. Noticeably, although *Os14-3-3b* and *Os14-3-3d* showed relatively low basal-level expression, enhanced expression was noted in young leaf tissue after salt (NSL) and cold (NCL) treatments.

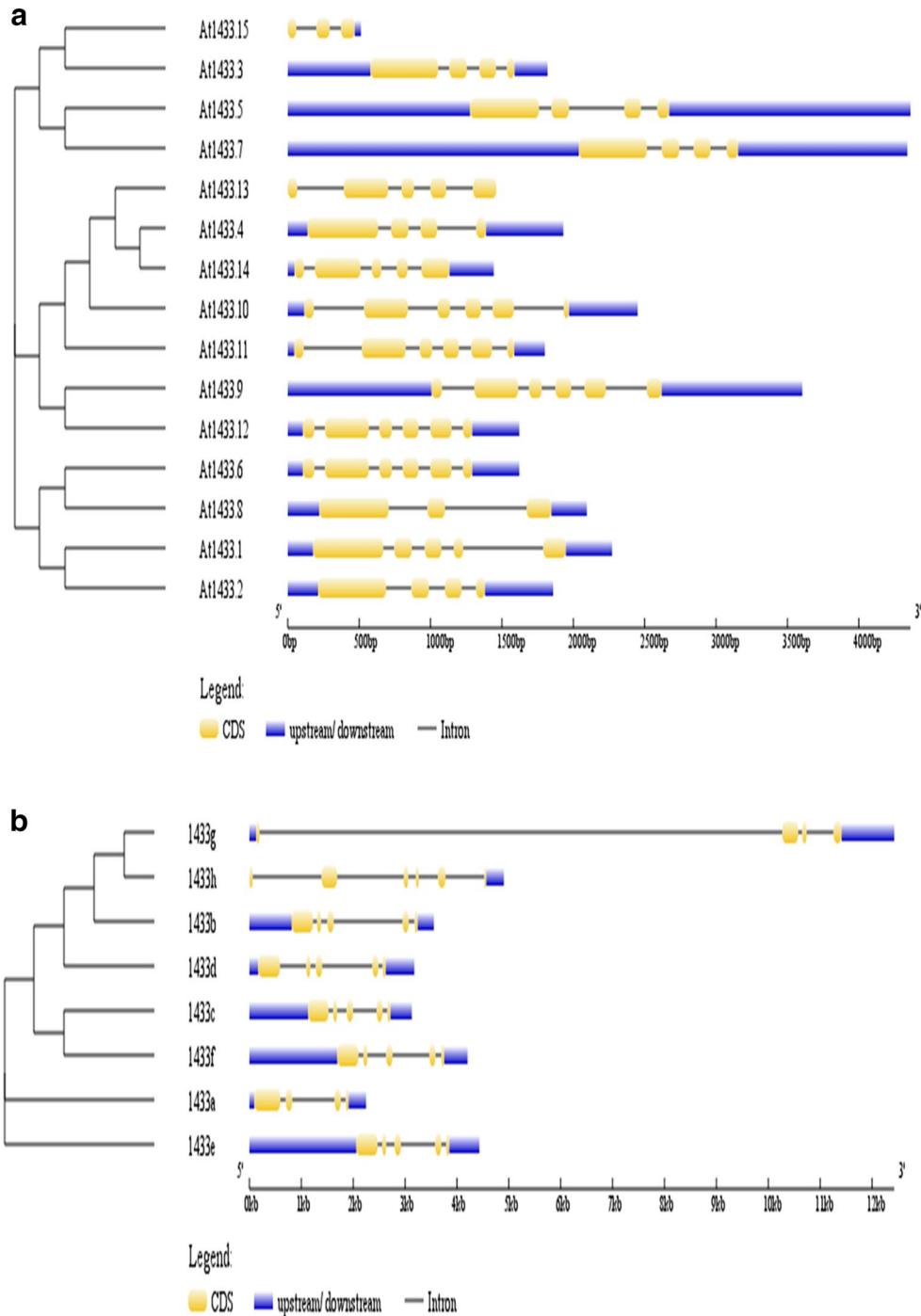
Microarray-based expression analysis of the 14-3-3 isoforms in *Arabidopsis* and rice

Microarray results also revealed variable expression profile of different isoforms for different tissue types. High gene expression in *At14-3-3.12* to *At14-3-3.15* was noted in shoots in response to salt, drought, cold and osmotic stress (Fig. 8). High expression of *At14-3-3.9* was recorded in *Arabidopsis* shoots for almost all the stress treatments tested.

Contrastingly, while almost no expression was recorded for shoots, isoforms *At14-3-3.1*, *At14-3-3.5*, *At14-3-3.7* and *At14-3-3.10* showed elevated gene expression levels in roots under drought treatment. Both *At14-3-3.5* and *At14-3-3.7* isoforms also showed high gene expression in root tissue under osmotic and heat stress (F). Unlike in shoots, *At14-3-3.9* was only found to increase under oxidative stress in *Arabidopsis* roots. In roots the isoform *At14-3-3.10* was found to be highly induced by dehydration, cold, heat, oxidative stress, genotoxicity, wounding and UV/B exposure. However, under salinity stress, the expression of *At14-3-3.10* was increased within 1 h and declined until 12 h after which it showed moderate increase in expression levels. Almost no gene expression was detected in roots under different stress regimes for isoforms *At14-3-3.11*, *At14-3-3.12*, *At14-3-3.13* and *At14-3-3*.

We performed expression analysis of 14-3-3 isoforms during the vegetative and reproductive stages using microarray data in *Arabidopsis* (Fig. 9). Microarray data information was found to be more detailed and rich compared to that in the MPSS database. However, in the majority of the cases, we got similar results in microarray analyses that corroborated with the MPSS data. A thorough analysis of the microarray results showed that *AtGRF1* to *AtGRF4* and *AtGRF8*

Fig. 3 Structural analysis of 14-3-3 genes. **a** Exon–intron structure of Arabidopsis 14-3-3s. **b** Exon–intron structure of rice 14-3-3s. Yellow bars represent exon, blue bars show upstream/downstream and black lines denote introns. The genes are shown according to their phylogenetic clustering and not on the basis of their numbers



and *AtGRF10* were highly expressed in roots. *AtGRF4* was expressed in high fold in almost all the developmental stages. Noticeably, the isoforms *AtGRF1*, *AtGRF13*, *AtGRF14* and *AtGRF15* were specifically expressed in 21-day-old flowers and stamen and in 6-week-old pollen. Similar observations for *AtGRF1* expression in roots of seedling and in petals and sepals of flower buds and siliques of mature plant were reported by Daugherty et al. (1996). Furthermore, *AtGRF13*, *AtGRF14* and *AtGRF15* were also found to be

highly expressed in 8-week-old seeds without siliques, indicating its role in early seed developmental processes. Similar to MPSS results, the isoforms *AtGRF8* and *AtGRF9* were highly expressed in leaves. However, unlike that indicated by MPSS results, *AtGRF8* and *AtGRF9* were also highly expressed in different stages of the seedling.

For rice microarray analysis, we explored Gene Expression Omnibus (GEO) database of the NCBI (<http://www.ncbi.nlm.nih.gov/geo/>) to obtain transcriptional data of

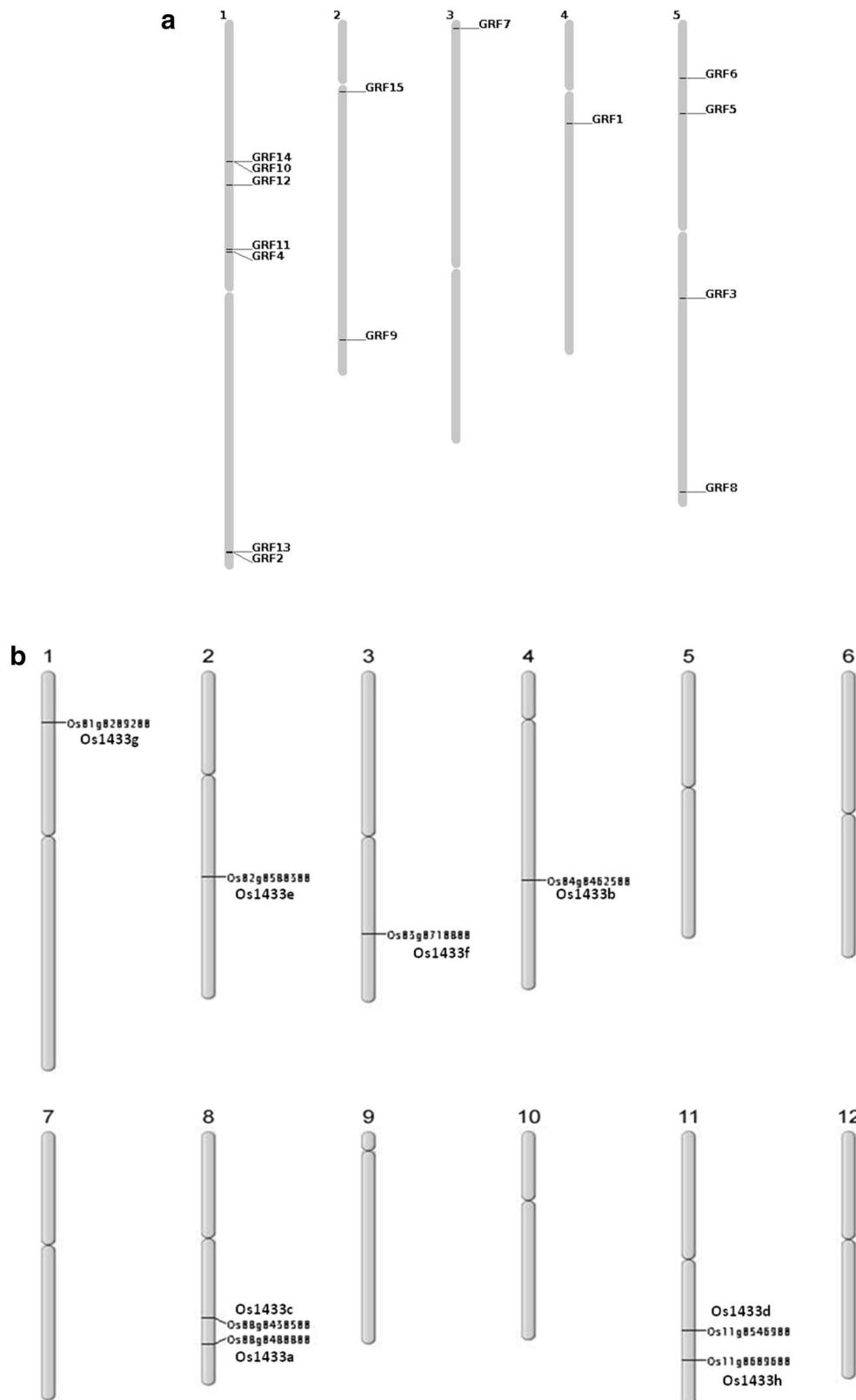


Fig. 4 Genomic distribution of 14-3-3 genes. **a** Genomic localization of *Arabidopsis* 14-3-3s. Fifteen 14-3-3s of *Arabidopsis* are present on all the five chromosomes of *Arabidopsis*. **b** Chromosomal localiza-

tion of rice 14-3-3s. The eight 14-3-3s present in rice were located on only 6 out of 12 chromosomes

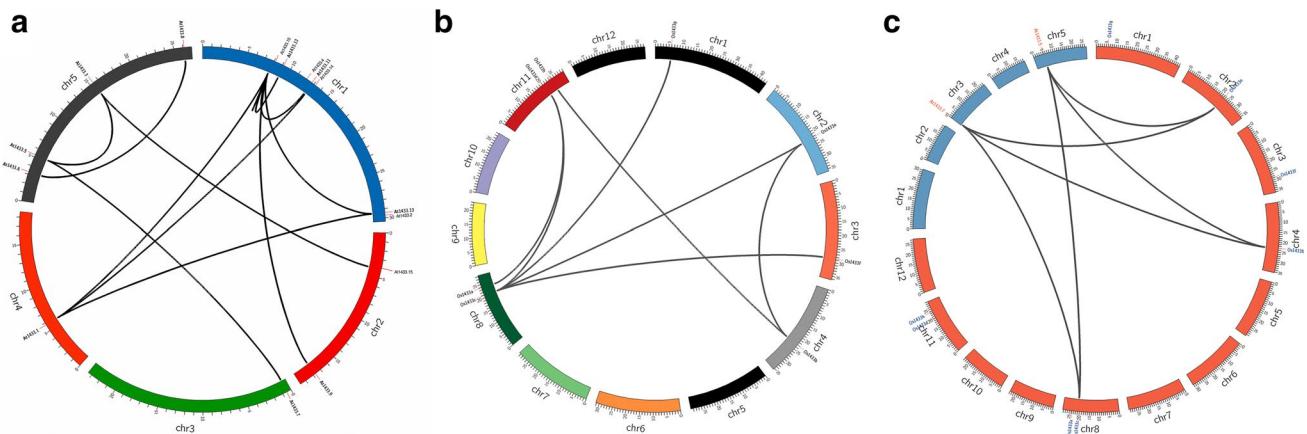


Fig. 5 Chromosomal distribution and synteny analyses of the 14-3 genes between *Arabidopsis* and rice. **a** Chromosomal distribution and synteny analysis of the 14-3-3 genes in *Arabidopsis*. **b** Chromosomal distribution and synteny analysis of the 14-3-3 genes in rice.

c Synteny analysis between *Arabidopsis* and rice 14-3-3s. The syntenic pairs were prepared according to the homology between them. The positions of all the 14-3-3s are depicted in the chromosomes. The black lines indicate the syntenic relationship between them

Table 1 Segmental duplications of 14-3-3 paralogous gene pairs in rice with the estimated duplication dates

Paralogous 14-3-3 pairs	Chr. location	Duplication type	Ka	Ks	Ka/Ks	Approximate duplication date (MYA)
14-3-3a	8	Segmental	0.092	0.38	0.242105	29.2
14-3-3d	11		0.057	0.223	0.255605	17.1
14-3-3b	4	Segmental	0.012	0.268	0.04476	20.6
14-3-3e	2		0.012	0.327	0.03667	25.1
14-3-3c	8	Segmental	0.031	0.43	0.072093	33.0
14-3-3f	3		0.038	0.443	0.085779	34.0
14-3-3 g	1	Segmental	0.168	0.454	0.37044	34.9
14-3-3 h	11		0.221	0.439	0.503417	33.7

14-3-3 isoforms under the different developmental stages of rice. Isoforms *OsGF14c* and *OsGF14f* had a constitutively high expression in all the different stages of development (Fig. 10), suggesting their broad functional attributes in all stages of the life cycle of a rice plant. Our analysis also suggested that *OsGRFa* was highly expressed in mature leaves and regenerating callus tissue, and moderately in seeds. We did not find any expression of *OsGRFg* and *OsGRFh* indicating no significant tissue-specific expression, but they might have other functions related to metabolism or environmental response.

Promoter and miRNA/target analysis of *Os14-3-3f* and *Os14-3-3g* isoforms in different indica rice cultivars

We restricted our expression analysis work to two different isoforms that belong to two different major cluster groups: epsilon (*Os14-3-3g*) and non-epsilon (*Os14-3-3f*). Moreover based on microarray and MPSS results, these two isoforms were found to differ widely in their expression pattern. Transcription factors (TF) affect gene expression by binding to

the corresponding TF-binding sites (TFBS) in the upstream of the target gene. The results obtained using Plant PAN and PLACE database revealed several abiotic stress-related cis-acting elements across 1.0 kb upstream in both *Os14-3-3f* and *Os14-3-3g* in the IR29, Nonabokra and N-22 cultivars (Table 2). These upstream elements play crucial roles in physiological processes like response to light, hormonal regulation and environmental effects. Promoter analysis revealed the presence of low-temperature responsive element (LTRE), both in *Os14-3-3f* and *Os14-3-3g*. Phytohormone-responsive elements like ARE (auxin responsive element) and ABRE (abscisic acid-responsive element) were also present, indicating a 14-3-3 role in phytohormone regulation. The presence of ABRE in *Os14-3-3f* (IR29 and Nonabokra) and a binding site for bZIP factor were detected indicating its role in ABA-mediated gene expression. In addition, binding sites of MYB transcription factor were found in Nonabokra and N-22 for both *Os14-3-3f* and *Os14-3-3g* and indicated its potential role in drought stress.

The evolutionary divergence of the 1 kb upstream and the coding sequence between different 14-3-3s of *Arabidopsis* and

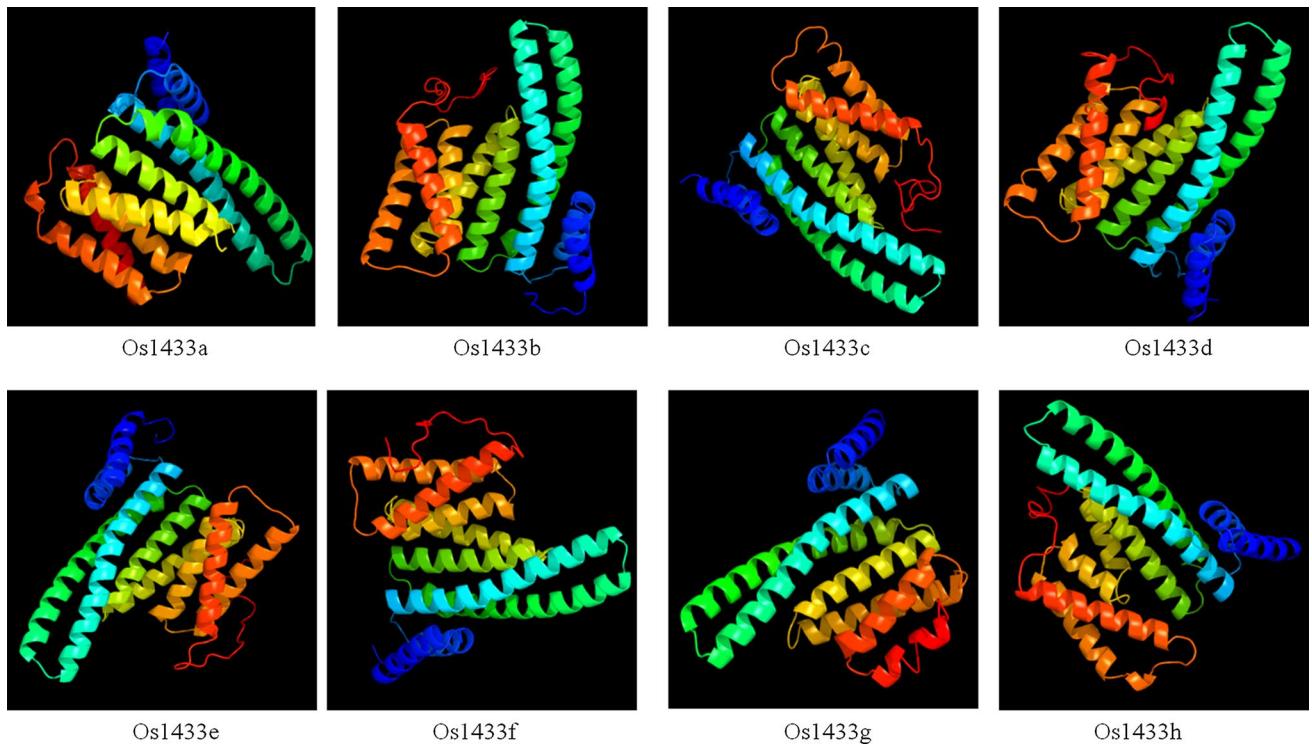


Fig. 6 The predicted 3D models of eight 14-3-3 proteins in rice. The models were prepared by Phyre server at intensive mode with an accuracy of 90% confidence of residues in the allowed region in the

Ramachandran plot analysis. Models were colored by rainbow from the N- to C-terminus

Oryza was calculated as indicated by their p-distance (Fig. 11), which is a measure of the number of nucleotide substitutions occurring between the sequences and indicates that the promoter in case of *Oryza* and coding region in *Arabidopsis* experienced high substitution rates. This is due to transitional and transversional substitutions in their sequences and these evolutionary distances are essential for molecular evolution studies, as they are useful in phylogenetic reconstructions and for estimating divergence time.

MicroRNAs (miRNAs) are endogenous non-coding RNAs that control the gene expression at the post-transcriptional level (Witkos et al. 2011). We searched an array of databases to find miRNA that target 8 *Os14-3-3* isoforms coding sequences (Supplementary Table S3). *Os14-3-3*/miRNA search was also performed in psRNATarget database with more stringent cut-off threshold value (0–2.0) to lower the false positive prediction rate. We detected only osa-MIR396 g that mainly targets GRFs (Growth Regulatory Factors) which are conserved (Debernardi et al. 2012).

Semi-quantitative RT-PCR of *Os14-3-3f* and *Os14-3-3g*

Salinity stress

Both, 14-3-3f and 14-3-3g expression in lamina, increased gradually (1.5 folds) after 3 and 6 h of NaCl stress (200 mM) and decreased after 12–24 h of treatment (Fig. 12a). Relatively high 14-3-3f expression in lamina was detected in both Nonabokra (salt tolerant) and N-22 (drought tolerant) cultivars at 3 and 6 h of NaCl treatment. Expression of 14-3-3f increased from the control up to 12 h of salt treatment and sharply decreased thereafter. The expression of 14-3-3g both in the lamina and the roots increased significantly in Nonabokra and N-22 after 3 and 6 h of NaCl treatment. Similarly, relatively high 14-3-3g transcript accumulation was recorded in Nonabokra and N-22 roots after 3 and 6 h of NaCl treatment and decreased thereafter. No significant fold change in expression of 14-3-3g occurred in IR29 even after 24 h of treatment.

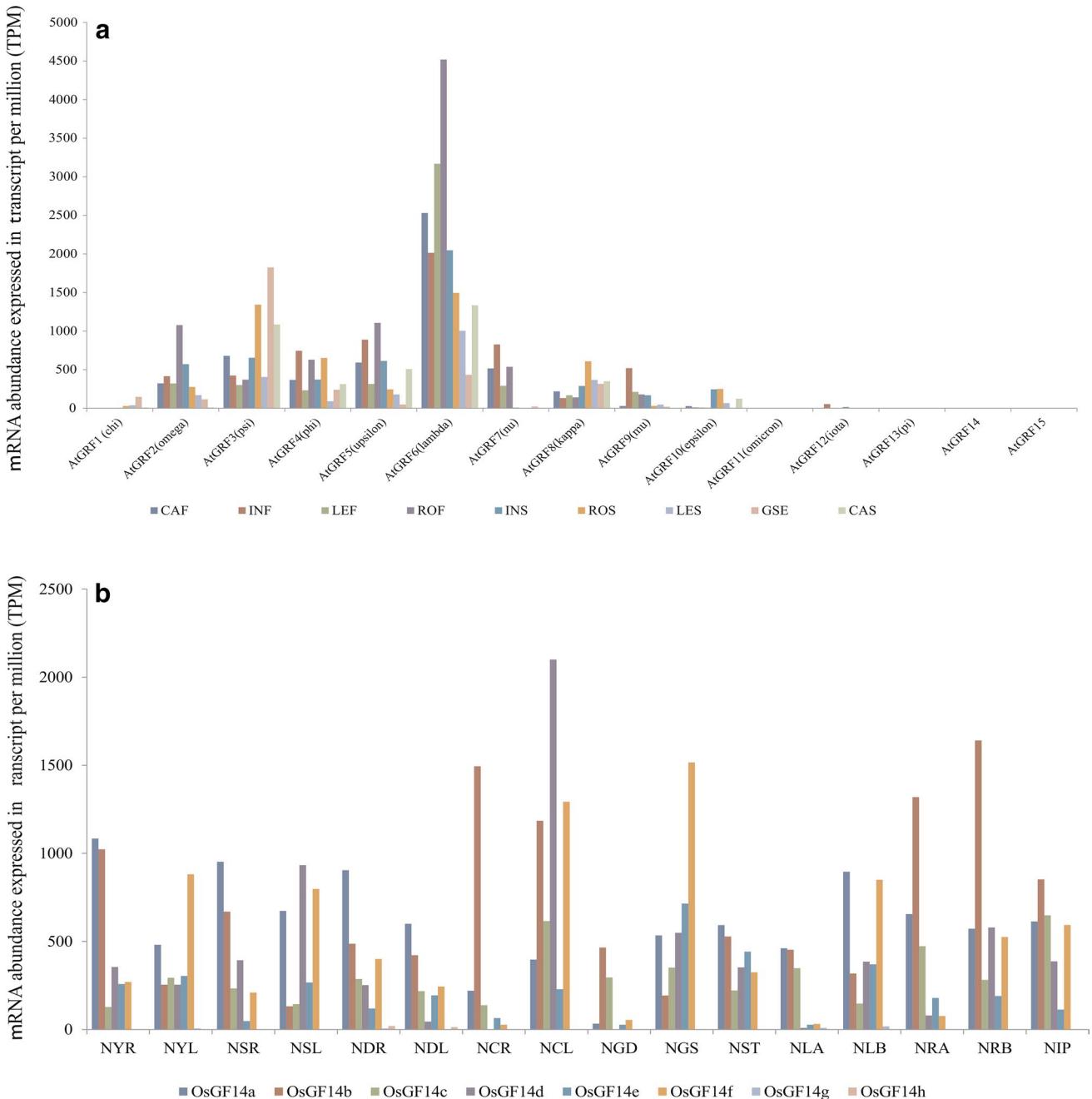


Fig. 7 a Transcript abundance of Arabidopsis 14-3-3s using MPSS. The libraries present in the MPSS database were analyzed for the expression level of all 14-3-3s in Arabidopsis-like CAF (callus actively growing), INF (inflorescence—mixed stage), LEF (leaves—21-day-old), ROF (root—21-day-old), INS (inflorescence—mixed stage, mature buds), ROS (root—21-day-old), LES (leaves—21-day-old), GSE (germinating seedlings) and CAS (callus—actively growing). **b** Transcript abundance of rice 14-3-3s in different tissue-specific libraries and different abiotic stress conditions in rice from the MPSS database such as NYR (14-day-young roots), NYL (14-day-

young leaves), NSR (14-day-young roots, 250 mM NaCl, 24 h), NSL (14-day-young leaves, 250 mM NaCl, 24 h), NDR (14-day-young roots, drought 5d), NDL (14-day-young leaves, drought 5d), NCR (14-day-young roots, cold treated, 4C, 24 h), NCL (14-day-young leaves, cold treated, 4C, 24 h), NGD (germinating seedlings grown in dark 10d), NGS (3-day-old germinating seeds), NST (60-day-old stem), NLA (60-day-old mature leaves, rep A), NLB (60-day-old mature leaves, rep B), NRA (60-day-old mature root, rep A), NRB (60-day-old mature root, rep B) and NIP (90-day-old immature panicle)

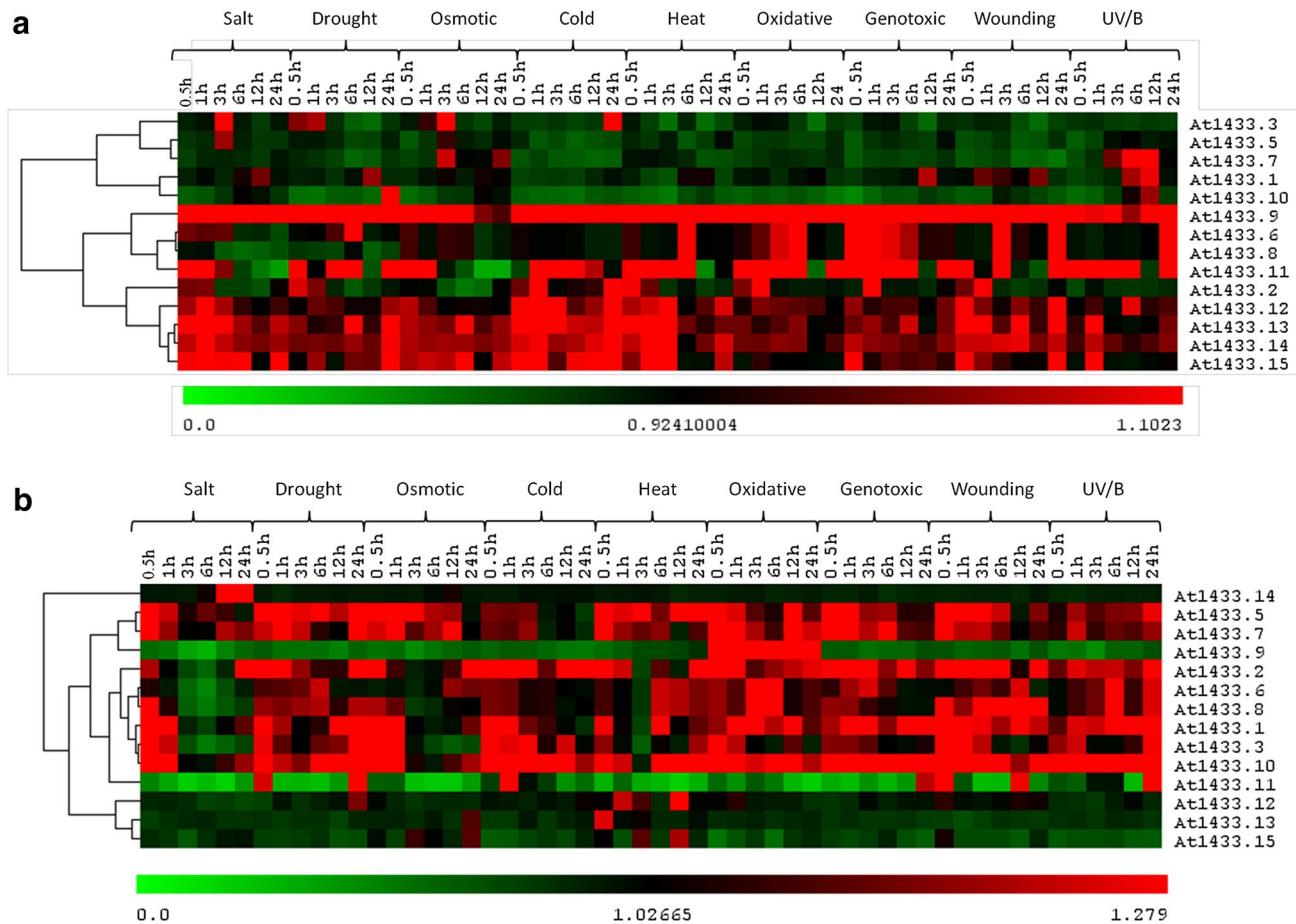


Fig. 8 Microarray-based expression profile of *Arabidopsis* 14-3-3 genes under several abiotic stress conditions. Heat maps show the fold change in expression pattern of 14-3-3 genes during different abiotic stress conditions such as salt, drought, osmotic, cold, heat, oxidative, genotoxic, wounding and UV/B in root (**a**) and shoot (**b**) for different time points, i.e., 0.5, 1, 3, 6, 12 and 24 h. The data were

obtained from the AtGenExpress database and the signal values are depicted by the color bar shown at the bottom of the heat map. The scale for the relative expression value is given below the heat map, in which green color shows downregulation, dark green shows no significant change in expression and red color shows upregulation

Dehydration stress

Expression of 14-3-3f in shoots and roots in IR29 increased steeply from 0 to 3 h (twofold) and recorded the highest at 6 h in lamina and 12 h in root tissue when exposed to 20% PEG6000 (Fig. 12b). Both Nonabokra and N-22 showed high 14-3-3f expression in roots at 12 h of stress. The highest expression of 14-3-3g transcript was recorded in shoots of Nonabokra and in roots of N-22. Low level expression of 14-3-3g was noted in roots of Nonabokra and N-22 as compared to control even up to 24 h of dehydration stress (Fig. 12b).

Cold stress

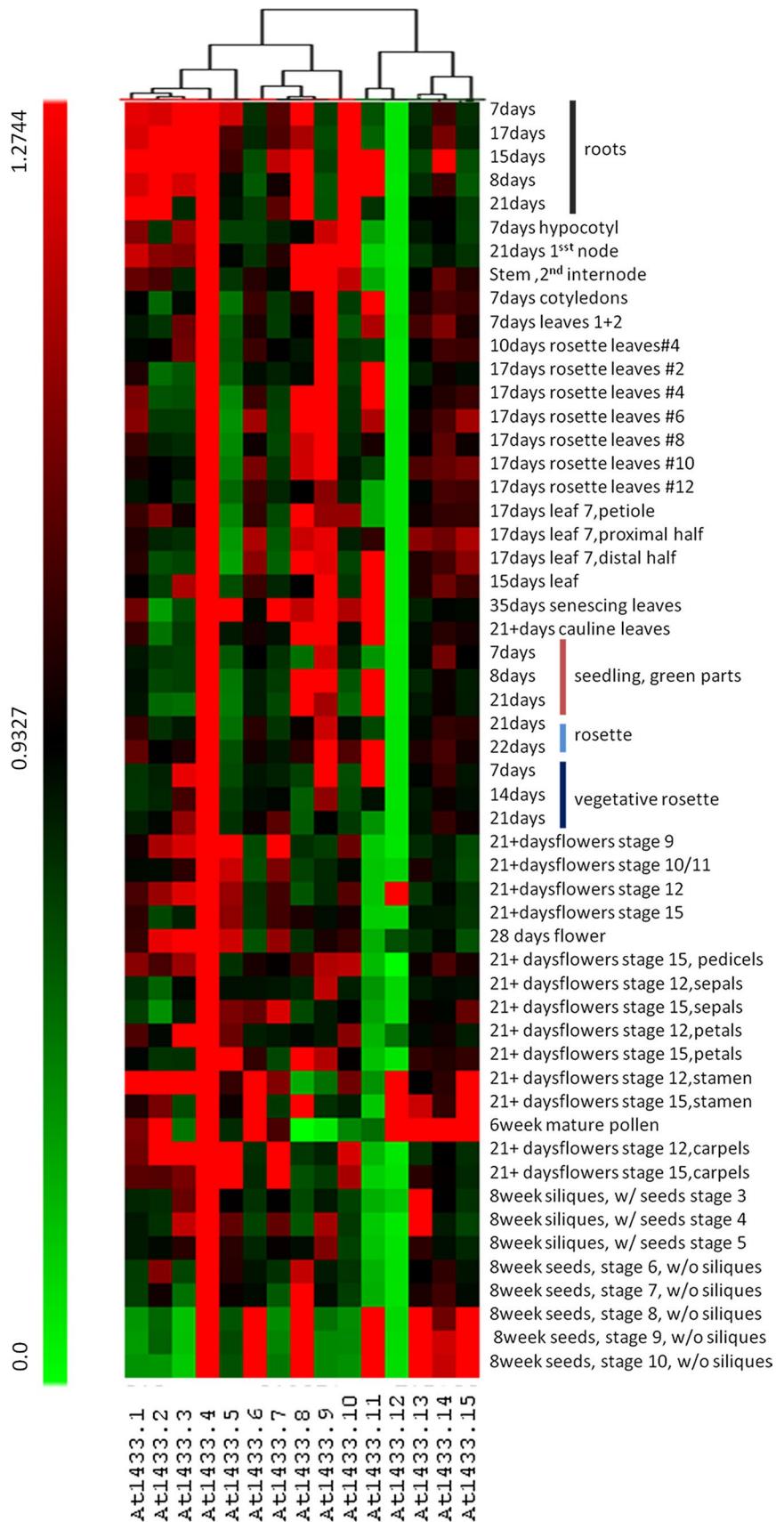
Both 14-3-3f and 14-3-3g expression was highly induced by cold stress in Nonabokra and N-22 (Fig. 12c). The expression of 14-3-3f was relatively higher in lamina than roots

in all three cultivars. Relatively higher expression levels of 14-3-3f in Nonabokra roots was noted at 6 and 24 h (twofold) of treatment with a sharp decrease at 12 h. The expression of 14-3-3g in roots after cold treatment increased marginally from control and remained steady till 24 h of treatment. However, we noted that the expression of 14-3-3g was very high in control roots in Nonabokra and N-22 and the expression decreased steadily after 24 h of cold treatment (Fig. 12c).

Exogenous ABA treatment

No apparent increase in the expression of 14-3-3f in the lamina of N-22 and Nonabokra was noted (Fig. 12d). However in IR29, 14-3-3f increased strongly after 3 and 6 h (four fold) of treatment in lamina and decreased sharply after 12–24 h of ABA treatment. Similarly, 14-3-3f expression increased in IR29 roots after 3 and 6 h and decreased at 24 h

Fig. 9 Microarray-based expression profile of *Arabidopsis* 14-3-3 genes at different developmental stages. Color bars represent the \log_{10} expression values. The different developmental stages used for expression profiling are shown on the right side of the heat map. The scale for relative expression values is given on the left of the heat map, in which green color represents the lowest expression levels, dark green medium and red denotes the highest expression level



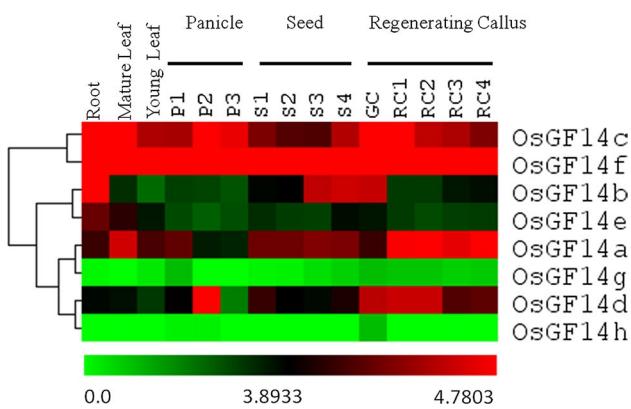


Fig. 10 Expression profile of rice 14-3-3s at different developmental stages using microarray data. The developmental stages used for analysis are depicted at the top of each column. The various stages used for expression profiling studies are roots of 7-day-old seedlings, mature leaf (collected before pollination), young leaf, 7-day-old seedlings, P(0–3 cm panicle), P2 (3–5 cm panicle), P3 (5–10 cm panicle), S1 (0–2 DAP), S2 (3–4 DAP), S3 (5–10 DAP), S4 (11–20 DAP), GC (growing callus), R1 (regenerating callus 2 days), R2 (regenerating callus 4 days), R3 (regenerating callus 6 days) and R4 (regenerating callus 8 days). The values are normalized and \log_{10} transformed. The scale for the relative expression value is shown at the bottom of the heat map in which green color denotes no significant change, while red color denotes the highest change in expression

of ABA exposure. Moderate increase in 14-3-3g expression was recorded in roots of IR29, and N-22 after ABA treatment. However, the expression of 14-3-3g in Nonabokra roots was relatively higher in the control set and thereafter decreased on increasing the ABA exposure time interval. Both Nonabokra and N-22 showed a marginal increase in the 14-3-3g expression in roots on increasing the ABA exposure time interval. Nonabokra exhibited the highest expression of 14-3-3g transcript in the lamina at 12 h of treatment (Fig. 12d).

Developmental stages of plant growth

14-3-3f was found to express in all tested tissue and stage type in all rice cultivars. Moderate expression of 14-3-3f was noted in 12-day-old shoots, 12-day-old roots, stems, panicles and mature roots of IR29 (Fig. 12e). The expression of 14-3-3f in Nonabokra was only relatively high in flag leaf, stems and panicles. A fairly high expression of 14-3-3f was noted in stems, panicles and mature roots in N-22. A high expression of 14-3-3g was recorded in N-22 across all stages. Relatively high 14-3-3g expression in developing seed was found in N-22 compared to IR29 and Nonabokra. The expression of 14-3-3g in the flag leaf was the highest in N-22. A high expression of 14-3-3g was recorded in mature stems, panicles and mature roots of N-22. A very weak expression level of 14-3-3g was detected in IR29. Low

Table 2 Upstream analysis of 1 kb upstream 14-3-3f (a) and 14-3-3g (b) genes from different indica rice cultivars differing in tolerance to abiotic stress

Element	IR-29	Nonabokra	N-22
a			
ARE	1	0	0
bHLH	1	2	1
MBS	2	2	2
Skn-1 motif	0	1	1
Sp1	2	1	0
AuxRR core	1	0	0
MBS (drought ind)	0	1	1
LTRE	0	1	0
TCA elements	0	1	0
ABRE	1	1	0
bZIP binding site	0	1	1
b			
ARE	1	0	0
bHLH	1	2	1
MBS	1	2	2
Skn-1 motif	0	1	1
Sp1	2	1	0
AuxRR core	1	0	0
MBS (drought ind)	0	1	1
LTRE	0	1	0
TCA elements	0	1	0
ABRE	0	0	0
bZIP binding site	0	0	1

Few abiotic stress-related cis-acting regulatory elements are shown

to moderate expression was noted in Nonabokra across all stages of plant development (Fig. 12e).

Expression profiling of 14-3-3f and 14-3-3g using quantitative real-time PCR under various abiotic stress and developmental stages

To validate the microarray and semi-quantitative RT-PCR data, we carried out an expression study using quantitative real-time reverse transcription-PCR (qRT-PCR) from lamina and root samples in IR29, Nonabokra and N-22 rice cultivars grown under salt, drought, cold and ABA treatments and in different stages of plant development (seed to mature plant). The relative transcript abundance of the *Os14-3-3f* and *Os14-3-3g* genes under various abiotic stresses, viz., salinity, dehydration, cold and exogenous ABA and in different stages of growth has been presented as bar graphs (Fig. 13). Our real-time PCR results were found to be consistent with semi-quantitative-PCR results. the expression of 14-3-3f as well as 14-3-3g increased significantly after 3 and 6 h of stress treatment. Relatively high expression of 14-3-3f

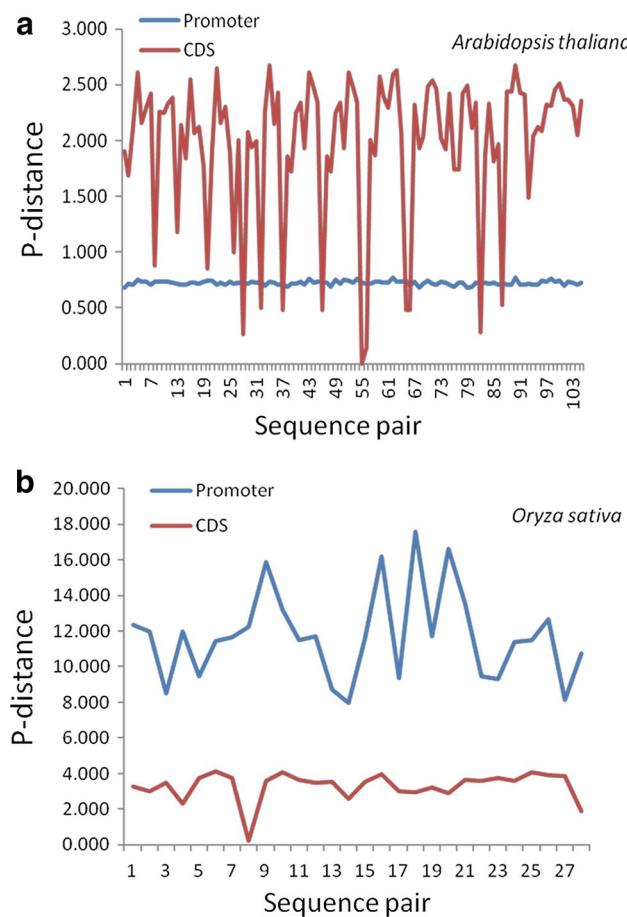


Fig. 11 Pairwise sequence divergence of 1 kb upstream of the coding region of all the sequence pairs estimated using p-distance in *Arabidopsis* (a) and rice (b)

occurred under salinity and dehydration stress in N-22 and Nonabokra cultivars compared to sensitive IR29, confirming its important role in salt and drought stress regulation in rice. A high transcript abundance was detected for 14-3-3f in 6 h salt-treated roots. Similarly, N-22 cultivars showed a high 14-3-3f expression after 6 h of dehydration stress in lamina and roots. An increase in transcript level was also recorded under 6 h cold stress treatment, particularly in N-22 shoots and N-22 and Nonabokra roots. Unlike other treatments, 14-3-3f was found to be upregulated in both lamina and roots of IR29 after 6 h of ABA treatment. Our data therefore confirmed that *Os14-3-3f* was found to be highly inducible by salt and dehydration stress, especially both in shoots and in roots of Nonabokra and N-22, respectively, and might have an important role in the mitigation of abiotic stress.

Similar to *Os14-3-3f*, the expression of *Os14-3-3g* also moderately increased up to 6 h and then declined. Relatively high expression of *Os14-3-3g* was detected in roots of N-22 and Nonabokra after 3 and 6 h of salt and drought treatment, respectively. In response to cold stress, 14-3-3g expression

was higher in Nonabokra cultivars than in N-22 and IR29. However, a very low expression level of 14-3-3 was detected in Nonabokra roots under cold stress. Moreover, 14-3-3g was upregulated in roots of IR29 and N-22 in cold stress. The expression of 14-3-3g increased after ABA treatment in lamina and root samples of all three rice cultivars up to 6 h of treatment. Relatively higher transcript abundance was noted in roots of IR29 after ABA treatment. A major pathway activated by stresses such as drought, temperature and salt stress is the abscisic acid (ABA) signaling pathway. High induction of *Os14-3-3f* and *Os14-3-3g* under ABA treatment supported their involvement in the ABA signal pathway by interaction with the AREB/ABF/ABI5-like transcription factors that bind to ABA-response elements (Schoonheim et al. 2007).

Our qRT-PCR demonstrated a differential expression of *Os14-3-3f* and *Os14-3-3g* across different tissues. High 14-3-3f transcript levels were found in panicles, flag leaf and dry seeds and mature roots particularly in N-22 cultivars. Relatively high 14-3-3f transcript abundance was recorded in 4-day-old lamina in IR29 compared to Nonabokra and N-22. Our results showed low 14-3-3f expression levels in 4-day-old and 12-day-old lamina and roots. The expression of 14-3-3g was also very high in panicles, dry seeds, 4-day-old lamina and 12-day-old roots in N-22 cultivars. Unlike *Os14-3-3f*, high 14-3-3g transcripts accumulated in stems and 4-day-old roots, especially in Nonabokra and IR29 (Fig. 13).

Inducible expression of *Os14-3-3f* protein in response to abiotic stress

Western-blot analysis detected a single protein band differentially expressed at 28 kDa in different tissue types in three rice cultivars under different abiotic stress with respect to anti-histone H3 antibody (Fig. 14a). Densitometry scanning and normalization of *Os14-3-3f* bands (28 kDa) revealed a significant fold increase in protein expression after 3 and 6 h and declining thereafter at 12 and 24 h of exposure to salinity, dehydration and ABA, except in cold when *Os14-3-3f* protein was expressed even after 24 h of cold stress in Nonabokra (Fig. 14b). Compared to IR29, Nonabokra and N-22 showed a nearly twofold increase in protein expression level after 3 h of exposure to stress. In addition, *Os14-3-3f* was relatively highly expressed under dehydration condition in N-22. Similarly in both Nonabokra and N-22, a relatively high *Os14-3-3f* protein expression in seeds, flag leaf, stems, panicles and mature roots was determined compared to that in IR-29. *Os14-3-3f* protein accumulation was highest in flag leaves, stems and panicles and lowest in seeds and mature roots. These results were in congruence with our gene expression data and further confirmed that *Os14-3-3f* was highly inducible by one or more abiotic stress conditions.

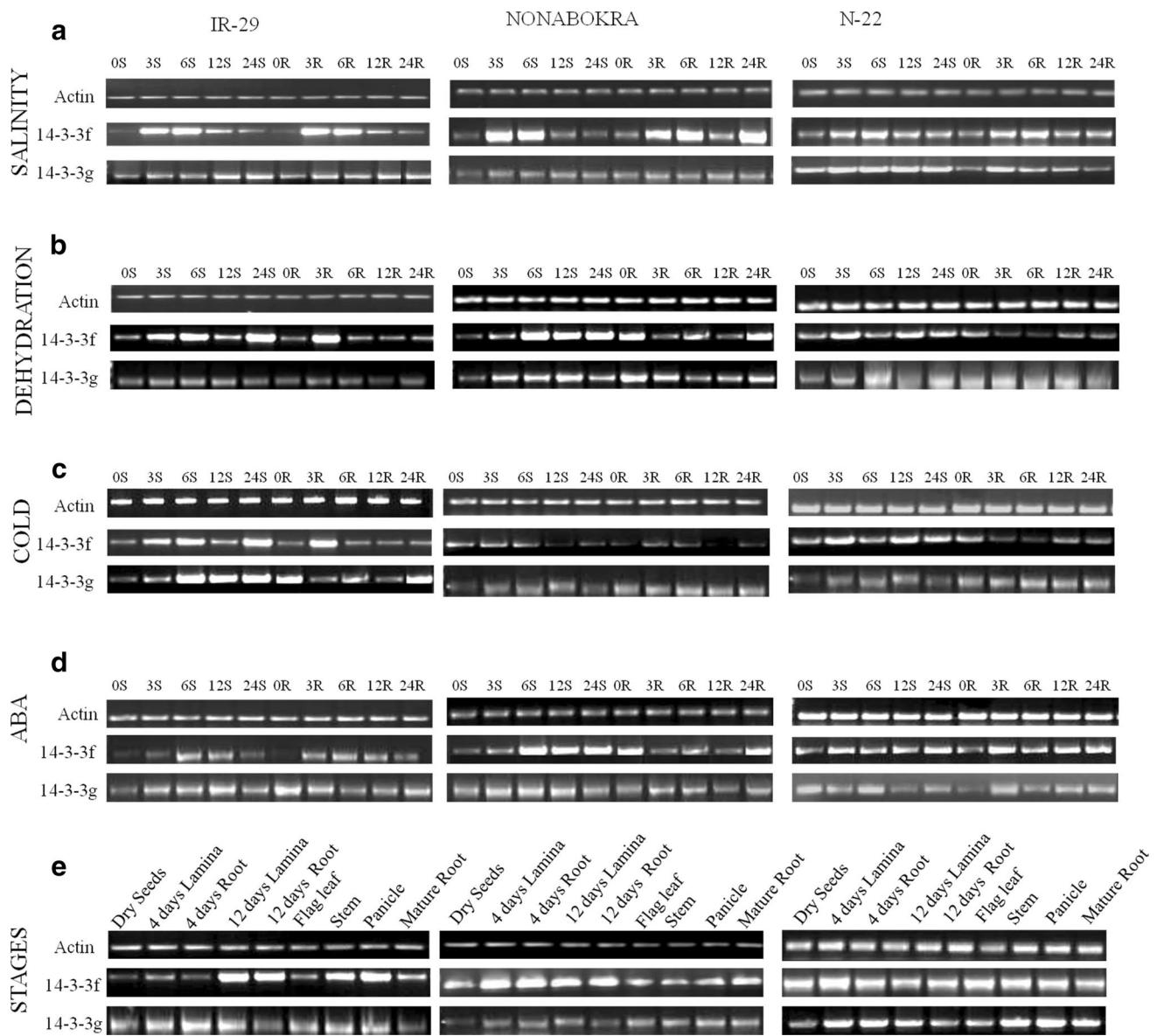


Fig. 12 Expression profile of 14-3-3f and g genes under different abiotic stress conditions using semi-quantitative RT-PCR. 14-3-3f and g genes expression under different abiotic stress conditions such as salinity (200 mM NaCl), dehydration (20% PEG), cold (4 °C) and

exogenous ABA (100 µM) for 0, 3, 6, 12 and 24 h and in different stages of development such as dry seeds, 4-day-old lamina and root, 12-day-old lamina and root, flag leaf, stems, panicles and mature roots of 90-day-old rice plants

Discussion

14-3-3 Proteins are key regulatory ubiquitous molecules involved in a plethora of metabolic and physiological pathways that are regulated by phosphorylation. Some recent reports have shown that plant 14-3-3s are regulated by multiple stress pathways, including cold, salinity and wound (Liu et al. 2016). Localization of the 14-3-3 family members inside organelles such as chloroplasts (Sehnke et al. 2000), nucleus (Bihl et al. 1997) and mitochondria (Sehnke and Ferl 2000), as well as in the cytoplasm (Bihl et al. 1997),

further demonstrates their global regulatory potential and their apparent diversity in expression and function. Identification and characterization of a large set of the 14-3-3 family in *Arabidopsis* (DeLille et al. 2001) followed by several other species have shown phylogenetically diverse epsilon and non-epsilon group of isoforms. Hence, comparative studies among isoforms within and among taxa are necessary to understand the evolution and further functional diversity in 14-3-3 genes. Our results provide the first comprehensive report for genome-wide molecular and phylogenetic characterization of 14-3-3 isoforms in some selected taxa

(lower to higher plant groups) along with *in silico* analysis of the expression pattern in rice and *Arabidopsis* using MPSS and microarray-based database.

The present analysis of 14-3-3 isoforms in five different taxa including *Arabidopsis* and rice revealed that 14-3-3 isoforms were phylogenetically conserved among plant groups, especially in the core region. However, amino acids facing the outside of the 14-3-3 molecule are relatively less conserved in all plant lineages as similarly reported earlier among vertebrates, yeast and plants (Liu et al. 1995). The amino acids in the dimerization domains are not completely conserved which might indicate differences in dimer formation between isoforms. From alignment results, it was noted that all 14-3-3 isoforms of different plant lineages showed the presence of α -helices, typical in the 14-3-3 structure. This reportedly serves as an amphipathic groove for attachment of the polypeptide chain of the target protein and NES (nuclear export signal) that interacts with chromosome maintenance region 1 (Crm1) and nuclear pore that further drives 14-3-3 and its target assemblage out of the nucleus (Fukuda et al. 1997).

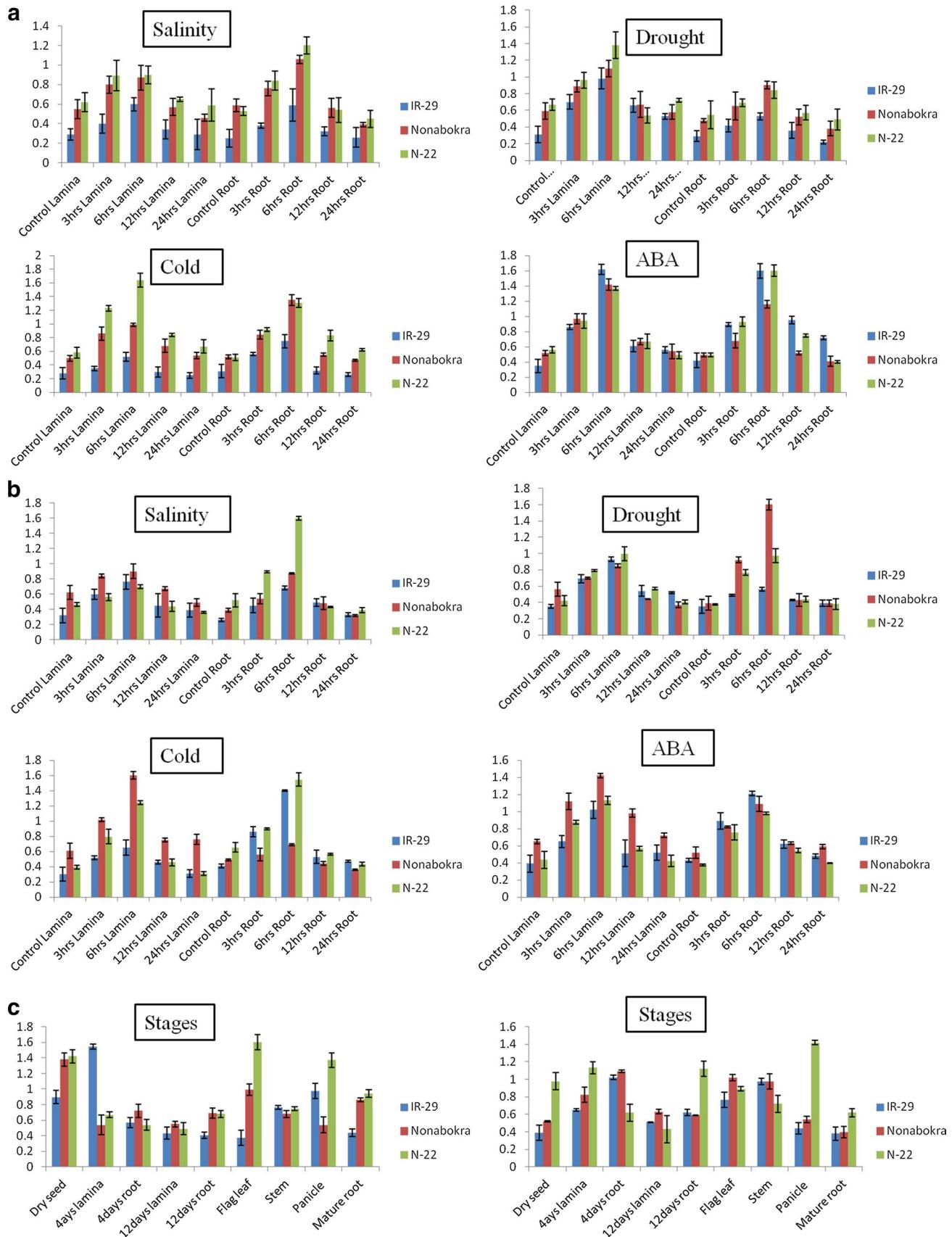
The majority of 14-3-3 isoforms in rice were included in the non-epsilon group except *Os14-3-3g* and *Os14-3-3h* that clustered in the epsilon group. This indicated that some 14-3-3 genes (isoforms) in monocotyledon plants might be lost during the course of evolution. The retained one or two group 14-3-3 genes might play a conserved function in monocotyledon plants as similarly reported by other researchers (Tian et al. 2015). The majority of non-epsilon isoforms of different plant groups clustered tightly in Clade 4, indicating major independent duplication events leading to isoform diversification after the separation of the epsilon and non-epsilon branches (Fig. 2). The non-epsilon isoforms in rice clustered separately from the dicotyledonous group, indicating that monocot 14-3-3 isoforms are sequentially divergent and arise from independent gene duplication events (Wu et al. 1997). Since the non-epsilon isoforms can be found in all organisms in greater numbers, it is thought that this phylogenetic branch might be involved in basal eukaryotic 14-3-3 functions, while the epsilon group is involved in organism-specific 14-3-3 functions (Jaspert et al. 2011).

Our synteny analysis showed only two *Arabidopsis* 14-3-3 orthologs (*At14-3-3.5* and *At14-3-3.7*) to have syntenic pairing (13%) with only three *O. sativa* 14-3-3 isoforms (14-3-3b, 14-3-3c, and 14-3-3e). The tandem duplication events were found to be extremely low in 14-3-3 gene diversification. Therefore, an examination of the orthologous and paralogous sequences in both *Arabidopsis* and rice indicated that *At14-3-3.5* and *At14-3-3.7* and *Os14-3-3b*, *Os14-3-3c* and *Os14-3-3e* isoforms (with segmental duplication in paralogous sequences in both genomes) might be derived from a common ancestor, but underwent rapid differentiation

and evolution after the separation of dicot and monocot species. The results also indicated that the timing of large-scale duplication events were less divergent, with values ranging from 17 to 35 million years ago (MYA), primarily in the past 30–40 MYA. These results were supported by a previous notion that *Arabidopsis At14-3-3* isoforms have undergone two major duplication events about 170 and 50 MYA that led to gene arrangement and loss of isoforms (Rosenquist et al. 2001). The results also showed that four segmental duplication events as recorded in rice 14-3-3 genes were probably recent and close to the origination time of monocots (Blanc and Wolfe 2004; Yao et al. 2007). From the results of the sequence alignment and homology modeling, it appeared that the less conserved C-terminal domain was responsible for functional diversities between different isoforms (Fig. 6).

Involvement of the 14-3-3 isoforms in mitigating abiotic stress in plants has been a primary focus of research. A wide array of recent reports identified and confirmed the role of 14-3-3 genes in several abiotic stress responses in major plant lineages (Shanko et al. 2003; Wang et al. 2009; Campo et al. 2011; Sun et al. 2011; Ho et al. 2013; Yang et al. 2017). Evidence that 14-3-3 plays a major functional role in environmental stress responses comes from the overexpression of *Arabidopsis* 14-3-3 in cotton, resulting in drought-tolerant phenotype characterized by less wilting and higher transpiration and photosynthesis rate than wild type due to increased stomatal opening (Yan et al. 2004). Similarly, the 14-3-3 gene (TFT4) from tomato when overexpressed in *Arabidopsis* caused a significant increase in H⁺ efflux and the activity of plasma membrane H⁺-ATPase, and was reportedly involved in the regulation of H⁺ efflux and basipetal IAA transport under alkaline stress (Xu et al. 2013). Overexpression of *Glycine soja* 14-3-3 protein *GsGF14o* in *Arabidopsis thaliana* was shown to be involved in plant development and drought response via induced reduction of plant tolerance at seed germination and seedling growth stages (Sun et al. 2013, 2016). Recently, transgenic *Arabidopsis* plants overexpressing *BdGF14a*, one of the 14-3-3 isoforms from *Brachypodium distachyon*, exhibited an improved tolerance to drought stress with increased leaf water content and reduced electrolyte leakage (Yang et al. 2017). A previous report based on semi-quantitative RT-PCR showed that 14-3-3s in japonica rice was differentially inducible by abiotic stress and heavy metals (Yao et al. 2007). The role of GF14c in drought tolerance was confirmed experimentally by the transgenic approach. Overexpression of GF14c enhanced drought tolerance in transgenic rice seedlings and GF14c was also demonstrated to function under the control of *OsCDPK1*, a calcium-dependent protein kinase gene (Ho et al. 2013).

In our present results, both microarray and MPSS analysis revealed that 14-3-3 isoforms in *Arabidopsis* and rice were differentially expressed under various abiotic stress



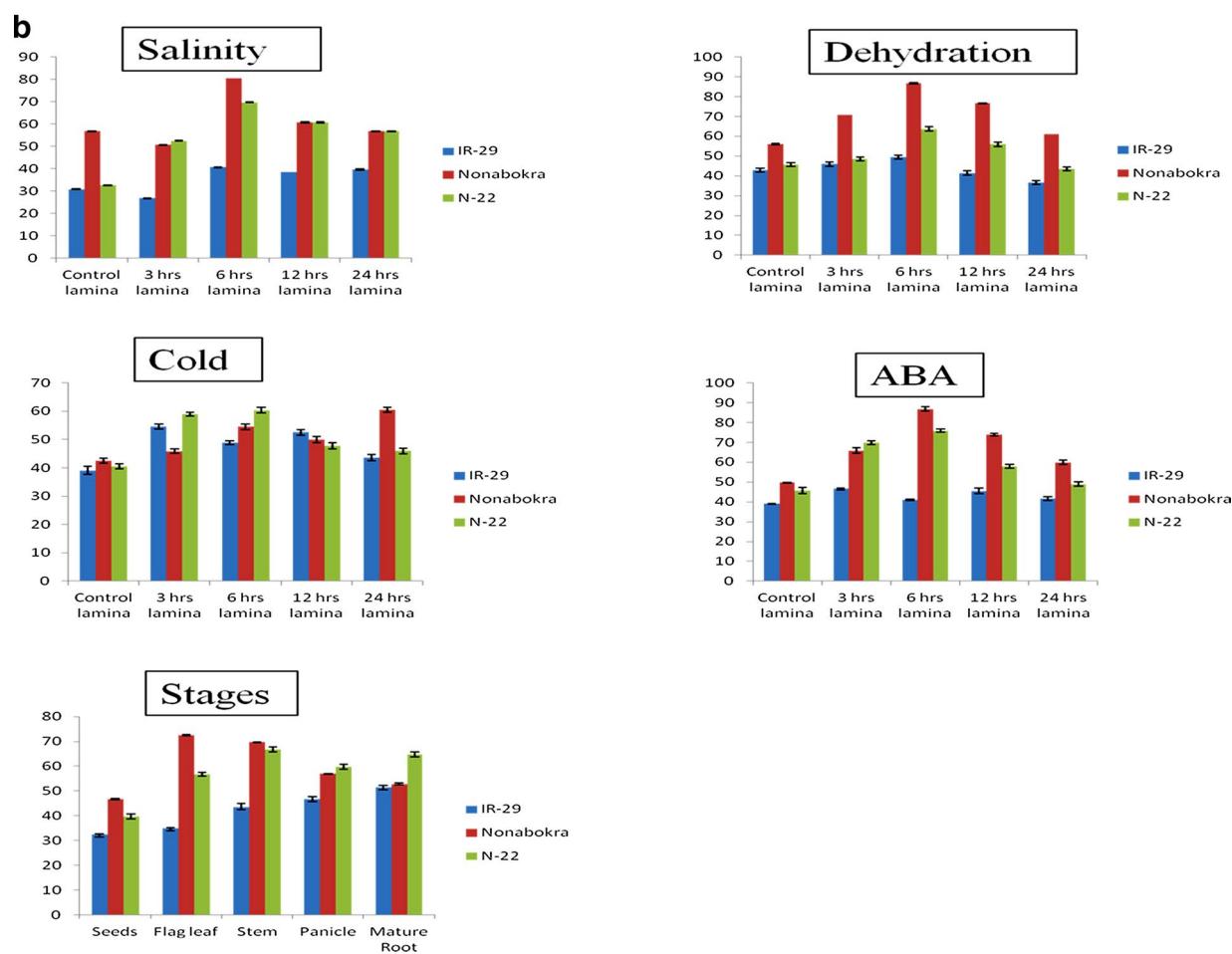
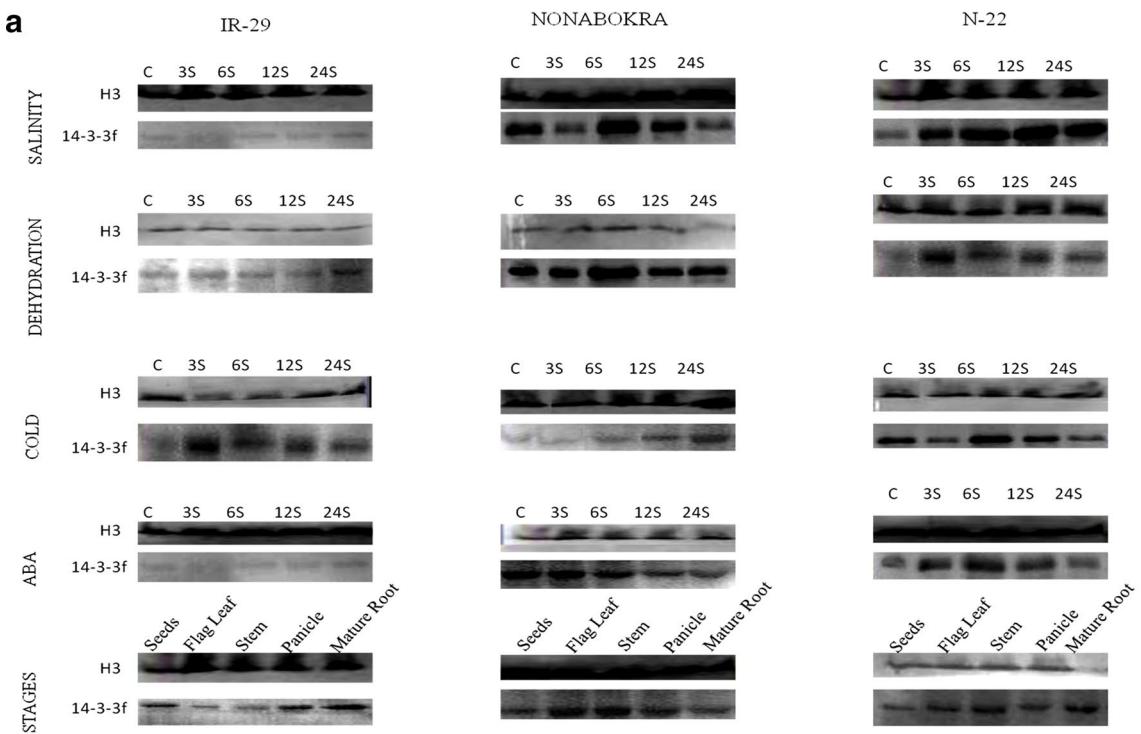
◀Fig. 13 Expression profile of 14-3-3f and g genes under different abiotic stress conditions and in different developmental stages of rice using real-time PCR. Bar graphs show the fold change in the expression of 14-3-3f (**a**) and 14-3-3 g (**b**) genes during abiotic stress such as salinity (200 mM NaCl), dehydration (20% PEG), cold (4 °C) and exogenous ABA (100 µM) for 0, 3, 6, 12 and 24 h. **c** Fold change in the expression of 14-3-3f and g genes in different stages of development such as dry seeds, 4-day-old lamina and root, 12-day-old lamina and root, flag leaf, stems, panicles and mature roots of 90-day-old rice plants

regimes in lamina and root tissue. Data from semi-quantitative RT-PCR and qPCR confirmed that both *Os14-3-3f* and *Os14-3-3g* were highly inducible by salinity, drought and cold stress in Nonabokra and N-22 cultivars, but with different magnitudes in the shoot and root tissue, especially after 3–6 h of exposure. However, 14-3-3f was found to be upregulated in both lamina and roots of IR29 after 6 h of ABA treatment. Relatively higher expression of 14-3-3g was found in roots than in shoots of Nonabokra and N-22 after salt and dehydration stress. Increased accumulation of *Os14-3-3f* protein in Nonabokra and N-22 compared to IR29 under salinity and dehydration and more intensely in cold stress condition confirmed our observation that *Os14-3-3f* could have diverse roles and might be a key element in abiotic stress physiology. Immunoblot assays demonstrated that *Os14-3-3f* protein accumulated nearly in all stages of plant growth with the highest expression in panicles, mature roots and stems, thereby indicating its requirement in plant growth and metabolism. We also noted that the expression of 14-3-3g increased after ABA treatment in the lamina and root samples of all three rice cultivars up to 6 h of treatment. A relatively high transcript abundance was detected in the roots of IR29 after ABA treatment. In addition, isoforms *Os14-3-3f* and *Os14-3-3g* had differential expression levels at different stages of growth. Low levels of 14-3-3 (f and g) gene expression under abiotic stress treatments, but an elevated response after exogenous ABA in IR29 (sensitive) indicated a strong correlation of gene expression and ABA level. Generally, the intercellular or endogenous ABA concentration in leaves increased 10- to 50-fold within a few hours of the onset of water deficit, caused by high osmoticum, high NaCl or drying (dehydration).

A major pathway activated by stresses such as drought, temperature and salt is the ABA signaling pathway that regulates the physiology from the seed to adult plant (Nakashima and Yamaguchi-Shinozaki 2013; Raghavendra et al. 2017). ABA is an essential mediator for triggering plant responses to adverse environmental stimuli (Yamaguchi-Shinozaki and Shinozaki 2006). Under drought stress conditions, ABA promotes stomatal closure to prevent water loss. Transcriptional monitoring of 14-3-3f and 14-3-3g and *in silico* analysis of promoter upstream confirmed its high expression in tolerant indica rice cultivars under salinity, dehydration and cold

stress. Higher gene expression in salt-sensitive IR29 under ABA treatment showed that ABA was a positive regulator of 14-3-3 expression at least in indica rice cultivars and might be an important mediator for the expression of downstream ABA-responsive genes in an ABA-dependent manner, as similarly proposed recently by Cao et al. 2016. Although only by limited reports, ABA has been shown to affect both the expression and protein levels of five 14-3-3 isoforms in embryonic roots of barley (Schoonheim et al. 2007). Transient co-expression of five 14-3-3 RNAi constructs along with an ABA-responsive promoter displayed that each of the five 14-3-3s plays an important regulatory role in generating an ABA response (Schoonheim et al. 2007). Moreover, binding of 14-3-3 to the phosphorylated ABF3 results in inhibition of proteasomal breakdown, an increase in ABF3 concentration and activation of ABA-inducible genes (Sirichandra et al. 2010). Similarly, transgenic tobacco lines expressing the 14-3-3 gene of *Brachypodium distachyon* (*BdGF14d*) exhibited adaptive responses to salt stress mediated by ABA signaling and elevated expression of related marker genes known to be involved in ABA signaling (He et al. 2017).

Under drought stress, 14-3-3 in *Vicia* was also reported to bind with a cytosolic protein of 61 kDa in guard cells when induced by ABA. AAPK (ABA-activated protein kinase) elicits the binding of the 14-3-3 protein to the 61 kDa protein in vitro when AAPK in guard cells is activated by ABA (Takahashi et al. 2007). One important effect of the ABA signaling pathway is to increase the expression of genes necessary to mitigate or tolerate these stresses (Chandler and Robertson 1994). 14-3-3s is also reported to interact with some of the transcription factors involved in ABA signal transduction (Chandler and Robertson 1994; Denison et al. 2011; He et al. 2017). These results indicated 14-3-3s to be an important candidate in ABA signal transduction, having a key role in abiotic stress response in plants and being possibly involved in both sensitive and tolerant rice cultivars, thus providing a new insight into the functional role of 14-3-3 isoforms in indica rice cultivars. In addition, gene expression results showed differential and isoform-specific expression of 14-3-3 genes at different developmental stages of plant growth. Our results of promoter analysis also showed the presence of abiotic stress-responsive elements such as MBS and bZIP binding site, indicating that 14-3-3s in rice is a potential candidate for stress-inducible gene expression under suitable environmental conditions. The presence of these elements in the upstream of 14-3-3 genes provides strong evidence about the role of 14-3-3s in diverse functions. These experimental results demonstrated that both *Os14-3-3f* and *Os14-3-3g* were highly inducible by salinity, drought and cold stress in abiotic stress-tolerant indica rice cultivars such as Nonabokra and N-22. These findings were supported by previous works that revealed the involvement



◀Fig. 14 Immunodetection of 14-3-3f in 12-day-old rice seedlings after different abiotic stress treatments and in different stages of rice plant. **a** Western-blot analysis was done using 14-3-3f specific antibody produced in rabbit at 1:20,000 dilution and also with H3 histone-specific antibody. The blots show the protein level expression of 14-3-3f in 12-day-old seedlings with different abiotic stress treatments such as salinity (200 mM NaCl), drought (20% PEG), cold (4 °C) and exogenous ABA for 0, 3, 6, 12 and 24 h and in different stages of the rice plant. **b** Relative change in expression levels which were prepared after densitometric scan of the bands; the values were normalized with that of histone

of 14-3-3 genes in abiotic stress in different plant species (Chen et al. 2006; Yang et al. 2014; Tian et al. 2015; Li et al. 2015; Cao et al. 2016) including japonica rice (Yao et al. 2007).

Conclusion

The present genome-wide study demonstrated that the 14-3-3 genes (isoforms) were relatively less conserved in the C-terminal region compared to the core region among the studied taxa. Phylogenetic studies revealed that segmental duplication events contributed to gene duplication, especially in rice, that might have occurred during evolution of monocot species. Transcriptional expression studies showed that 14-3-3f and 14-3-3g isoforms were inducible by salinity, dehydration and cold stress in stress-tolerant indica rice cultivars with differential expression levels. These results were indicative of the presence of a more complex cross talk between different signaling pathways regulated by 14-3-3-ligand complexes in response to abiotic stress in rice. Global interactome studies coupled with functional analysis of interaction complex under different abiotic stress regime in stress-tolerant rice cultivars would reveal isoform(s)-specific putative interacting partners of 14-3-3s. The present work not only provided a better understanding of the evolutionary processes of 14-3-3 genes in *Arabidopsis* and rice, but also offered a new insight into the role of 14-3-3 genes in response to abiotic stresses in indica rice cultivars.

Author contribution statement Conceived and designed the experiments: NY, SB, SC and DNSG. Performed the experiments: NY and SB. Analyzed the data: NY, SB, DNSG and SC. Wrote the paper: NY and SB. All authors read and approved the final manuscript.

Acknowledgements We gratefully acknowledge the Director, Bose Institute, for providing fellowship to NY and for infrastructural support. We are also thankful to Mr. Jadab Ghosh, Mrs. Kaberi Ghosh and Mr. Mrinal Das, Bose Institute, for their technical help. We also thank Dr. Subarna Thakur (Bioinformatics Centre, Bose Institute) for helping in synteny map preparation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical statement regarding use of plant material The authors state that rice seeds, leaves and other planting materials were procured as per local/national regulations. All experimental materials were grown in a contained facility without causing any harm to the natural resource and biodiversity.

References

- Bihl EA, Paul A-L, Wang SW et al (1997) Localization of 14-3-3 proteins in the nuclei of *Arabidopsis* and maize. *Plant J* 12:1439–1445. doi:[10.1046/j.1365-313x.1997.12061439.x](https://doi.org/10.1046/j.1365-313x.1997.12061439.x)
- Blanc G, Wolfe KH (2004) Widespread paleopolyploidy in model plant species inferred from age distributions of duplicate genes. *Plant Cell* 16:1667–1678. doi:[10.1105/tpc.021345](https://doi.org/10.1105/tpc.021345)
- Brenner S, Johnson M, Bridgham J et al (2000) Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat Biotech* 18:630–634
- Campo S, Peris-Peris C, Montesinos L et al (2011) Expression of the maize *ZmGF14-6* gene in rice confers tolerance to drought stress while enhancing susceptibility to pathogen infection. *J Exp Bot* 63:983–999
- Cao H, Xu Y, Yuan L et al (2016) Molecular characterization of the 14-3-3 gene family in *Brachypodium distachyon* L. reveals high evolutionary conservation and diverse responses to abiotic stresses. *Front Plant Sci* 7:1099. doi:[10.3389/fpls.2016.01099](https://doi.org/10.3389/fpls.2016.01099)
- Chandler P, Robertson M (1994) Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol* 45:113–141. doi:[10.1146/annurev.pp.45.060194.000553](https://doi.org/10.1146/annurev.pp.45.060194.000553)
- Chen F, Li Q, Sun L, He Z (2006) The rice 14-3-3 gene family and its involvement in responses to biotic and abiotic stress. *DNA Res* 13:53–63
- Daugherty CJ, Rooney MF, Miller PW, Ferl RJ (1996) Molecular organization and tissue-specific expression of an *Arabidopsis* 14-3-3 gene. *Plant Cell* 8:1239–1248
- Debernardi JM, Rodriguez RE, Mecchia MA, Palatnik JF (2012) Functional specialization of the plant miR396 regulatory network through distinct microRNA–target interactions. *PLoS Genet* 8:e1002419
- Degenkolbe T, Do PT, Zuther E et al (2009) Expression profiling of rice cultivars differing in their tolerance to long-term drought stress. *Plant Mol Biol* 69:133–153. doi:[10.1007/s11103-008-9412-7](https://doi.org/10.1007/s11103-008-9412-7)
- DeLille JM, Schenke PC, Ferl RJ (2001) The *Arabidopsis* 14-3-3 family of signaling regulators. *Plant Physiol* 126:35–38. doi:[10.1104/pp.126.1.35](https://doi.org/10.1104/pp.126.1.35)
- Denison FC, Paul A-L, Zupanska AK, Ferl RJ (2011) 14-3-3 proteins in plant physiology. *Semin Cell Dev Biol* 22:720–727. doi:[10.1016/j.semcd.2011.08.006](https://doi.org/10.1016/j.semcd.2011.08.006)
- Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 95:14863–14868
- Ferl RJ (2004) 14-3-3 proteins: regulation of signal-induced events. *Physiol Plant* 120:173–178. doi:[10.1111/j.0031-9317.2004.0239.x](https://doi.org/10.1111/j.0031-9317.2004.0239.x)
- Ferl RJ, Manak MS, Reyes MF (2002) The 14-3-3s. *Genome Biol* 3:reviews3010.1–reviews3010.7
- Fu H, Subramanian RR, Masters SC (2000) 14-3-3 Proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol* 40:617–647. doi:[10.1146/annurev.pharmtox.40.1.617](https://doi.org/10.1146/annurev.pharmtox.40.1.617)

- Fukuda M, Asano S, Nakamura T et al (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* 390:308–311
- Ganguly M, Datta K, Roychoudhury A et al (2012) Overexpression of *Rab16A* gene in indica rice variety for generating enhanced salt tolerance. *Plant Signal Behav* 7:502–509. doi:[10.4161/psb.19646](https://doi.org/10.4161/psb.19646)
- Gaut BS, Morton BR, McCaig BC, Clegg MT (1996) Substitution rate comparisons between grasses and palms: synonymous rate differences at the nuclear gene *Adh* parallel rate differences at the plastid gene *rbcL*. *Proc Natl Acad Sci USA* 93:10274–10279
- He Y, Zhang Y, Chen L et al (2017) A member of the 14-3-3 gene family in *Brachypodium distachyon*, *BdGF14d*, confers salt tolerance in transgenic tobacco plants. *Front Plant Sci* 8:340
- Ho S-L, Huang L-F, Lu C-A et al (2013) Sugar starvation-and GA-inducible calcium-dependent protein kinase 1 feedback regulates GA biosynthesis and activates a 14-3-3 protein to confer drought tolerance in rice seedlings. *Plant Mol Biol* 81:347–361
- Jaspert N, Throm C, Oecking C (2011) *Arabidopsis* 14-3-3 proteins: fascinating and less fascinating aspects. *Front Plant Sci* 2:96
- Kelley LA, Mezulis S, Yates CM et al (2015) The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* 10:845–858
- Krzywinski M, Schein J, Birol I et al (2009) Circos: an information aesthetic for comparative genomics. *Genome Res* 19:1639–1645. doi:[10.1101/gr.092759.109](https://doi.org/10.1101/gr.092759.109)
- Li R, Jiang X, Jin D et al (2015) Identification of 14-3-3 family in common bean and their response to abiotic stress. *PLoS ONE* 10:e0143280
- Liu D, Bienkowska J, Petosa C et al (1995) Crystal structure of the zeta isoform of the 14-3-3 protein. *Nature* 376:191–194
- Liu Q, Zhang S, Liu B (2016) 14-3-3 proteins: macro-regulators with great potential for improving abiotic stress tolerance in plants. *Biochem Biophys Res Commun* 477:9–13. doi:[10.1016/j.bbrc.2016.05.120](https://doi.org/10.1016/j.bbrc.2016.05.120)
- Lovell SC, Davis IW, Arendall III WB et al (2003) Structure validation by $\text{C}\alpha$ geometry: ϕ, ψ and $\text{C}\beta$ deviation. *Proteins Str Func Bioinfo* 50(3):437–450. doi:[10.1002/prot.10286](https://doi.org/10.1002/prot.10286)
- Muthuramalingam P, Krishnan SR, Pothiraj R, Ramesh M (2017) Global transcriptome analysis of combined abiotic stress signaling genes unravels key players in *Oryza sativa* L.: an in silico approach. *Front Plant Sci* 8:759
- Nakano M, Nobuta K, Vemaraju K et al (2006) Plant MPSS databases: signature-based transcriptional resources for analyses of mRNA and small RNA. *Nucleic Acids Res* 34:D731–D735. doi:[10.1093/nar/gkj077](https://doi.org/10.1093/nar/gkj077)
- Nakashima K, Yamaguchi-Shinozaki K (2013) ABA signaling in stress-response and seed development. *Plant Cell Rep* 32:959–970. doi:[10.1007/s00299-013-1418-1](https://doi.org/10.1007/s00299-013-1418-1)
- Paul A-L, Liu L, McClung S et al (2009) Comparative interactomics: analysis of *Arabidopsis* 14-3-3 complexes reveals highly conserved 14-3-3 interactions between humans and plants. *J Proteome Res* 8:1913–1924. doi:[10.1021/pr8008644](https://doi.org/10.1021/pr8008644)
- Purwestri YA, Ogaki Y, Tamaki S et al (2009) The 14-3-3 protein GF14c acts as a negative regulator of flowering in rice by interacting with the florigen Hd3a. *Plant Cell Physiol* 50:429–438
- Raghavendra AS, Gonugunta VK, Christmann A, Grill E (2017) ABA perception and signalling. *Trends Plant Sci* 15:395–401. doi:[10.1016/j.tplants.2010.04.006](https://doi.org/10.1016/j.tplants.2010.04.006)
- Reddy INBL, Kim B-K, Yoon I-S et al (2017) Salt tolerance in rice: focus on mechanisms and approaches. *Rice Sci* 24:123–144. doi:[10.1016/j.rsci.2016.09.004](https://doi.org/10.1016/j.rsci.2016.09.004)
- Roberts MR (2017) 14-3-3 Proteins find new partners in plant cell signalling. *Trends Plant Sci* 8:218–223. doi:[10.1016/S1360-1385\(03\)00056-6](https://doi.org/10.1016/S1360-1385(03)00056-6)
- Roberts MR, Salinas J, Collinge DB (2002) 14-3-3 proteins and the response to abiotic and biotic stress. *Plant Mol Biol* 50:1031–1039. doi:[10.1023/A:1021261614491](https://doi.org/10.1023/A:1021261614491)
- Rosenquist M, Alsterfjord M, Larsson C, Sommarin M (2001) Data mining the *Arabidopsis* genome reveals fifteen 14-3-3 genes expression is demonstrated for two out of five novel genes. *Plant Physiol* 127:142–149. doi:[10.1104/pp.127.1.142](https://doi.org/10.1104/pp.127.1.142)
- Schmid M, Davison TS, Henz SR et al (2005) A gene expression map of *Arabidopsis thaliana* development. *Nat Genet* 37:501–506
- Schoonheim PJ, Sinnige MP, Casaretto JA et al (2007) 14-3-3 adaptor proteins are intermediates in ABA signal transduction during barley seed germination. *Plant J* 49:289–301. doi:[10.1111/j.1365-313X.2006.02955.x](https://doi.org/10.1111/j.1365-313X.2006.02955.x)
- Sehnke PC, Ferl RJ (2000) Plant 14-3-3s: omnipotent metabolic phosphopartners? *Sci STKE* 2000(56):pe1
- Sehnke PC, Henry R, Cline K, Ferl RJ (2000) Interaction of a plant 14-3-3 protein with the signal peptide of a thylakoid-targeted chloroplast precursor protein and the presence of 14-3-3 isoforms in the chloroplast stroma. *Plant Physiol* 122:235–242
- Shankar R, Bhattacharjee A, Jain M (2016) Transcriptome analysis in different rice cultivars provides novel insights into desiccation and salinity stress responses. *Sci Rep* 6:23719. doi:[10.1038/srep23719](https://doi.org/10.1038/srep23719)
- Shanko AV, Mesenko MM, Klychnikov OI et al (2003) Proton pumping in growing part of maize root: its correlation with 14-3-3 protein content and changes in response to osmotic stress. *Biochemistry* 68:1320–1326
- Shin R, Alvarez S, Burch AY et al (2007) Phosphoproteomic identification of targets of the *Arabidopsis* sucrose nonfermenting-like kinase SnRK2.8 reveals a connection to metabolic processes. *Proc Natl Acad Sci USA* 104:6460–6465. doi:[10.1073/pnas.0610208104](https://doi.org/10.1073/pnas.0610208104)
- Shin R, Jez JM, Basra A et al (2011) 14-3-3 proteins fine-tune plant nutrient metabolism. *FEBS Lett* 585:143–147. doi:[10.1016/j.febslet.2010.11.025](https://doi.org/10.1016/j.febslet.2010.11.025)
- Sirichandra C, Davanture M, Turk BE et al (2010) The *Arabidopsis* ABA-activated kinase OST1 phosphorylates the bZIP transcription factor ABF3 and creates a 14-3-3 binding site involved in its turnover. *PLoS ONE* 5:e13935
- Sun G, Xie F, Zhang B (2011) Transcriptome-wide identification and stress properties of the 14-3-3 gene family in cotton (*Gossypium hirsutum* L.). *Funct Integr Genomics* 11:627–636. doi:[10.1007/s10142-011-0242-3](https://doi.org/10.1007/s10142-011-0242-3)
- Sun X, Luo X, Sun M et al (2013) A *Glycine soja* 14-3-3 protein GsGF14o participates in stomatal and root hair development and drought tolerance in *Arabidopsis thaliana*. *Plant Cell Physiol* 55:99–118
- Sun X, Sun M, Jia B et al (2016) A 14-3-3 family protein from wild soybean (*Glycine Soja*) regulates ABA sensitivity in *Arabidopsis*. *PLoS ONE* 10:e0146163
- Takahashi Y, Kinoshita T, Shimazaki K (2007) Protein phosphorylation and binding of a 14-3-3 protein in *Vicia* guard cells in response to ABA. *Plant Cell Physiol* 48:1182–1191
- Tamura K, Peterson D, Peterson N et al (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739. doi:[10.1093/molbev/msr121](https://doi.org/10.1093/molbev/msr121)
- Taoka K, Ohki I, Tsuji H et al (2011) 14-3-3 proteins act as intracellular receptors for rice Hd3a florigen. *Nature* 476:332–335
- Tian F, Wang T, Xie Y et al (2015) Genome-wide identification, classification, and expression analysis of 14-3-3 gene family in *Populus*. *PLoS ONE* 10:e0123225
- Walia H, Wilson C, Condamine P et al (2005) Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. *Plant Physiol* 139:822–835. doi:[10.1104/pp.105.065961](https://doi.org/10.1104/pp.105.065961)
- Wang X, Yang P, Zhang X et al (2009) Proteomic analysis of the cold stress response in the moss, *Physcomitrella patens*. *Proteomics* 9:4529–4538

- Witkos TM, Koscińska E, Krzyzosiak WJ (2011) Practical aspects of microRNA target prediction. *Curr Mol Med* 11:93–109. doi:[10.2174/156652411794859250](https://doi.org/10.2174/156652411794859250)
- Wu K, Rooney MF, Ferl RJ (1997) The *Arabidopsis* 14-3-3 multigene family. *Plant Physiol* 114:1421–1431
- Xu WF, Shi WM (2006) Expression profiling of the 14-3-3 gene family in response to salt stress and potassium and iron deficiencies in young tomato (*Solanum lycopersicum*) roots: analysis by real-time RT-PCR. *Ann Bot* 98:965–974
- Xu W, Jia L, Shi W et al (2012) Smart role of plant 14-3-3 proteins in response to phosphate deficiency. *Plant Signal Behav* 7:1047–1048. doi:[10.4161/psb.20997](https://doi.org/10.4161/psb.20997)
- Xu W, Jia L, Shi W et al (2013) The tomato 14-3-3 protein TFT4 modulates H⁺ efflux, basipetal auxin transport, and the PKS5-J3 pathway in the root growth response to alkaline stress. *Plant Physiol* 163:1817–1828
- Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu Rev Plant Biol* 57:781–803. doi:[10.1146/annurev.arplant.57.032905.105444](https://doi.org/10.1146/annurev.arplant.57.032905.105444)
- Yan J, He C, Wang J et al (2004) Overexpression of the *Arabidopsis* 14-3-3 protein GF14λ in cotton leads to a “stay-green” phenotype and improves stress tolerance under moderate drought conditions. *Plant Cell Physiol* 45:1007–1014
- Yang Z-P, Li H-L, Guo D et al (2014) Identification and characterization of the 14-3-3 gene family in *Hevea brasiliensis*. *Plant Physiol Biochem* 80:121–127. doi:[10.1016/j.plaphy.2014.03.034](https://doi.org/10.1016/j.plaphy.2014.03.034)
- Yang L, You J, Wang Y et al (2017) Systematic analysis of the G-box factor 14-3-3 gene family and functional characterization of *GF14a* in *Brachypodium distachyon*. *Plant Physiol Biochem* 117:1–11. doi:[10.1016/j.plaphy.2017.05.013](https://doi.org/10.1016/j.plaphy.2017.05.013)
- Yao Y, Du Y, Jiang L, Liu J-Y (2007) Molecular analysis and expression patterns of the 14-3-3 gene family from *Oryza Sativa*. *J Biochem Mol Biol* 40(3):349–357
- Zeng L, Shannon MC, Lesch SM (2001) Timing of salinity stress affects rice growth and yield components. *Agric Water Manag* 48:191–206. doi:[10.1016/S0378-3774\(00\)00146-3](https://doi.org/10.1016/S0378-3774(00)00146-3)
- Zhang Z, Li J, Zhao X-Q et al (2006) KaKs_Calculator: calculating Ka and Ks through model selection and model averaging. *Genomics Proteomics Bioinform* 4:259–263. doi:[10.1016/S1672-0229\(07\)60007-2](https://doi.org/10.1016/S1672-0229(07)60007-2)
- Zhang Z, Yu J, Li D et al (2010) PMRD: plant microRNA database. *Nucleic Acids Res* 38:D806–D813. doi:[10.1093/nar/gkp818](https://doi.org/10.1093/nar/gkp818)