



Comparative Analysis of the Expression of Candidate Genes Governing Salt Tolerance and Yield Attributes in Two Contrasting Rice Genotypes, Encountering Salt Stress During Grain Development

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Abstract

The rice grain filling process is of inordinate importance as it is directly associated with productivity and rice quality. Salt stress occurring during early grain development hinders seed development, resulting in yield penalty. To dissect transcriptional responses and to identify promising candidate genes, comparative expression profiling of key stress-responsive and yield-related genes was performed in developing (10 DAP, 20 DAP, and 30 DAP) and matured grains of salt-sensitive (IR-64) and salt-tolerant (Nonabokra) rice cultivars under salt stress (250 mM NaCl) along with the analyses of grain yield parameters. The phenotypic values of most of the tested yield attributes were significantly reduced under salt stress and the effect of stress was more pronounced in IR-64. Gene expression through semi-quantitative reverse transcriptase-polymerase chain reaction followed by statistical analyses identified that members of the TFs and LEA family (that is, *TRAB-1*, *RITA-1*, *RISBZI*, *WRKY-21*, and *Osem*), osmolytes and polyamine metabolic genes (*BADHI* and *SAMDC*) as well as the yield-related gene *GIFI*, were significantly induced by salt stress. Statistical analyses further revealed a significant correlation between the expression of these genes and grain yield under salt stress. In IR-64, the *TRAB-1*, *RITA-1*, and *Osem* transcripts were more up regulated during the early to mid-phase of seed development, suggesting an adaptive response of the sensitive cultivar to salt stress. The TFs along with *BADHI*, *SAMDC*, and *GIFI* transcripts were mostly up regulated in Nonabokra during the early phase, and the level was maintained even after the mid-phase under stress. The heat map analysis also revealed the differential expression of genes between the two cultivars throughout the seed developmental stages. Our result indicated a possible interplay between ABA-inducible TFs and grain filling-related genes, allowing Nonabokra to maintain the grain filling process under stress condition. This is also evident by comparatively lower reduction of grain weight and filled-grain number in Nonabokra under stress. The role of different TFs in ABA-signaling in matured grains is clear by the accumulation of transcripts, especially in dry and ABA-imbibed seeds. Overall, our data established the correlation of grain yield with tolerance or susceptibility, accompanied by the expression of effector or regulatory genes.

Keywords Rice · IR-64 · Nonabokra · Developing grains · Salt stress · Gene expression · Transcription factor · Productivity · Developing grain · Microarray · ANOVA · Semi-quantitative RT-PCR

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Introduction

Natural resources and agriculture are currently facing incessant demands from continuous population growth, so that productivity and yield of major food crops is a matter of chief concern. Rice, being one of the most important staple cereals consumed globally, is a major target for crop improvement as its yield is severely affected by several abiotic stress factors, of which soil salinity is of major environmental concern. The decrease in the arable land area due to soil salinization is reported to be 190 million ha of the global land area (FAO 2010), especially in South East

Asian countries including India (Kumar et al. 2015), which imposes enormous pressure on productivity (Rengasamy 2010).

Considering the developmental stages in rice, grain filling is of particular interest, because parameters of yield like grain weight can be directly correlated with the formation of caryopsis from the fertilized embryo. The process of grain filling is a continuous and complex process that requires the close coordination of expression of many genes. Salt stress occurring during early grain development affects grain sink potential, thereby diminishing the capacity of endosperm cells to accumulate starch. The factors contributing to yield loss include the synergistic effects of the modified cell wall flexibility, reduced effectiveness of turgor pressure, membrane damage, interrupted photosynthesis and nutrient imbalance (Mahmood et al. 2009; Mohammadi-Nejad et al. 2012; Hakim et al. 2014). The difference in grain yield in indigenous rice cultivars is due to differential tolerance to salinity, as well as other environmental stresses. The high-yielding rice varieties such as IR-64, IR-29, IR-28, M-1-48 are found to be more susceptible than low-yielding tolerant genotypes like Nonabokra and Pokkali (Negrão et al. 2011; Soda et al. 2013) which rely on physiological, molecular and epigenetic mechanism for salt tolerance (Anil et al. 2005; Banerjee and Roychoudhury 2017).

Abscisic acid (ABA) is the key regulatory phytohormone in the stress response (Gurmani et al. 2013), as well as being involved in regulating grain development (Yang et al. 2001). In developing grains, the concentration of ABA was observed to increase after the heading stage, whereas maximum concentration was reported during the middle stage and subsequently decreased and maintained a lower level during the later phase of grain filling period (Kato et al. 1993). ABA accumulation, being stage-specific, is indicative of its role in aiding grain development through promotion of desiccation tolerance (Nakashima and Yamaguchi-Shinozaki 2013). The positive correlation between ABA level and grain filling rate at the early grain filling stage was reported in wheat (Bai et al. 1989), whereas poor grain filling was associated with low ABA content in rice grains (Zhao et al. 2007; Zhang et al. 2009). Endogenous ABA levels spike in developing grains (Ahmadi and Baker 1999; Yang et al. 2001, 2006) in response to soil drying, with higher accumulation noted in the tolerant cultivars as compared to the sensitive ones (Moons et al. 1995; Yang et al. 2014).

During the maturation phase in rice, several ABA-inducible genes are expressed, such as genes encoding late embryogenesis abundant (LEA) proteins like *Osem* (Hattori et al. 1995). *Osem* was detectable at 8 days after flowering (DAF) and its level increased as maturation proceeded, the highest transcript level was observed in the dry mature embryo (Hattori et al. 1995), being regulated by the TF, VP1 in response to ABA (Miyoshi et al. 2002). In addition, the

accumulation of transcripts of transcription factors (TFs) such as basic leucine zipper (bZIP) TFs, that is, RITA-1 and TRAB-1 (Izawa et al. 1994; Hobo et al. 1999) that bind to the abscisic acid responsive elements (ABREs), is also noteworthy. TRAB-1 is expressed in rice embryos at 8–14 DAF and the dry matured stages of seed, and regulates the transcription of its downstream genes in an ABA-dependent manner through interaction with VP1 during seed development (Hattori et al. 1992, 1994). The bZIP factor RITA-1 is expressed in aleurone and endosperm cells of developing rice seeds (Izawa et al. 1994). Another bZIP TF RISBZ1 (Rice Seed b-Zipper 1) regulates various processes of grain filling and development by co-ordinately expressing with rice seed-specific protein (SSP) and activating the transcription of *SSP* gene in the mature endosperm (Yamamoto et al. 2006). The WRKY TFs have also been known to be involved in multiple developmental and physiological processes by mediating ABA responses in aleurone cells (Alexandrova and Conger 2002; Xie et al. 2005; Wang et al. 2016). The WRKY-21 interacts synergistically with ABA and transcriptional activators VP1 and ABI5, thereby mediating ABA-signaling cascades during abiotic stress (Zou et al. 2004).

To override osmotic stress, plants subjected to salt stress produce compatible solutes (osmolytes) such as proline (Pro) and glycine betaine (GB), as well as polyamines (PAs), and the tolerance mechanism depends on the increased expression of genes encoding osmolyte biosynthetic enzymes such as Δ^1 -pyrroline-5-carboxylate synthetase (P5CS), involved in Pro synthesis (Roychoudhury et al. 2015) and betaine aldehyde dehydrogenase 1 (BADH1), that participates in GB synthesis (Roychoudhury and Banerjee 2016). Both Pro and GB regulate tolerance to stresses like salinity, drought and cold, when accumulated at higher levels, following overexpression of the genes like *P5CS* and *BADH1* respectively (Kumar et al. 2010; Kim and Nam 2013; Chen et al. 2013a; Ibragimova et al. 2015; Chen and Murata 2011; Hasthanasombut et al. 2010, 2011). The PAs, including tetra-amine spermine (Spm^{4+}), tri-amine spermidine (Spd^{3+}) and their di-amine precursor, putrescine (Put^{2+}) are polycationic, ubiquitous molecules that play an important role in conferring salt tolerance of indica rice cultivars. Induced expression of genes encoding the PA-biosynthetic pathway, that is, *S-adenosylmethionine decarboxylase (SAMDC)*, *spermidine synthase (SPDS)* and *spermine synthase (SPMS)* have been noted under saline conditions (Roychoudhury et al. 2009; Paul et al. 2017; Paul and Roychoudhury 2017a, b).

Rice yield is a multigenic quantitative trait, and the contribution of each of these regulatory genes is quite significant. Several yield-related traits such as tiller number, grain size and number, as well as panicle development have been studied in rice. The *grain incomplete filling 1 (GIF1)*, known to be a positive regulator of grain filling, encodes a cell wall invertase which is required for carbon partitioning during

early grain filling (Wang et al. 2008). The *Grain Width and Weight 2 (GW2)*, encoding a RING-type E3 ubiquitin ligase, negatively regulates rice grain width and weight (Song et al. 2007). *SPL14*, a member of the Squamosa promoter binding protein (SBP)-like (SPL) family of plant-specific TFs, is involved in panicle development (Miura et al. 2010). The gene encoding *Dense and Erect Panicle 1 (DEP1)* regulates the rate of spikelet formation by accelerating the cell division of rice, leading to higher grain number (GN) (Huang et al. 2009). The role of *LRK1*, a plasma membrane protein, in increasing the number of panicles and spikelets per panicle, as well as weight per grain has been reported earlier (Zha et al. 2009).

In the present communication, we focused on the relationship between the expression of ABA-inducible genes or yield-related genes and grain yield under salt stress. The tolerance of rice to salinity varies with its stage of development. The salt-tolerant phenotype at the seedling stage or vegetative phase depends on the regulation of gene expression distinct from that occurring during flowering and the reproductive stage (Das et al. 2015; Mohammadi-Nejad et al. 2012). Lutts et al. (1995) reported that rice is more sensitive to salinity at the reproductive stage, showing high sensitivity during pollination, fertilization and the early seedling stage (Abdullah et al. 2001; Khan and Abdullah 2003). To date, there is indeed a dearth of adequate studies on the effect of salt stress during panicle formation or grain development in rice. A comprehensive experimental observation is absolutely essential to determine the effect of salt stress on grain yield as well as to monitor the differential expression of ABA-inducible genes or genes contributing to seed development in developing and matured grains. Therefore, a systematic analysis of the effect of salt stress was carried out at the grain filling stages, using salt-sensitive (IR-64) and salt-tolerant (Nonabokra) rice varieties. The major objectives of this study were to determine (i) impact of salt stress on key ABA-inducible, stress-responsive genes like *Osem*, *TRAB-1*, *RITA-1*, *VPI*, *RISBZ1*, and *WRKY-21*, genes encoding enzymes for osmolytes and PAs determining stress tolerance, as well as genes governing yield attributes under salt stress, (ii) effect of salt stress on grain yield and spikelet sterility as affected by salinity, and (iii) the correlation between grain yield and transcriptional regulation of diverse groups of stress-responsive and yield-related genes under salt stress.

Materials and Methods

Plant Materials, Growth Conditions, and Stress Treatments

The seeds of IR-64 were procured from Chinsurah Rice Research Station, Hooghly, West Bengal, India, and the

seeds of Nonabokra from Central Soil Salinity Research Institute, Canning, West Bengal, India. For analysis of the effect of salt stress on gene expression in the grains of IR-64 and Nonabokra, the rice plants were grown on Soilrite in earthen pots during rice growing season (may–october). The germinated seedlings were initially grown in a Petri plate for 21 days, followed by their transfer to earthen pots (30 cm in height and 25 cm in diameter) filled with Soilrite. For each treatment, four separate pots were maintained for each variety, with each pot containing two seedlings. After anthesis of first panicles, 250 mM NaCl solution was used to impose salt stress to the potted plants. A separate set of potted plants without stress was maintained as a control. The treatment sets were as follows: (i) Set 1 (control set): plants maintained without NaCl stress continuously for 30 days, the time required for grain maturation and (ii) Set 2 (salt stress): 250 mM of NaCl was applied to the potted plants and the plants maintained continuously under salt stress for 30 days. Fresh NaCl solution was added from time to time to make sure that rice plants were maintained under continuous stress condition. The grains of IR-64 and Nonabokra were harvested 10, 20 and 30 days after pollination (DAP) from both the above sets and total RNA was isolated from all the samples for gene expression study. Three independent biological replicates were used for gene expression study.

Measuring Yield-Related Attributes

To examine the effect of a high concentration of salt on rice growth and development at the reproductive stage, yield-related traits, that is, panicle length (PL), rachis length (RL), primary branch length (PBL) and secondary branch length (SBL), total number of spikelets, GN per panicle, filled grain (FG) and empty grains (EG), 1000-grain weight (TGW), grain length (GL), and grain breadth (GB) were measured at physiological maturity of both the cultivars. For measuring RL, PBL and SBL, three matured rice panicles from each pot of both the cultivars were cut and manually spread out on paper; the RL, PBL, and SBL were marked with lines and measured in a centimeter scale. The total number of spikelets and GN per panicle was counted manually. The hand-threshing method followed by winnowing was applied for separating out FG from unfilled grains of spikelets. FG and EG were collected from respective spikelets. A total of ten panicles from eight separate plants was used for measuring GN, FG, and EG for each genotype and each treatment. Harvested rice grains were dried (moisture between 13 and 14%) prior to measurement of traits related to grain shape (GL and GB). A total of ten fully FGs from eight separate plants were used for measuring GL and GB. For measuring TGW, the total weight of 100 grains from each plant was measured and converted to its TGW by using an analytical balance.

Expression Analysis Using Microarrays

To analyze the transcript profiling of genes pertaining to stress tolerance, the microarray data (CEL file) for rice were retrieved from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI-GEO) database (Barrett et al. 2013). The expression profile for rice under salt (GSE6901) (Jain et al. 2007) and ABA (GSE37557) (Garg et al. 2012) were obtained and the expression was analyzed. The rice microarray raw data were processed with *ricecdf* respectively as reported earlier (Movahedi et al. 2011; Paul and Roychoudhury 2018). Briefly, the MA plot was used for assessing the quality of individual arrays. The raw data were normalized by the Robust Multi-array Average (RMA) algorithm with the following steps; background correction, log transformation and quantile normalization and summarization. To perform rice data analysis, the average signal intensities of replicated slides were \log_{10} transformed, heat maps were produced and hierarchical clustering was done by the average linkage method using the *Mev* software package (Saeed et al. 2003).

Semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction (Semi-qRT-PCR) Analyses

The primers for *TRAB-1*, *RITA-1*, *WRKY-21*, *RISBZ1*, *VP1*, *Osem*, *BADH1*, *P5CS*, *SAMDC*, *SPDS*, *SPMS*, *GIF1*, *GW2*, *SPL14*, *DEP1*, *LRK1*, and *actin* genes were designed using online NCBI Primer-blast (<http://www.ncbi.nlm.gov/tools/primer-blast/index>) software. *Actin* was used as a reference gene based on the earlier report where *actin* has actually been used for gene expression study during grain filling stages (Huang et al. 2009; Chen et al. 2013b). Salt stress tolerance being a multigenic trait, several genes have been identified and characterized for their protective role. In the present work, we have selected only some key representative or candidate genes involved in either stress tolerance or regulating yield attributes and analyzed their expression pattern (Table 1). RNA extraction, cDNA preparation, and semi-qRT-PCR were carried out using the method as described earlier (Paul and Roychoudhury 2018). For semi-qRT-PCR analysis from developing grains, three independent biological replicates were collected for each of the developing stages of both the genotypes and for each treatment, resulting in total 36 samples (three biological replicates \times two genotypes \times two treatments \times three grain-developing stages = 36 samples). Total RNA was isolated from the developing (10 DAP, 20 DAP, and 30 DAP) and matured [dry, water-imbibed (10 h) and ABA (100 μ M)-imbibed (10 h)] grains of IR-64 and Nonabokra, using RNAiso plus (Takara, Japan) following the manufacturer's instructions and the RNA concentration and purity was checked spectrophotometrically. Total RNA was treated with DNase I (Thermo Scientific) to

remove DNA contamination. About 5 μ g of total RNA was reverse-transcribed using Maxima First Strand cDNA synthesis kit (Thermo Scientific). About 100 ng of cDNA was used as template for semi-qRT-PCR using gene-specific primers, and *actin* as an internal standard with three biological replicates in the thermal cycler (2720 Applied Biosystem). The primer sequences and corresponding gene accession IDs are shown in Table 1. The number of PCR cycles for each gene was separately validated by stopping the reaction at different cycle numbers to show that it was still in the exponential phase. The primer efficiency was also tested by creating a standard curve of template concentration to rule out any variation between samples caused by primer efficiency (data not shown). Following densitometric scanning of band intensity of each transcript with Quantity One software (Bio Rad), the derived value for gene expression level at the respective treatment was normalized by dividing the value with that of the internal standard. The average data from semi-qRT-PCR analyses were imported into the TM4 microarray software suite for heat map analysis (Saeed et al. 2003).

Statistical Analyses

All statistical analyses were performed using the R statistical program. The residuals were first checked to confirm that the assumptions of linear models were met. To identify the significantly expressed genes, a three-way analysis of variance (ANOVA) was conducted on a linear model to dissect out the independent effects of all three main factors (genotype, G; treatment, T; and developing stage, S) and their interactions. Three independent biological replicates for each of the developing stages of both the genotypes and for each treatment were used for this analysis. The resulting *P* values were corrected for multiple testing using the stringent Bonferroni method. Partial eta-squared (η_p^2) values were used to estimate the effect size of each factor from the ANOVA for each gene. A two-way factorial ANOVA was run for each yield attribute on all the biological replicates ($n = 8$) for rice cultivars and conditions (for example, control and salt stress) to look for significant genotype, treatment, and genotype by treatment effects. The Tukey's honestly significant difference (HSD) test was conducted to identify the mean values that were significantly different from each other at the $P < 0.05$ level.

Result

Physiological Effects of Salt Stress on Yield-Related Traits

To evaluate the effect of salt stress on the reproductive development of the two rice genotypes, several yield-related traits,

Table 1 Primer sequences used for semi-qRT-PCR

Gene	Primer name	Sequence	Gene accession ID	References
<i>TRAB-1</i>	TRAB-1 F	CGATGAATTCTCTAGAATGA- -ACATGGACGAGCTGCT	LOC_Os08g36790	Paul and Roychoudhury (2017b)
	TRAB-1 R	ATGAAGCTTGAGCTCTTACC- -AGGGACCTGTCAATGTT		
<i>RITA-1</i>	RITA1 F	ATGAAGAAGTGCCCGTCGGAGC	LOC_Os02g16680	Izawa et al. (1994)
	RITA1 R	TCACTTGGACATGGCGCCATTG		
<i>VP1</i>	VP1 F	GCGAAGACGGACAAGAACCTG	LOC_Os01g68370	Hobo et al. (1999)
	VP1 R	TCAGATGCTAACCGCCATCTGG		
<i>RISBZ1</i>	RISBZ1 F	AAGGGAGCCATCACCATCAGAGG	LOC_Os07g08420	Yamamoto et al. (2006)
	RISBZ1 R	CGGCATTGTTAGCGTTGGAGAAGT		
<i>WRKY-21</i>	WRKY-21 F	TGAGAGTTGTCGTGCGAGAG	LOC_Os01g60640	Xie et al. (2005)
	WRKY-21 R	CCAGTCAAGCTGCCATTCA		
<i>Osem</i>	Osem F	ATGGCGTCCGGGCAGCAGCAG	LOC_Os05g28210	Hattori et al. (1995)
	Osem R	CTAGGACTTGGTCTTGACTTGG		
<i>BADH1</i>	BADH1 F	TCTTTGGACCGGTCACTGC	LOC_Os04g39020	Hasthanasombut et al. (2010)
	BADH1 R	CTTGGTGACTTGCTTCACGC		
<i>P5CS</i>	P5CS F	AGTCCCACCTGATGCCTTG	LOC_Os05g38150	Igarashi et al. (1997)
	P5CS R	TGGTCCACGGGCATGGATAC		
<i>SAMDC</i>	SAMDC F	TCTTGCTTATGGCGACCTGG	LOC_Os04g42090	Paul and Roychoudhury (2017a)
	SAMDC R	AGCAACAGGTACGTCTTCGG		
<i>SPDS</i>	SPDS F	CCTGGTGAGGCACACTCATT	LOC_Os07g22600	Paul and Roychoudhury (2018)
	SPDS R	TTGCTCCACCGAGGAATGTC		
<i>SPMS</i>	SPMS F	ATCCTTTGCTGACACCTGGG	LOC_Os02g14190	Do et al. (2013)
	SPMS R	GCGTCTCATTCAACAGCGAC		
<i>GW2</i>	GW2 F	TCAGTGCCGTCATACCGATG	LOC_Os02g14720	Song et al. (2007)
	GW2 R	ACTTCCCTGCTCCTGAATGG		
<i>GIF1</i>	GIF1 F	AAGAACTGGATCAATGGTAATGCG	LOC_Os04g33740	Wang et al. (2008)
	GIF1 R	CGTAGAACGTCTTGAGGCG		
<i>LRK1</i>	LRK1 F	AATGTTGCAGCAAGGCAAGG	LOC_Os02g05980	Zha et al. (2009)
	LRK1 R	AGTATCGGAACTGGCCTCCT		
<i>SPL14</i>	SPL14 F	GCAGCGTAAGGAATGCATGG	LOC_Os08g39890	Miura et al. (2010)
	SPL14 R	CCATCGTGTGCTGGTTTGG		
<i>DEP1</i>	DEP1 F	AATGCAAACCCGATTGTGGC	LOC_Os09g26999	Huang et al. (2009)
	DEP1 R	AACCACTGAGACAGCATGGG		
<i>actin</i>	actin F	GAACTGGCATGGTCAAGGCTG	LOC_Os11g06390	Chen et al. (2007)
	actin R	ACACGGAGCTCGTTGTAGAAG		

F forward primer, R reverse primer

that is, PL, RL, PBL and SBL, total number of spikelets, TGW, FG and EG, GL and GB were measured at maturity. We analyzed the effects of genotype (G), treatment (T), and their interaction on the yield attributes under salt stress. The two-way ANOVA indicated that PL, RL, PBL, SBL, GN, and TGW were influenced significantly by genotype ($P < 0.001$), by treatment ($P < 0.001$), and also by their interaction ($P < 0.001$) (Supplementary Table S1). Salt stress decreased PL to 38% in the sensitive cultivar as compared to the control condition (Fig. 1a). The RL, PBL, SBL, and GN were reduced in both the cultivars; however, the effect

was more pronounced in IR-64 (Fig. 1b–d, f). Salt treatment significantly decreased TGW to 41% and 12.4% for IR-64 and Nonabokra respectively as compared to control plants (Fig. 1i). Significant $G \times T$ ($P < 0.001$) effect was recorded for the total number of spikelets, with decreasing total number of spikelets, by 15.6% in IR-64 and 4.6% in Nonabokra under salt stress (Fig. 1e). Significant genotype ($P < 0.05$), treatment ($P < 0.001$) and $G \times T$ ($P < 0.001$) effects were noted for FG (Supplementary Table S1), with decreasing FG number by 78.4% in IR-64 under salt stress (Fig. 1g). Significant genotype ($P < 0.001$) and treatment ($P < 0.01$)

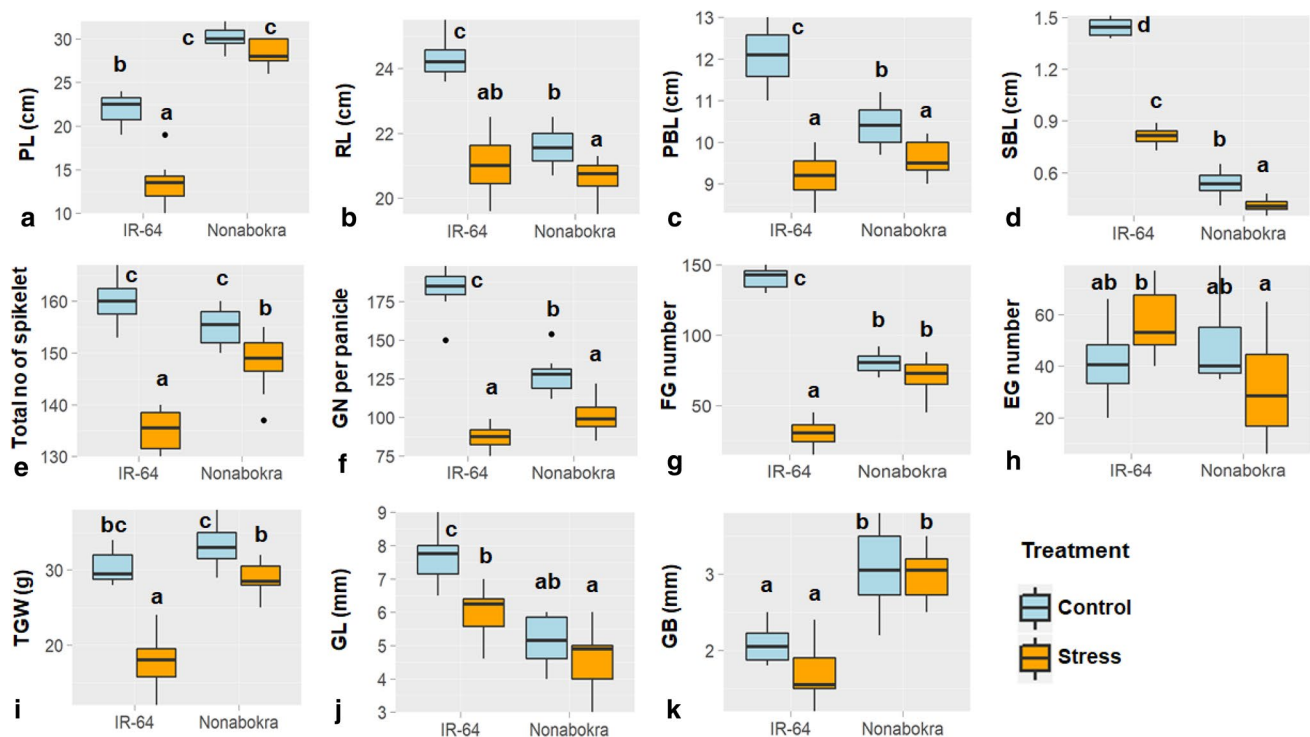


Fig. 1 Yield attributes of IR-64 and Nonabokra (NB) under control and salt stress (250 mM NaCl). The box plots showing PL (a), RL (b), PBL (c), SBL (d), total number of spikelets (e), GN per panicle (f), FG number (g), EG number (h), TGW (i), GL (j) and GB (k) in rice growing season (may–october). For all box plots, the central black bold line indicates median; boxes delineate the first and third

quartiles, the lower and upper lines refer to the minimum and maximum values respectively, while the black dots indicate outliers. Two rice plants of IR-64 and NB varieties were grown under control (blue) and stress (orange) conditions in four separate pots ($n=8$). Similar letters indicate no significant difference ($P<0.05$) using Tukey's HSD test. (Color figure online)

effects were recorded for GL, with decreasing GL by 22% in IR-64 under stress (Fig. 1j). Overall, the deleterious effect of salt stress on yield parameters was higher in IR-64 as compared to Nonabokra.

Microarray-Based Expression Profiling of Stress-Responsive and Yield-Related Genes

Because there are no previous reports regarding the expression pattern of ABA-inducible, stress-responsive and yield-related genes during rice grain development under salt stress conditions, we carried out expression profiling of key representative genes using a publicly available microarray database under salt and ABA treatment (Fig. 2). For microarray study, 7-day-old seedlings of the rice variety IR-64 were used for salt stress (GSE6901) and ABA treatment (GSE37557). Because gene expression data in the database under salt (200 mM) and ABA (100 μ M) treatment are available only for the seedling stage, these data served as a blueprint for gene expression analysis from developing grains under salt stress in our experiments. The microarray study actually seemed to be relevant and significant to confirm the ABA inducibility and expression pattern of the candidate

genes under salt stress. The results from the microarray data showed that among the TFs, the expression of *TRAB1*, *RITA1*, and *WRKY21* was up regulated under both salt stress and ABA treatment; however, the expression was higher with ABA (Fig. 2). The *RISBZ1* and *VPI* transcripts were up regulated only by ABA. The *LEA* gene, *Osem* was induced under both salt and ABA; however, the expression was higher with ABA treatment. Among osmolytes and PA-biosynthetic genes, the *P5CS* and *SAMDC* were highly up regulated with ABA. The expression of *SPMS* was down regulated under salt stress, but up regulated with ABA treatment. The *BADH1* and *SPDS* transcripts were more up regulated under ABA treatment than salt stress. Among yield-related genes, *SPL14*, *GIF1*, *GW2*, and *DEP1* were found to be up regulated during ABA treatment (Fig. 2).

Expression Profiling of ABA-Inducible Stress-Responsive and Yield-Related Genes under Salt Stress

To gain insight into the molecular mechanism of grain development under salt stress, we carried out comparative transcript profiling of ABA-inducible, stress-responsive genes

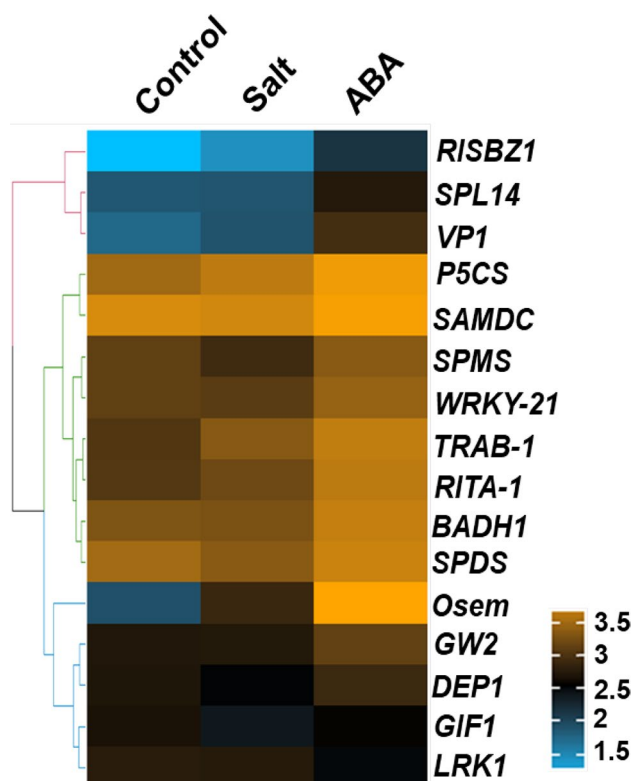


Fig. 2 Microarray-based expression analysis of genes in IR-64 rice under salt and ABA treatment. The differential transcript profile of stress-responsive and yield-related genes is represented as a heat map and hierarchical clustering was done by average linkage method. The color bar represents the relative signal values in log₁₀ scale; the heat maps were prepared with the help MeV software. (Color figure online)

between IR-64 and Nonabokra. An earlier report has shown that the salt-tolerant variety Nonabokra has a higher endogenous ABA level than the salt-sensitive varieties (Moons et al. 1995). The relative transcript level of the selected candidate genes has been extensively studied in the present communication. In addition, we also checked the expression pattern of those genes in three phases of seed development (10 DAP, 20 DAP, and 30 DAP) under stress. The heat map and hierarchical clustering showed the differential transcript abundance of the candidate genes in developing grains of IR-64 and Nonabokra under salt stress (Fig. 3). The genes were clustered into two separate groups when analyzed by hierarchical clustering. In the sensitive cultivar, the first cluster included genes (*P5CS*, *RISBZ1*, *BADH1*, *WRKY-21*, and *SPDS*) that tended to decrease or showed relatively lower expression under salt stress. The second cluster included genes (*Osem*, *VP1*, *TRAB-1*, *RITA-1*, and *SAMDC*) that accumulated to higher levels under stress condition. The expression of these genes varied between genotypes with different tolerance levels under stress conditions, suggesting a relationship with stress tolerance (Fig. 3). We utilized

a three-way factorial analysis of variance (ANOVA) on the expression levels of each gene obtained from semi-qRT-PCR. This parses expression variation due to genotype (G), treatment (T), developing stage (S), and their interactions. The three-way ANOVA indicated that out of 16 studied genes, the expressions of 12, 14 and 12 genes were influenced significantly by the effects of genotype, treatment and grain-developing stage respectively. On the other hand, the expressions of 8, 11, 12, and 11 genes were influenced by the effects of the interaction of G×T, G×S, T×S and, G×T×S respectively (Fig. 4). A visualization of the amount of variation (η_p^2 values) explained by G, T, S, and their interaction reveals that the genes were mostly affected by genotype, treatment and developing stage or with a strong interaction of G×T×S and T×S (Fig. S1). Out of 14 salt-affected genes, 71% were also influenced by both G and S. Further, 42% of genes were influenced by the two-way interaction of G×T, whereas 64% and 78% of salt-responsive genes were influenced by G×S and T×S interactions respectively. Seventy-one percent of salt-responsive genes were influenced by the three-way interactions of G×T×S (Supplementary Table S2). This analysis revealed high order complexity and intricacy with respect to all the three variables.

Genes Encoding TFs and LEA

The analysis of variance showed that there is a significant G, T and S ($P < 0.001$) effect for *TRAB-1* and *RITA-1*, with G×S and T×S interaction for *TRAB-1* and T×S interaction for *RITA-1* expression (Supplementary Table S2). The maximum up regulation of *TRAB-1* and *RITA-1* transcripts were recorded in IR-64 under salt stress at 10 DAP as compared to the respective control condition. Both *TRAB-1* and *RITA-1* transcript levels increased significantly under stress in both the cultivars (Fig. 5 and Supplementary Table S3). Similarly, salt stress increased the expression of *TRAB-1* and *RITA-1* in both IR-64 and Nonabokra throughout the developing stages, except 30 DAP for Nonabokra where the expression of *TRAB-1* was found to be non-significant. Moreover, the *TRAB-1* and *RITA-1* transcripts were found to be more up regulated in the sensitive cultivar than the tolerant one at 20 DAP under stress conditions. A significant three-way interaction (G×T×S) was recorded for *VP1* ($P < 0.001$), *RISBZ1* ($P < 0.05$), *WRKY-21* ($P < 0.01$), and *Osem* ($P < 0.01$) (Supplementary Table S2). Salt stress significantly up regulated the expression of *VP1* at 10 DAP in both the cultivars, with higher induction in Nonabokra. IR-64 recorded the maximum up regulation of the *VP1* transcript at 20 DAP under stress as compared to Nonabokra (Fig. 5 and Supplementary Table S3). The expression of *RISBZ1* in IR-64 was found to be significantly down regulated at all the stages of seed development under salt stress, as compared to the control condition. In Nonabokra, the

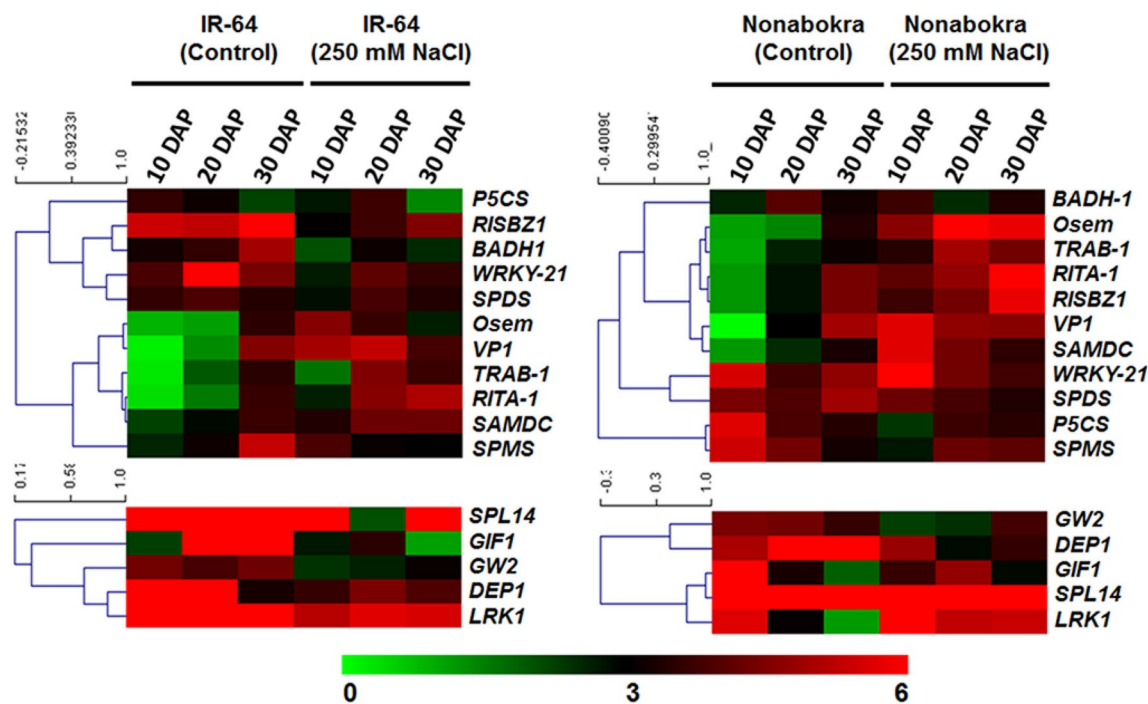


Fig. 3 Heat map and hierarchical cluster displaying differential expression profile of various stress-responsive genes which encode TFs (*TRAB-1*, *RITA-1*, *WRKY-21*, *RISBZ1*, and *OsVP1*), LEA (*Osem*), osmolyte regulatory enzymes (*P5CS* and *BADH1*), PA regulatory enzymes (*SAMDC*, *SPDS* and *SPMS*) and genes governing yield attributes (*GIF1*, *GW2*, *SPL14*, *DEPI*, and *LRK1*) in develop-

ing (10 DAP, 20 DAP and 30 DAP) grains of salt-sensitive (IR-64) and salt-tolerant (Nonabokra) rice cultivars under salt stress (250 mM NaCl). The color bar at the bottom represents the expression level in log₂ scale; green, black and red colors represent the lowest, medium and highest expression level respectively. (Color figure online)

expression of these genes was significantly up regulated during the early phase of grain development and remained higher even at 30 DAP. Salt stress significantly down regulated the expression of *WRKY-21* at 20 DAP in the sensitive cultivar; however, the expression was non-significant for the tolerant cultivar throughout the developing stages (Fig. 5). Salt stress significantly increased the expression of *Osem* in both the cultivars and throughout the developing stages as compared to the control condition. Under salt stress, the maximum induction of *Osem* was observed in IR-64 at 10 DAP, as compared to Nonabokra (Fig. 5).

Genes Encoding Osmolytes and PAs

A significant $G \times T \times S$ effect was recorded for *P5CS* ($P < 0.01$) and *BADH1* ($P < 0.001$). However, the genotype response was non-significant for *P5CS* expression (Supplementary Table S2). The *P5CS* transcript in Nonabokra was significantly down regulated at 10 DAP (Fig. 6). IR-64 did not show significant *P5CS* expression across the treatment and developing stages. Upon salt stress, the expression of *BADH1* in IR-64 was significantly down regulated at 10 DAP and 30 DAP; the expression was significantly up regulated at 10 DAP in Nonabokra seeds, whereas down regulated as

maturation proceeded under stress conditions (Fig. 6). A significant ($P < 0.001$) three-way interaction ($G \times T \times S$) was recorded for *SAMDC* and *SPMS* expression. However, the genotype and developing stage response was non-significant for *SAMDC* expression (Supplementary Table S2). The maximum induction of *SAMDC* transcript in Nonabokra was recorded at 10 DAP, followed by a slight increase in expression at 20 DAP, whereas the expression was significantly up regulated at 20 DAP in IR-64 seeds in response to salt stress (Fig. 6). In IR-64, the expression of *SPMS* was significantly up regulated at 10 DAP, followed by down regulation at 30 DAP under stress condition. However, in Nonabokra, the *SPMS* transcript was down regulated during the early phase of seed development. Significant genotype ($P < 0.001$), treatment ($P < 0.05$) and $G \times S$ ($P < 0.05$) interactions were noted for *SPDS* expression (Supplementary Table S2). Salt stress down regulated the *SPDS* expression across the cultivars and developing stages compared to the control condition (Fig. 6).

Genes Governing Yield-Related Attributes

We also carried out expression profiling of some key yield-related genes in salt-sensitive and salt-tolerant cultivars to check the effect of salt stress on the transcript level

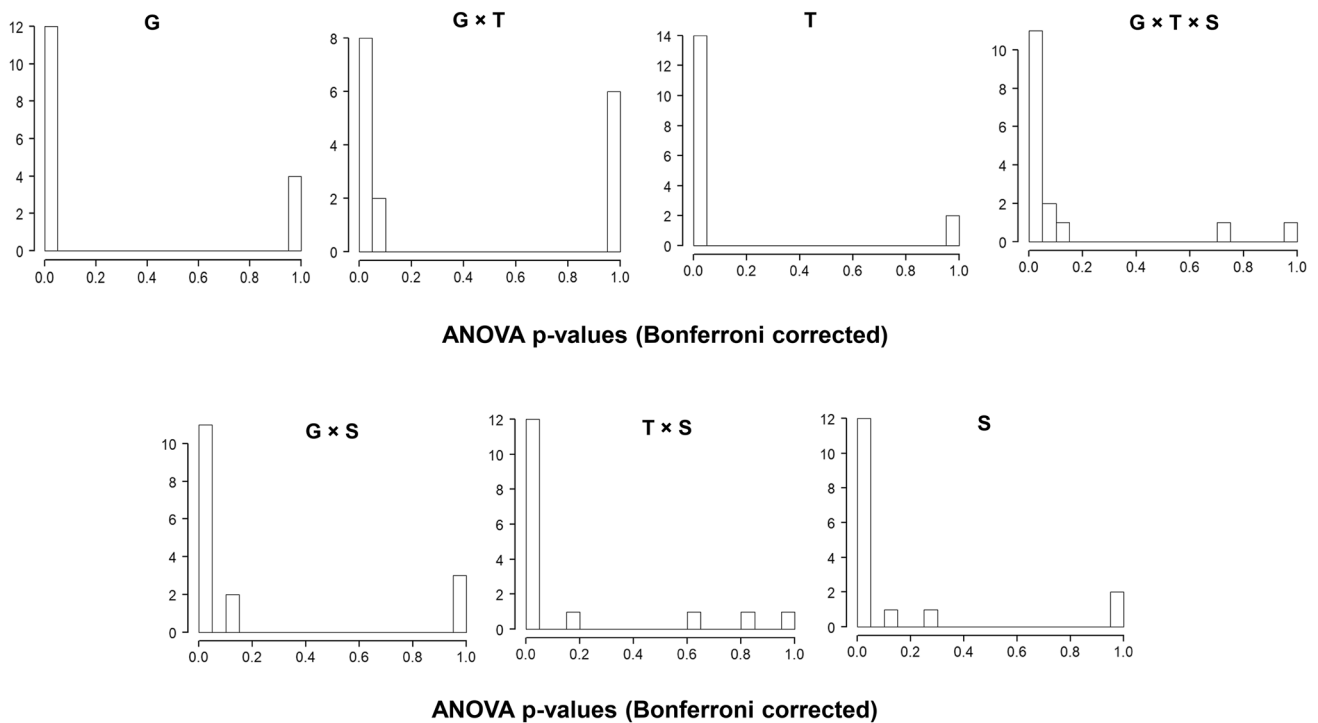


Fig. 4 Effects of genotypes (G), treatments (T) and grain-developing stages (S) on the expression levels of individual genes. Histograms show the number of genes whose expression levels were altered, with indicated *P* values by Bonferroni-corrected three-way ANOVA ana-

lyzing the effects of G, T and S. Each bar indicates a range of 0.05. The transcript levels from three biological replicates ($n=3$) were used for analysis

of genes governing yield attributes (Fig. 7). The ANOVA result revealed significant ($P < 0.001$) three-way interaction ($G \times T \times S$) for *GIF1*, *DEP1* and *SPL14* expression (Supplementary Table S2). Our observation showed that under stress condition, the *GIF1* expression was drastically inhibited at 20 DAP and 30 DAP in IR-64, whereas the transcript level was significantly inhibited at the early phase of seed development, followed by increased expression during 20 DAP and 30 DAP in Nonabokra, as compared to the control condition (Fig. 7). Salt stress caused significant inhibition of *DEP1* expression in both the cultivars throughout the developing stages; with a higher inhibition at 10 DAP in IR-64. Under stress condition, the expression was significantly down regulated during 20 DAP and up regulated at 30 DAP in IR-64, whereas the transcript level was down regulated at the early phase (10 DAP) and again up regulated after 30 DAP in Nonabokra (Fig. 7). Significant treatment ($P < 0.001$) and $T \times S$ ($P < 0.01$) interactions were recorded for *GW2* expression (Supplementary Table S2). Under stress, the *GW2* transcript was significantly down regulated throughout seed development in IR-64; however, in Nonabokra, the expression was down regulated at 10 DAP and 20 DAP (Fig. 7) as compared to the control condition. Significant ($P < 0.001$) $G \times T$, $G \times S$ and $T \times S$ interactions were noted for *LTK1* expression (Supplementary Table S2). In IR-64, the *LTK1* transcript was significantly down regulated

throughout the developing stage under stress condition, whereas the transcript was significantly up regulated in Nonabokra with maximum induction at 30 DAP (Fig. 7).

Expression Analysis of TFs in Dry, Water-Imbibed and ABA-Imbibed Mature Seeds

To investigate whether the TFs in seeds are induced following germination and ABA treatment, we analyzed the expression pattern of *TRAB-1*, *RITA-1*, *VPI*, and *WRKY-21* in dry, water-imbibed, and ABA-imbibed seeds (Fig. 8). The transcript profiling with matured grains showed that *TRAB-1* and *RITA-1* were expressed in dry and ABA-imbibed seeds, whereas no corresponding transcripts were detected upon water imbibition. The transcript level of *WRKY-21* was the highest in ABA-imbibed seeds of the salt-tolerant variety Nonabokra. The *VPI* expression was uniform under all the conditions in both the varieties (Fig. 8).

Correlation Analysis Revealed a Close Relationship Between Transcript Levels and Grain Yield Under Stress Conditions

To investigate the relationship between gene expression and grain yield under stress condition, the correlation between

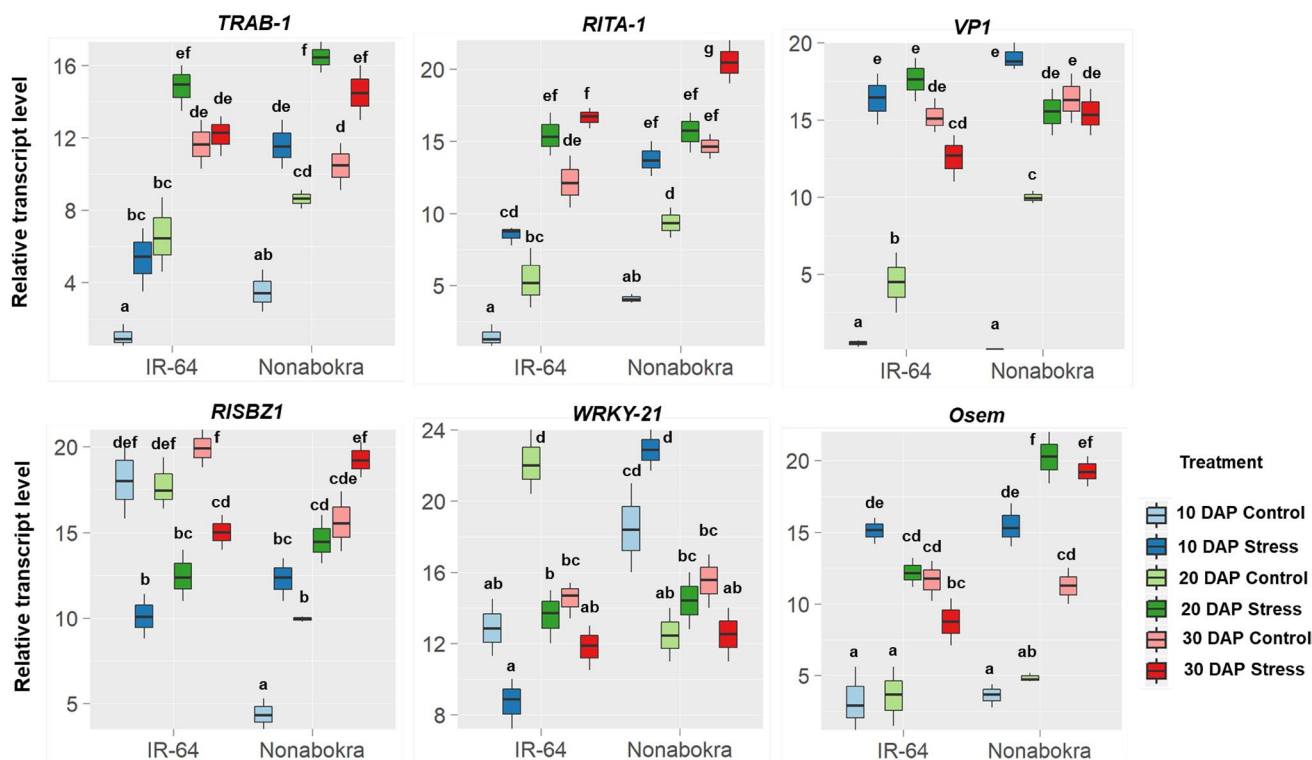


Fig. 5 Relative expression of genes encoding TFs (*TRAB-1*, *RITA-1*, *VPI*, *RISBZ1*, and *WRKY-21*) and LEA (*Osem*) under salt stress (250 mM NaCl) in developing grains (10 DAP, 20 DAP, and 30 DAP) of salt-sensitive (IR-64) and salt-tolerant (Nonabokra) rice. The expression level of each gene was normalized with *actin*, which was used as an internal control and the transcript level was expressed

relative to *actin*. For all box plots, the central black bold line indicates median; boxes delineate the first and third quartiles, while the lower and upper lines refer to the minimum and maximum values respectively. The transcript levels from three biological replicates ($n=3$) were used for analysis. Similar letters indicate no significant difference ($P<0.05$) using Tukey's HSD test

expression levels of each gene and grain yield parameters under stress condition was tested by Pearson's correlation analysis (Fig. 9a–c and Supplementary Table S4). TGW has been considered a key factor indicating the sensitive or tolerant nature of plants under stress conditions. In our study, we analyzed the correlation between genes and TGW (Fig. 9a–c). Under salt stress, the expression levels of *TRAB-1*, *WRKY-21*, *Osem* ($P<0.01$), *RITA-1*, and *RISBZ1* ($P<0.05$) were positively correlated with TGW (Fig. 9a). Among osmolytes and PA, *BADHI* ($P<0.05$), *SAMDC* ($P<0.05$), and *SPDS* ($P<0.01$) (Fig. 9b) and *GIF1* ($P<0.01$) among yield-related genes showed a positive correlation with TGW (Fig. 9c).

Discussion

Plant abiotic stress tolerance is a complex mechanism, depending on the coordinated actions of a wide range of genes belonging to diverse metabolic pathways. The aim of this work was to understand how salt stress affects the expression of stress-responsive and yield-related genes during rice reproductive development and their correlation

with yield under salt stress. This study is extremely crucial, because the grain filling step is the ultimate determining factor governing final productivity and crop yield. Such study can provide new insight into the distinct processes of salt stress response during rice grain development and the potential genetic variation at the transcript level. To the best of our knowledge, this is the first study reporting the transcriptional regulation of stress-responsive and yield-related genes during rice grain development under salt stress.

The plant response to environmental stress during the reproductive stage is extremely vital because it is a determinant of grain yield. The factors responsible for the reduction in rice grain yield under stress mostly include modified cell wall flexibility, along with the reduced effectiveness of turgor pressure responsible for cell growth (Hakim et al. 2014). In our study, we noted that PL, total number of spikelets, TGW, GN per panicle and filled GN decreased significantly in the salt-sensitive cultivar in response to salt stress which is in accordance with previous reports (Abdullah et al. 2001; Rad et al. 2012; Rao et al. 2013; Thitisaksakul et al. 2015; Panda et al. 2016). This result was further supported by the two-way ANOVA where a significant $G \times T$ effect was observed for these yield attributes (Supplementary

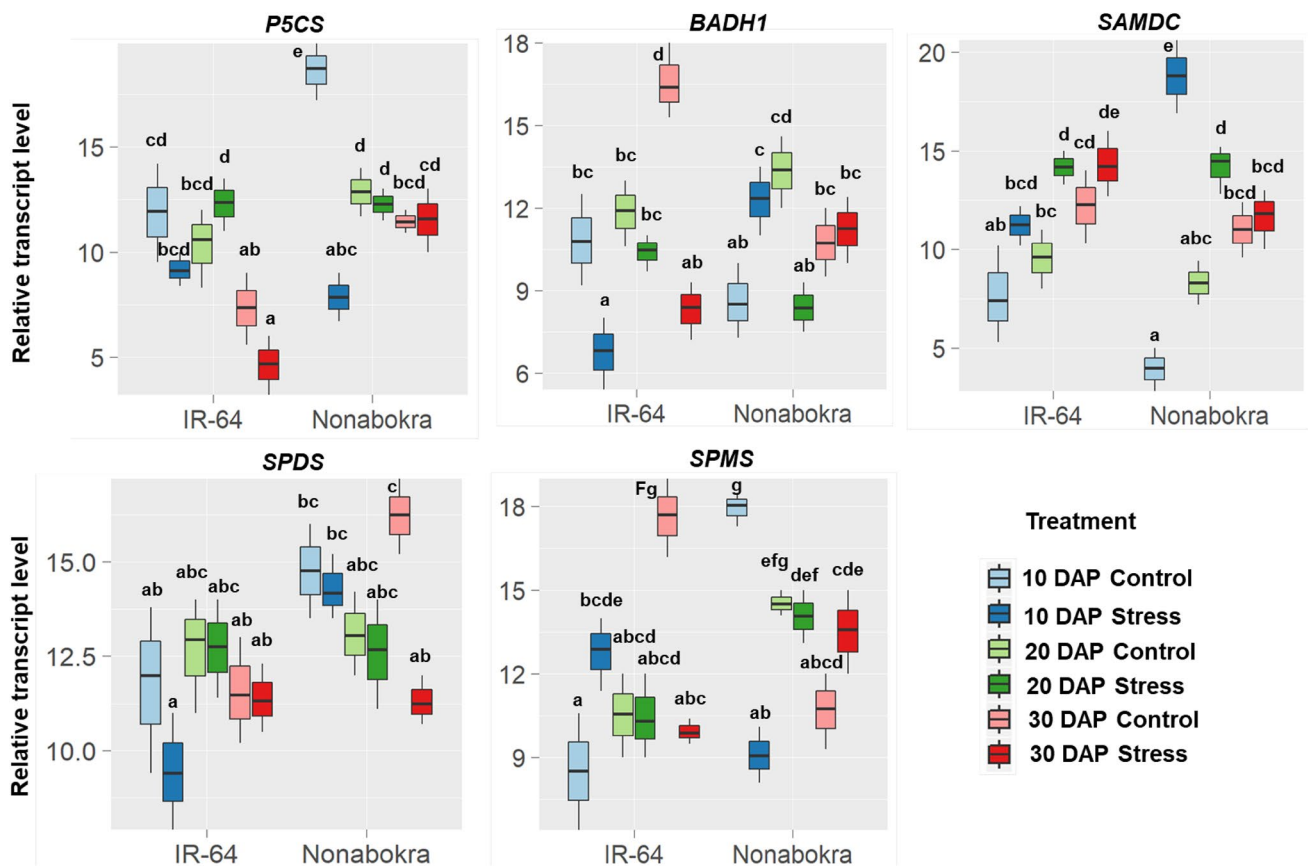


Fig. 6 Relative expression of genes encoding osmolytes (*P5CS* and *BADH1*) and PA regulatory enzymes (*SAMDC*, *SPDS*, and *SPMS*) under salt stress (250 mM NaCl) in developing grains (10 DAP, 20 DAP, and 30 DAP) of salt-sensitive (IR-64) and salt-tolerant (Nonabokra) rice. The expression level of each gene was normalized with *actin*, which was used as an internal control and the transcript level

was expressed relative to *actin*. For all box plots, the central black bold line indicates median; boxes delineate the first and third quartiles, whereas the lower and upper lines refer to the minimum and maximum values respectively. The transcript levels from three biological replicates ($n=3$) were used for analysis. Similar letters indicate no significant difference ($P < 0.05$) using Tukey's HSD test

Table S1). We also noted that salt stress significantly decreased GL in the sensitive cultivar. Previous reports have shown that salinity decreases both GN and grain size (Fabre et al. 2005; Rao et al. 2013). An earlier report by Asch et al. (2000) has shown that during the reproductive stage, salinity decreases the number of filled panicles, fertile panicle, TGW, percentage of fertile grains and increases sterility in rice cultivars, with panicle initiation being the most sensitive stage. The data obtained from our study suggested that early exposure to salt stress prior to reproductive development significantly affected seed development of IR-64 which ultimately alters crop yield.

Microarray analysis was performed prior to semi-qRT-PCR analysis to confirm whether the selected candidate genes would be induced at all during rice seed development under salt stress. The microarray data suggested the salt- and ABA-inducible nature of these genes (Fig. 2). The up regulation of yield-related genes, that is, *SPL14*, *GIF1*, *GW2*, and *DEP1*, upon the application of ABA is indicative

of the possible adaptive response conferred by these genes during reproductive development of salt-treated rice. Analysis of the expression patterns of these genes through semi-qRT-PCR showed that the TFs like *TRAB-1*, *RITA-1*, *RISBZ1*, *VPI*, and *WRKY-21* (members of bZIP, ABI3/VP1, and WRKY families) were differentially expressed in developing seeds of salt-sensitive and salt-tolerant rice cultivars, which is consistent with the previous studies in which these TF families were shown to be associated with seed development. The bZIP TFs that show aleurone- and endosperm cell-specific expression in developing rice seeds (Izawa et al. 1994), associate with α -globulin promoters to drive the expression of genes encoding storage proteins (Nakase et al. 1997); such factors are also known to mediate ABA-responsive gene expression by binding to ABA-responsive elements (ABREs) (Hattori et al. 1992, 1994; Hobo et al. 1999). The three-way ANOVA showed that out of 16 studied genes, 14 genes were influenced by salt treatment in developing grains, suggesting the salt-responsive

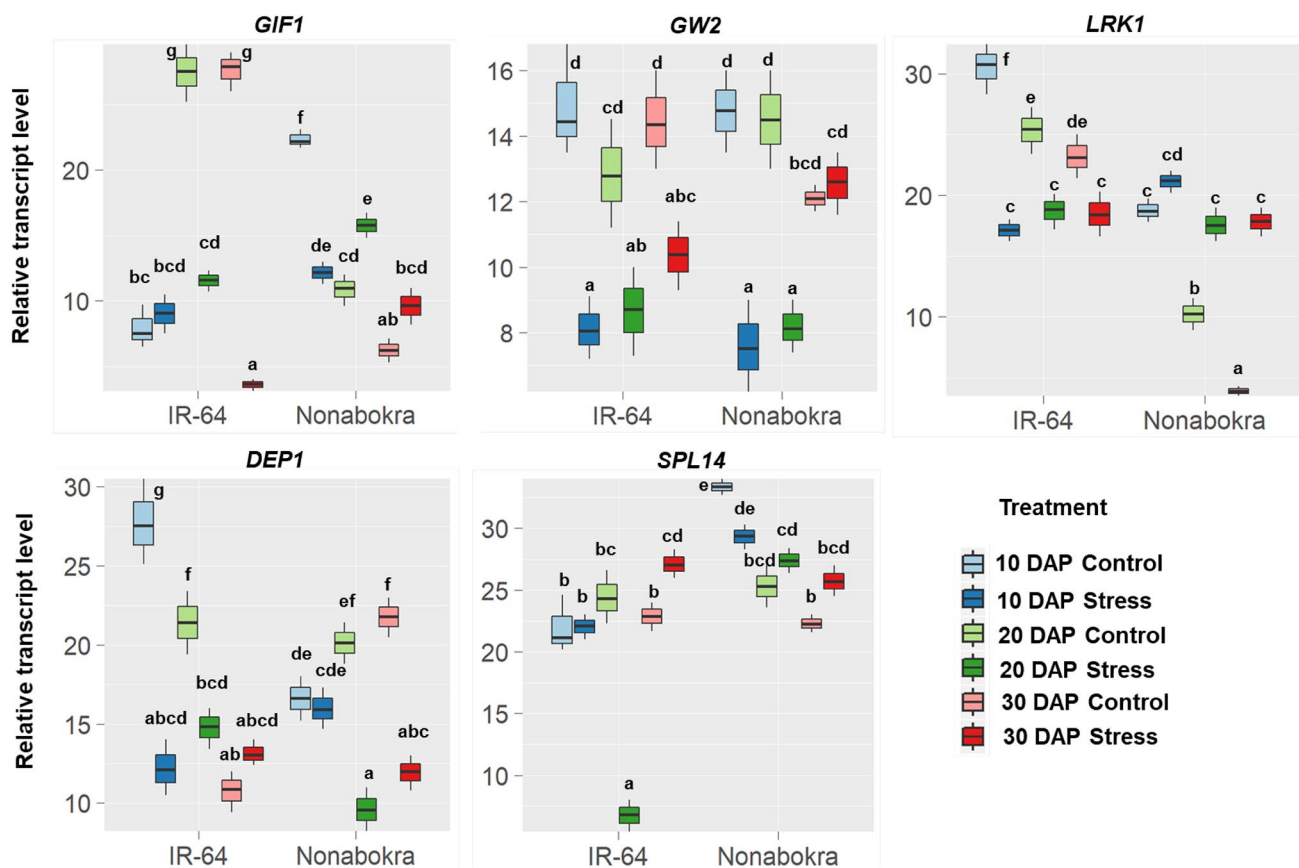


Fig. 7 Relative expression of genes governing yield attributes (*GIF1*, *GW2*, *LRK1*, *DEP1*, and *SPL14*) under salt stress (250 mM NaCl) in developing grains (10 DAP, 20 DAP, and 30 DAP) of salt-sensitive (IR-64) and salt-tolerant (Nonabokra) rice. The expression level of each gene was normalized with *actin*, which was used as an internal control and the transcript level was expressed relative to *actin*. For all

box plots, the central black bold line indicates median; boxes delineate the first and third quartiles, whereas the lower and upper lines refer to the minimum and maximum values respectively. The transcript levels from three biological replicates ($n=3$) were used for analysis. Similar letters indicate no significant difference ($P < 0.05$) using Tukey's HSD test

nature of these genes. This result also justifies the selection of these candidate genes for the present study. Further, ANOVA results revealed that the interaction of $G \times T \times S$ effect was significant for most of the genes (Supplementary Table S2), indicating the differential regulation of the genes with respect to developing stages of seeds or exposure to salt stress. The expression of *TRAB-1* and *RITA-1* was predominantly up regulated at the early to mid-phase of seed development in the sensitive cultivar, as compared to the tolerant one under stress condition. The expression of these genes in Nonabokra was mostly up regulated at the early phase of seed development (Supplementary Table S3). This result was further confirmed by ANOVA, which showed that the effects of $G \times T$ and $T \times S$ were significant for *TRAB-1* and $T \times S$ interaction for *RITA-1* expression (Supplementary Table S2). The higher expression level of *TRAB-1* and *RITA-1* in IR-64 during early to mid-phases of seed development under salt stress represents a protective measure of the salt-sensitive cultivar to deal with early exposure of salt stress.

Among TFs and *LEA* genes, the *VPI*, *RISBZ1*, *WRKY-21*, and *Osem* showed a significant effect of $G \times T \times S$ interaction (Supplementary Table S2), indicating differential expression between the sensitive and tolerant genotype and the influence of developing stages on the expression of these genes under stress condition. IR-64 recorded the maximum up regulation of *VPI* transcript at 20 DAP as compared to Nonabokra, whereas at 10 DAP for Nonabokra under stress. The role of *VPI* in ABA-regulated gene expression during seed development has been reported earlier (Hattori et al. 1992, 1994). The expression of *RISBZ1* was found to be significantly down regulated at all the stages of seed development in IR-64 under salt stress. However, in Nonabokra, the expression of *RISBZ1* was significantly up regulated during the early phase of grain development and remained higher even at 30 DAP. The *WRKY-21* transcript was down regulated by salt stress at 20 DAP in the sensitive cultivar (Fig. 5 and Supplementary Table S3). Earlier study showed that *RISBZ1* plays a regulatory role in various processes of

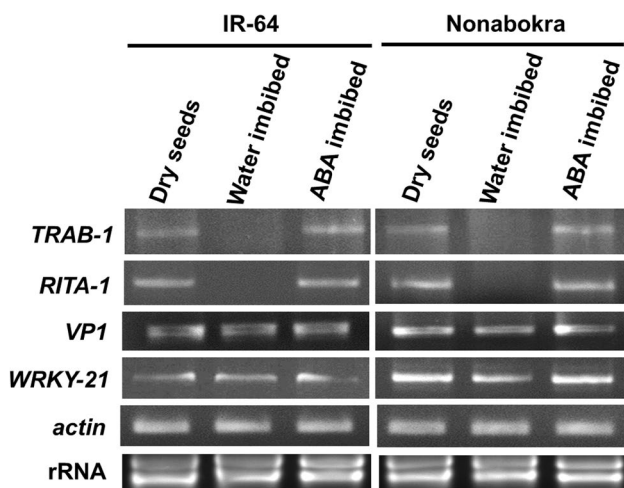


Fig. 8 Expression profile of TFs (*TRAB-1*, *RITA-1*, *OsVP1*, and *WRKY-21*) in dry, water-imbibed (10 h) and ABA (100 μM)-imbibed (10 h) seeds of salt-sensitive (IR-64) and salt-tolerant (Nonabokra) rice cultivars derived from RT-PCR analysis. 25 PCR cycles were carried out for each gene and the experiment was repeated thrice; *actin* was used as the reference gene

grain filling and development by activating the transcription of SSP in the mature endosperm (Yamamoto et al. 2006). The regulatory role of *WRKY-21* in multiple developmental and physiological processes by mediating ABA responses in aleurone cells has been reported earlier (Alexandrova and Conger 2002; Xie et al. 2005). The higher expression of TFs in Nonabokra suggested the inherent tolerant property which enables them to maintain grain development under salt stress. On the other hand, the down regulation of *RISBZ1* and *WRKY-21* in IR-64 throughout seed development causes lower grain weight under stress. The role of these TFs in maintaining better yield performance under stress is evident from the correlation analysis with TGW (Fig. 9a). The LEA protein which is hydrophilic in nature is highly accumulated during the mid to late stages of seed development and also under drought stress (Jin et al. 2013; Yu et al. 2016). In our study, the *Osem* transcript was more accumulated under salt stress, especially in the tolerant cultivar throughout seed development which helps in maintaining the grain weight even under stress condition, as evident by the significant correlation with yield parameter (Fig. 9a). The *Osem* and *VP1* transcripts showed a similar expression pattern under stress

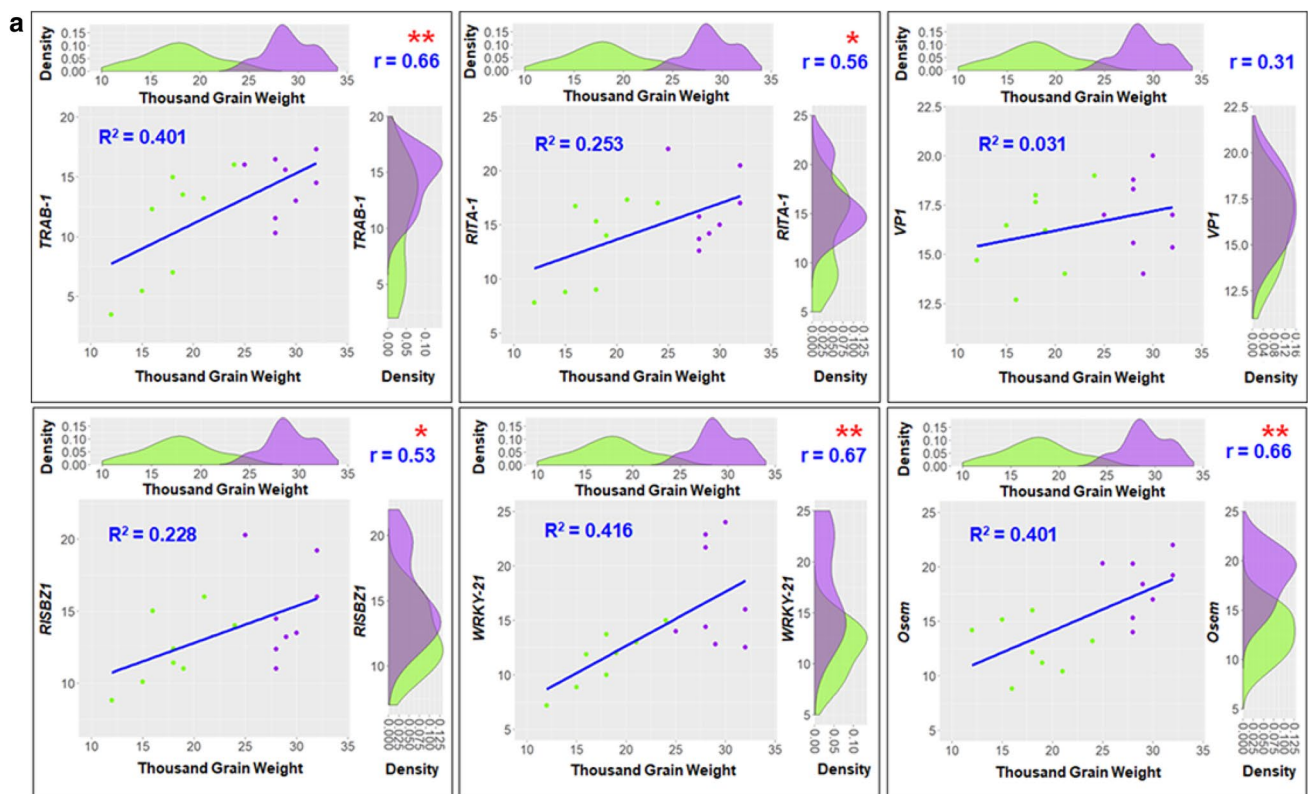


Fig. 9 Correlation of TGW with expression of genes encoding TFs (*TRAB-1*, *RITA-1*, *WRKY-21*, *RISBZ1*, and *OsVP1*) and LEA (*Osem*) (a), osmolyte regulatory enzymes (*P5CS* and *BADH1*) and PA regulatory enzymes (*SAMDC*, *SPDS* and *SPMS*) (b), and genes governing yield attributes (*GIF1*, *GW2*, *SPL14*, *DEP1*, and *LRK1*) (c) under salt

stress (250 mM NaCl). The corresponding distribution curves of salt-sensitive (IR-64) and salt-tolerant (Nonabokra) rice cultivars are also shown for each trait. The Pearson's correlation coefficient (r), regression values (R^2) and their significance level (** $P < 0.01$, * $P < 0.05$) are displayed on top right of each segment

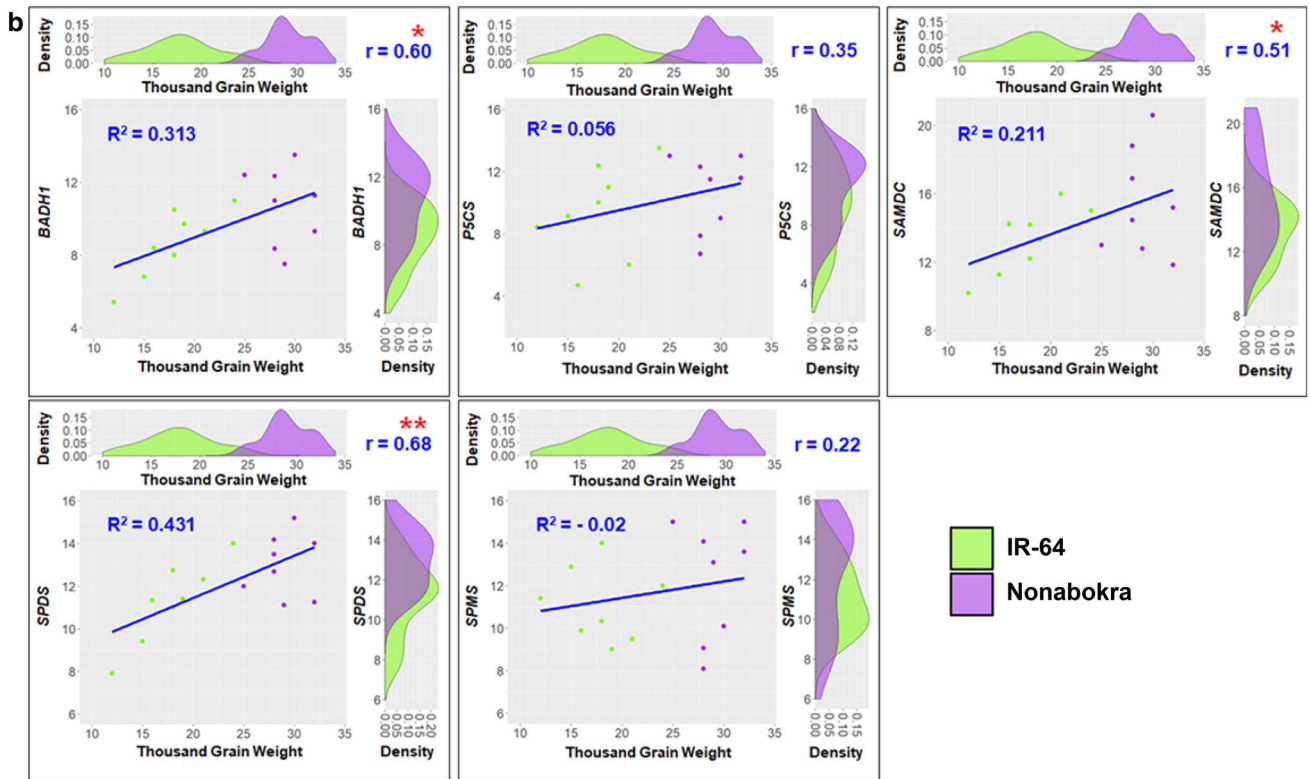


Fig. 9 (continued)

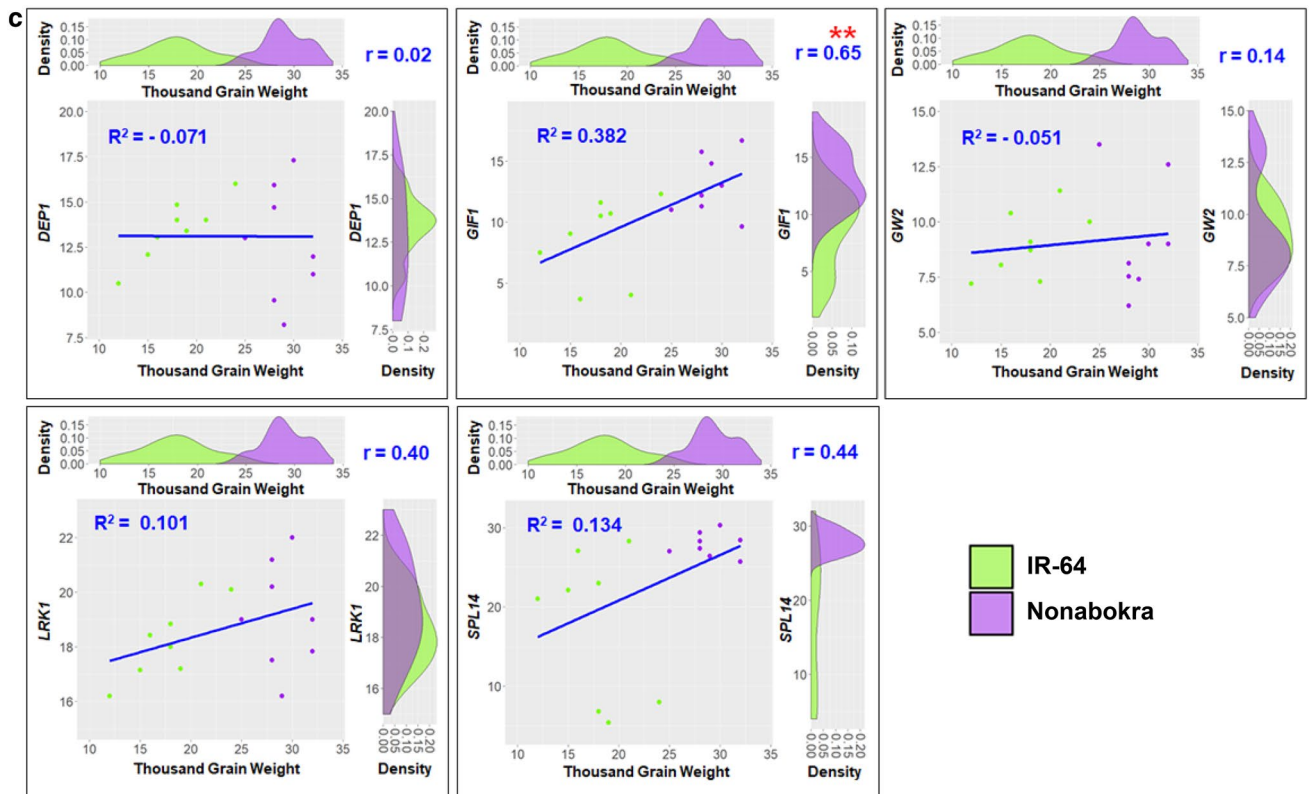


Fig. 9 (continued)

condition, being up regulated at 10 DAP to 20 DAP which is the active grain filling period for rice. The regulation of *Osem* expression by *VP1* in response to ABA during seed development has also been reported earlier (Hattori et al. 1995; Miyoshi et al. 2002).

Pro and GB are one of the many well-known osmolytes that provide protection to osmotic stress (Kumar et al. 2010; Chen and Murata 2011), although comparative assessment of the function of the genes encoding these metabolites during grain development under salt stress remains elusive. In our study, we observed that the *P5CS* transcript was only down regulated at the early phase of seed development in Nonabokra, whereas IR-64 did not show significant expression throughout the developing stages under stress condition. The expression of *BADHI* was significantly down regulated in IR-64 throughout the grain-developing stage, whereas the expression in Nonabokra seed was significantly up regulated at 10 DAP which is the active phase of the grain filling process (Fig. 6). This differential expression was further supported by the three-way ANOVA where a significant $G \times T \times S$ effect was recorded for *P5CS* and *BADHI* (Supplementary Table S2). A positive correlation of the *BADHI* transcript level and TGW under salt stress is connected with the accumulation of GB in developing grains which leads to the variation in yield performance under salt stress in the two genotypes (Fig. 9b). The role of PAs in grain filling and milling has been reported earlier (Yang et al. 1997; Wang et al. 2007). The higher levels of PAs like Spd and Spm have been reported to promote grain filling and increase seed weight. However, little is known about the role of PAs in rice grain filling under salt stress. In our study, we observed a differential expression pattern of PA-biosynthetic genes in the sensitive and tolerant cultivars. Among the PAs, *SAMDC* has been found to be significantly up regulated at the early to mid-phase of seed development, that is, the active phase of grain filling, in the tolerant cultivar. ABA-regulated *SAMDC* expression in the matured rice grains has been reported earlier (Roychoudhury et al. 2009). Further, positive correlation between TGW and expression of *SAMDC* under salt stress connected the protective role of PAs in grain development of Nonabokra during salt stress. Application of Spd or Spm to rice panicles at the early grain filling stage significantly enhanced the activities of several regulatory enzymes in grains (Wang et al. 2012). A similar mechanism of PA action is involved in rice grain development under salt stress in our case.

We also studied the effect of salt stress on the expression of some key yield-related genes during grain formation. Our result showed that among the five yield-related genes, only *GIF1* showed significant correlation with final grain weight under stress condition. The *GIF1* gene, known to be a positive regulator of grain filling, encodes a cell wall invertase which is required for carbon partitioning during grain filling

(Wang et al. 2008). In our study, the *GIF1* was found to be significantly up regulated in the tolerant cultivar during the mid to late phases of seed development, whereas the expression was down regulated in IR-64. This dissimilarity of expression between cultivars was further supported by ANOVA, where significant $G \times T \times S$ effect was noted for *GIF1* expression (Supplementary Table S2). The higher expression of *GIF1* in developing grains of Nonabokra in response to salt stress enables the tolerant cultivar to maintain better grain filling rate under salt stress. This is further supported by the strong correlation of *GIF1* expression with final grain weight and GN measured under salt stress (Fig. 9c).

The dynamic equilibrium between ABA and gibberellin metabolism acts antagonistically and controls seed dormancy and germination. ABA promotes dormancy induction, but inhibits seed germination at low concentrations and this process gets initiated with the perception of ABA which subsequently triggers downstream genes involved in a complex regulatory and signaling network. In the signaling pathway, TFs play a nodal role because they act as an upstream signaling component by binding to the conserved cis-acting elements of the promoter region of their target genes, thereby controlling their expression in response to ABA during environmental stress. In our study, the higher induction of TF transcripts in dry and ABA-imbibed seeds of the tolerant cultivar indicated their role in the ABA-signaling pathway during seed development and maturity (Fig. 8). The ABA-inducible nature of these TFs was further supported by our microarray analysis where the expression of *TRAB-1*, *RITA-1*, *VP1*, and *WRKY-21* were up regulated by ABA treatment (Fig. 2). The up regulation of TFs in ABA-imbibed seeds, whereas down regulation upon water imbibition of seeds has been reported earlier (Nakagawa et al. 1996), which is in accordance with our observation. Zou et al. (2004) reported earlier that the complex of *WRKY-21*, *VP1* and *ABI5* functions downstream of *ABI1* in the ABA-mediated response cascade during abiotic stress. Hattori et al. (1994) have also shown that the *VP1* transcript was detected in dry as well as ABA-imbibed mature embryos. This is because of the fact that the function of *VP1* is to mediate ABA-mediated transcription in seeds, via its interaction with *TRAB-1* (Hobo et al. 1999).

Overall, in the present communication, we carried out comprehensive expression profiling of ABA-inducible, stress-responsive and yield-related genes during grain formation under salt stress in two indica rice cultivars with contrasting levels of salt tolerance and correlated the expression profile with grain yield. The detailed transcript profiling study during rice grain development under salt stress is lacking, and to the best of our knowledge, this is the first novel report highlighting the transcriptional regulation of stress-responsive and yield-related genes during grain development

under salt stress. This really speaks of the importance of the present work, which will pave the way for further investigation. Our study concludes that salt stress has a significant and negative impact on the reproductive and grain-developing stages, translating into a higher spikelet sterility and loss of grain weight. Based on the extensive statistical model and correlation analysis, we identified that among the examined genes, certain members of the TFs and LEA (that is, *TRAB-1*, *RITA-1*, *RISBZ1*, *WRKY-21*, and *Osem*), osmolytes and PA metabolic genes (that is, *BADH1*, and *SAMDC*) as well as the yield-related gene *GIF1* were more closely related to the grain yield under salt stress. Co-expression of ABA-inducible TFs and PA-biosynthetic genes along with *GIF1* under stress conditions indicated a possible interplay between ABA-inducible TFs, PAs and genes responsible for grain filling, which enable Nonabokra to maintain its grain filling process even under harsh saline conditions, so that it can produce enough seeds and proceed towards the next generation. The salt-tolerant trait of Nonabokra at the reproductive stage is therefore attributed to the PA-mediated regulation of grain filling, via regulation of the *GIF1* transcript and ABA-dependent TFs which subsequently modulate PA metabolism and regulates grain filling processes under stress condition. The role of endogenous ABA in salt and chilling tolerance by modulating PA metabolism has been reported earlier in maize and rice seedlings (Lee et al. 1997; Liu et al. 2005). The information derived in this communication will help in regulating the salt tolerance mechanism of rice, especially the sensitive cultivars, at the reproductive or grain filling phase.

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