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Identification of novel genes potentially involved in rice (*Oryza sativa* L.) drought tolerance

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Abstract

Drought is a major constraint affecting rice production and causing yield reduction of up to 60% in the major growing areas of Asia. Developing drought-tolerant cultivars in rice is an appropriate strategy to provide food security and hinder the harmful effects of drought. Therefore, particular attention must be directed toward identifying drought-responsive genes. In the present study, based on the microarray analysis results of two rice genotypes with contrasting response to drought stress, 308 probe sets are uniquely upregulated with equal to or greater than 3 symmetric fold changes in drought-tolerant genotype upon exposure to drought stress. As the next step, mapping of the corresponding genes of these probe sets via the web-based tool "QlicRice" is expected to reveal the genes within the drought stress-associated QTLs (quantitative trait loci). To determine the number of probe sets annotated to the transcription factors in various families, the plant transcription factor database (PlnTFDB) is relatively utilized. Finally, the biclustering analysis using Genevestigator is at hand to unveil the biclusters along with the embedded probe sets annotated to 3 transcription factors in different drought stress studies. The survey is also aimed at determining the possible relationships between up- and co-regulated genes and the transcription factors in the obtained biclusters through plant promoter analysis navigator (PlantPAN). To substantiate how the exploration of transcriptomic changes of the genotypes with contrasting drought tolerance could uncover a number of genes associated with rice drought stress is the ultimate goal of the present study.

Key words: biclustering analysis, drought, transcription factor, *Oryza sativa* L., transcriptomic analysis

Introduction

Rice is one of the most important crops, which has a worldwide reputation for a stable food supply with considerable economic standing. Drought, with different intensities, however, is one of the main constraints hampering the rice productivity in 20% of the total rice growing areas in Asia (Pandey, 2007) and causing yield reduction of up to 60% (Sarris, 2004; Farooq et al., 2009). Various researches carried out to understand the mechanism and genes involved in rice drought tolerance bear considerable significance when addressing concerns associated with the increasing demand for food. In an effort to improve the food security and avert the adverse effects of drought and water deficit, such attempts will most likely help in finding effective strategies (like breeding for water saving and drought tolerance purposes) for rice cultivation (Luo, 2010). It should be highlighted that the drought tolerance is a complicated trait embodying changes at biochemical, physiological, and morphological levels (Hadiarto and Tran, 2011), including accumulation of osmoprotectants such as proline and trehalose, reduced leaf area, leaf rolling, reduced tillering, reduced transpiration, developing efficient rooting system, stomatal closure, and earliness (Guo et al., 2006; Nakashima et al., 2007; Wang et al., 2007; Islam et al., 2009; Jin et al., 2009). In recent years, fortunately enough substantial attention has been devoted to the escalation of the molecular genetic basis of drought tolerance in plants, which has led to the discovery of novel drought-responsive genes. At the same time, the overexpression of stress-responsive genes in plants is an important strategy for enhancing the tolerance to abiotic stresses (Liu et al., 2013).

Microarray-based transcriptome studies are being used to generate the expression profiles of thousands of genes and identify those whose expression is altered by

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stresses, e.g., biotic and abiotic stresses (Takahash et al., 2004). Moreover, comparative transcriptome analysis is a potent approach for unraveling the molecular basis that underlies the specific biological events (Roy et al., 2011; Ward et al., 2012; Halimaa et al., 2014). This approach, not only allows us to unearth the gene expression changes under various conditions, but it also contributes to the uncovering of unique transcripts in organisms (Dhaubhadel et al., 2007; Wei et al., 2010; Huan et al., 2013). The main objective of the present study was to identify and explore genes involved in response to drought stress in 2 contrasting rice genotypes. The hypothesis was based on the theory that genes, which are uniquely upregulated in a tolerant genotype would provide the strongest candidates for drought tolerance in rice. The procedure was formulated on the basis of microarray data from 2 rice genotypes with contrasting responses to drought stress. The probe sets consisting of a drought-tolerant genotype with symmetric fold changes equal to or greater than 3 and a drought-sensitive genotype with symmetric fold changes less than 1, exposed to drought, were selected for further analysis. A biclustering analysis was used to mine the statistically significant biclusters in the identified probe sets. Furthermore, it was assumed that the location of the genes identified within the important QTLs (Quantitative Trait Loci), which is associated with the drought stress, robustly supports their potential efficacy in the drought tolerance. Accordingly, the genes were assessed in terms of their location within the previously identified drought-associated QTLs. In addition, the genes encoding the transcription factors (TFs) were also identified and placed under scrutiny. Generally speaking, the present study has uncovered a number of genes involved in drought responsiveness in rice, which is a promising attempt for the engineering of rice cultivars and the enhancement of their drought-tolerance.

Materials and methods

Data collection

To gather the required data, the raw CFX expression language (CEL) data provided by Rai and Singh (2011) were downloaded from the gene expression omnibus (GEO) deposited at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov), and the GEO accession number of the primary microarray data was 21651 gene series (GSE21651). The GSE21651 file contained 8 GEO samples (GSM) including GSM540080, GSM540081, GSM540082, GSM540083, GSM540084, GSM540085, GSM540086, and GSM540087. The experimental condition consisted of 2 rice varieties IR64 (drought-sensitive) and Vandana (drought-tolerant), tested under non-stressed (control) and drought-stressed conditions. Seeds of 2 varieties were grown hydroponically in Hoagland's nutrient solution (Hoagland and Arnon, 1950). After 14 days, to induce the drought stress response in 1 set, the seedlings were blot dried and kept outside the medium for 24 hours, while another set of seedlings was kept as a control in Hoagland's nutrient solution. Total RNAs were extracted from the leaf samples using TRIzol reagent (Sigma) and processed according to the Affymetrix GeneChip expression analysis technical manual. The cDNA was synthesized from poly (A)+ mRNA present in 8 μg of total RNA using Superscript double-stranded cDNA synthesis kit and poly (T) nucleotide primers that contained a sequence recognized by T7 RNA polymerase. A portion of the resulting double-stranded cDNA served as a template to generate biotin-tagged cRNA in an *in vitro* transcription reaction, using Affymetrix GeneChip IVT labeling kit. Fifteen micrograms of biotin-tagged cRNA was fragmented in to strands of 35-200 bases in length, following the Affymetrix protocols. Subsequently, 10 μg of the fragmented was hybridized at 45 °C with rotation for 16 h in an Affymetrix GeneChip hybridization oven 450 using probes present on the Affymetrix rice genome array. The GeneChip was washed and then stained with streptavidin-phycoerythrin in Affymetrix Fluidics station 400, followed by a scanning on a GeneChip Scanner 3000. Sixteen GeneChip arrays were used for 8 RNA samples extracted from 2 biological replicates of 2 rice varieties with contrasting drought tolerance, each grown under control and drought stress conditions.

Microarray analysis

To start with the analysis, 8 downloaded CEL files of 3′ expression array were imported into Affymetrix expression console software and normalized by robust multichip analysis (RMA) algorithm, as described previously (Alisoltani et al., 2014). In the given experiment, 8 GeneChip arrays were used for 8 RNA samples extracted from 2 biological replicates of 2 rice genotypes

with contrasting drought tolerance, each grown under control and drought stress conditions. According to the procedure by Alisoltani and coworkers (2014), the analyses have been carried out by FlexArray package (McGill University, Canada).

Finally, the Bayesian two-sample *t*-test was used to select the genes with significant differential expression at corrected *P*-value ≤ 0.05 and using this test, a symmetric fold change was determined. To comply with the objective of the research, the genes that were selected showed a significant differential expression (corrected *P*-value ≤ 0.05) and remarkably upregulated (equal to or greater than 2 symmetric fold change) in a tolerant genotype and downregulated (less than 1 symmetric fold change) in a sensitive one under drought stress.

Identified genes and statistical discovery of significant biclusters

In the gene expression data, a bicluster was considered to be a subset of genes, exhibiting similar expression patterns over a subset of the conditions. Such a capacity of the biclustering tool embedded in Genevestigator (Zimmermann et al., 2004) was used to detect the biclusters in the identified probe sets that uniquely upregulated in a tolerant rice genotype. The Genevestigator contains thousands of public microarray and RNAseq experiments that are manually curated and described. The database content for rice incorporated into the Genevestigator includes 130 studies, 2527 samples, 324 conditions, 368 genotypes, and 38 anatomy (organs, tissues, and cell cultures from primary cells) (https://genevestigator.com/gv/doc/intro_plant.jsp). Genevestigator visualizes gene expression in various biological contexts such as chemicals, nutrients, diseases, tissues, genotypes, or biotic and abiotic stress conditions. In order to serve biclustering, "Perturbations" were chosen as the type of profile to bicluster. The threshold value and the minimum size of biclusters used for biclustering analysis were shown to be 0.7 and 5 × 5, respectively.

Gene Mapping within the drought-associated QTLs

In identifying the mapping of the genes within the drought-associated QTLs, the internet-based tool "QlicRice" (http://nabg.iasri.res.in:8080/qlic-rice/qtlbrowser.html) was utilized. Having been used in the process, the QlicRice could directly map the gene IDs (MSU-Locus IDs) within the corresponding QTLs (Smita et al., 2011).

Identification of genes encoding transcription factors

In this phase, a homology-based search against the plant transcription factor database (PlnTFDB) (Pérez-Rodríguez et al., 2010) was implemented to detect genes encoding the transcription factors in the probe sets. The factor is characterized by a symmetric fold change equal to or greater than 3 in drought-tolerant rice genotype and less than 1 in drought-sensitive rice genotype under drought stress. Affymetrix probe sets were converted to MSU gene locus (a locus identifier from Michigan State University rice database) and used as queries.

Investigation of genes with identified transcription factors in biclusters

In order to find any possible link between genes with the identified transcription factors in biclusters, the prediction of corresponding TFs, in each gene, capable of creating bonds with genes in biclusters was followed by mining-identified transcription factors in bicluster among the corresponding transcription factors.

Results and discussion

Understanding the drought stress responses provides new insights into the stabilization and protection of crop performance under water-deficit conditions (Hadiarto and Tran, 2011). Transcriptome data have been applied to unravel the responses of contrasting genotypes of rice to a drought stress. The differences in the degree of drought tolerance of the 2 tested genotypes makes them ideally fitting to comprehensive functional genomics analyses. The upregulation of some genes in a tolerant genotype and their downregulation in a sensitive genotype strongly suggests that under drought stress conditions, particular genes have been selected to enable the tolerant genotype to cope with the imposing drought conditions. That is why making a progress in developing the drought-tolerant transgenics in rice and other crops necessitates the identification of such genes. Based on the microarray analysis results, 527 probe sets had a symmetric fold change equal to or greater than 2 in a drought-tolerant rice genotype and symmetric fold change less than 1 in a drought-sensitive rice genotype under drought stress. Among 527 probe sets, a set of 308 probe sets had a symmetric fold change equal to or greater than 3 in a drought-tolerant rice genotype and a symmetric fold change less than 1 in a drought-sensi-

tive rice genotype under drought stress. In addition, there were 35 probe sets with a symmetric fold change equal to or greater than 30 in a drought-tolerant rice genotype and a symmetric fold change less than 1 in a drought-sensitive rice genotype under drought stress. The study was designed to include the probe sets with a symmetric fold change equal to or greater than 3 in a drought-tolerant rice genotype and a symmetric fold change less than 1 in a drought-sensitive rice genotype under drought stress.

Identified genes and a statistical discovery of significant biclusters

The identification of co-regulated genes is a significant step toward understanding the regulation of transcription (Qin et al., 2003). Based on the database incorporated into Genevestigator, in order to group the genes with similar expression levels under drought conditions irrespective of their expression profiles in other conditions, biclustering was performed in 308 probe sets that are uniquely upregulated in the tolerant rice variety. The biclustering analysis created 2 biclusters (bicluster I and bicluster II). Each bicluster consisted of 20 probe sets, which were co- and upregulated in different drought studies (Fig. 1A and Fig. 1B). It was also found that 18 out of 20 probe sets were common between bicluster I and bicluster II. In other word, two probe sets were specific for each bicluster. To be more specific, 2 probe sets (Os. 11808.2.S1_at and Os.51651.1.A1_at) belonged to bicluster I and 2 probe sets (Os.27482.1.A1 _at and Os.623.2.S1_x_at) were specifically traced in bicluster II (Table 1). Co-regulation of these probe sets could be a genetic factor for the drought tolerance in *Oryza sativa*. These genes seemed to constitute the regulatory backbone for the drought tolerance. Based on that new candidate genes in these biclusters might be considered to play important roles in the drought tolerance of *O. sativa* and to facilitate breeding programs that are targeted to improve the drought tolerance.

Moreover, drought stress affected the expression of drought-responsive transcription factors that control the expression of downstream genes involved in the drought stress tolerance (Kudo et al., 2016). Interestingly, in bicluster I and II, the probe sets 4 and 5, respectively, were annotated to Os01g0192300 (Myb transcription factor, putative, expressed), LOC_Os02g13800 (Heat stress transcription factor C-2a), and LOC_Os08g02070 (OsMADS26 – MADS-box family gene with MIKCc type-box, expressed) transcription factors. As it has been hypothesized that there might be some relationship between these transcription factors and other genes in biclusters, using computational biology techniques, the possible relationships between the up- and co-regulated genes and the transcription factors in the biclusters were analyzed. Strikingly, Os01g0192300 (Myb transcription factor, putative, expressed) was found to be bound to almost all genes (LOC_Os02g13800, LOC_Os01g03320, LOC_Os08g02070, LOC_Os01g03340, LOC_Os01g15340, LOC_Os02g37590, LOC_Os01g09640, LOC_Os06g11980, LOC_Os12g43450) in both biclusters, controlling their expression. In this respect, Os01g0192300 was the central transcription factor in biclusters (Table 1).

Among the up- and co-regulated genes in biclusters, a number of novel genes with unknown functions, such as Os01g0915000, Os05g0519300, Os06g0223700, and Os04g0586100, have been found. However, the unique upregulation of these genes in the tolerant rice genotype upon the imposition of drought stress insinuates that these functionally unknown genes may also play crucial roles in the drought stress tolerance. Additionally, the lack of sequence similarities to other well-characterized genes and proteins is suggestive of the feasibility of these genes possessing special roles and functions in crucial pathways (Singh et al., 2012). With the progress in genome sequencing and functional annotation, specific roles of those genes shall be unearthed, which is more likely to culminate in the discovery of novel candidates and new alternate pathways and it may further broaden our understanding on stress tolerance (Luhua et al., 2008; Pawlowski, 2008).

Gene mapping within the QTLs marked with the drought stress

Microarrays in combination with the QTL mapping and the expression analysis of genes, embedded in these QTLs, can potentially lead to the identification of candidate genes within the shortest possible time (Krzywinski et al., 2009; Deshmukh et al., 2010; Pandit et al., 2010; Cotsaftis et al., 2011). This survey unveiled that 29 out of 308 Affymetrix probe sets did not match the specific MSU gene loci. Among the remaining 279 MSU gene loci, the genes falling within the important drought-stress-related QTLs were identified, among them: LOC_Os05g41490 (circadian clock coupling factor ZGT,

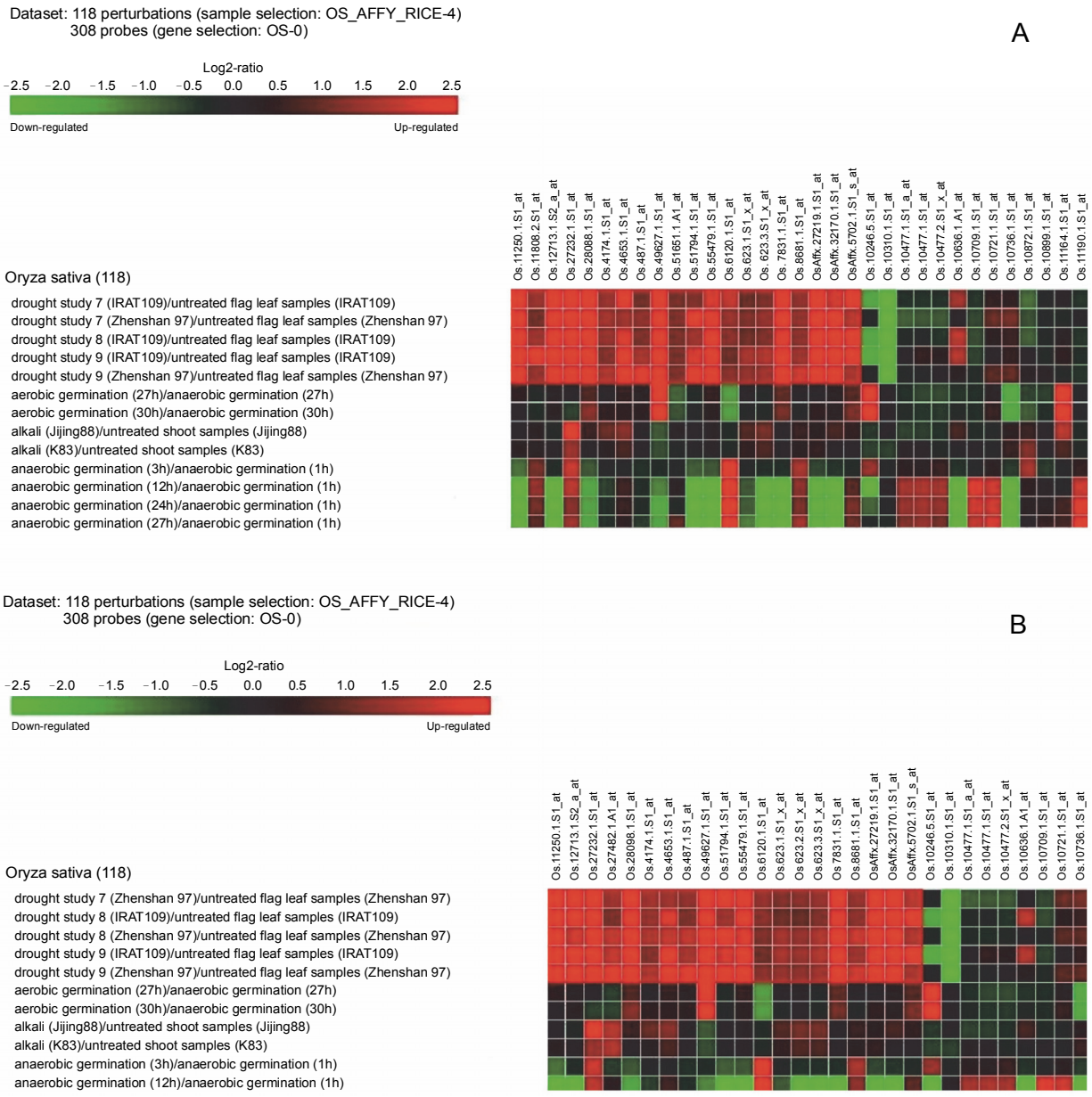

Fig. 1. A) The biclustering analysis of 308 probesets revealed two biclusters including bicluster I and B) bicluster II. Each biclusters consist 20 probesets co- and up-regulated under different drought studies

putative, expressed) located within the DQE50 (relative water content), AQDZ002 (lodging incidence), AQDZ013 (lodging incidence), CQAA19 (relative phosphorus distribution between shoot and root), and LOC_Os05g38290 (protein phosphatase 2C, putative, expressed) located within the DQE50 (relative water content), AQDZ002 (lodging incidence), AQDZ013 (lodging incidence), CQAA19 (relative phosphorus distribution between shoot and root) in chromosome 5, LOC_Os09g36680 (ribonuclease T2 family domain containing protein, expressed) located within the AQAA017 (relative root length) in chromosome 9, LOC_Os10g32810 (beta-amylase, putative, expressed) located within the AQAC027 (root dry weight), AQF103 (potassium chlorate resistance), CQG8 (leaf rolling time) in chromosome 10. An analytical overview showed that 3 out of 4 loci namely LOC_Os10g32810, LOC_Os05g41490, and LOC_ Os05g 38290 were among the common genes in 2 biclusters, which highlight the significance of these ge nes in conferring the drought tolerance. Tracing the locations of LOC_Os05g41490 (circadian clock coupling factor ZGT, putative, expressed), LOC_Os05g38290 (protein phos-

Table 1. Information about probesets present in bicluster I and II

Bicluster I	Bicluster II	MSU	TF family	Corresponding TF	Description	Sensitive genotype					Tolerant genotype				
						fold change	symmetrical raw fold change	T statistic	*P*-value	adjusted *P*-value	fold change	symmetrical raw fold change	T statistic	*P*-value	adjusted *P*-value
Os.11250.1.S1_at	Os.11250.1.S1_at	LOC_Os02g13800	HSF	Os01g0192300	Heat stress transcription factor C-2a	0.8258015	−1.210945	4.231827	0.001738757	0.007250845	4.245346	4.245346	−14.37934	5.24E-08	1.22E-06
Os.11808.2.S1_at		LOC_Os03g06580			MTN26L2 – MtN26 family protein precursor, expressed	0.2882052	−3.46975	9.010717	4.09E-06	3.74E-05	3.496182	3.496182	−5.385948	0.000307485	0.001734034
Os.12713.1.S2_a_at	Os.12713.1.S2_a_at	LOC_Os01g03320		Os01g0192300	BBTI2 – Bowman-Birk type bran trypsin inhibitor precursor, expressed	0.4723257	−2.117183	16.83516	1.15E-08	2.88E-07	4.48942	4.48942	−14.48279	4.90E-08	1.16E-06
Os.27232.1.S1_at	Os.27232.1.S1_at	LOC_Os01g68650			plant-specific domain TIGR01615 family protein, expressed	0.7869875	−1.270668	4.810758	0.000712033	0.003308273	4.313299	4.313299	−13.95551	6.98E-08	1.53E-06
	Os.27482.1.A1_at	LOC_Os05g44300			plant-specific domain TIGR01615 family protein, expressed	0.6676312	−1.497833	4.875757	0.000646037	0.003037051	3.353348	3.353348	−15.89637	2.00E-08	5.76E-07
Os.28098.1.S1_at	Os.28098.1.S1_at	LOC_Os10g32810			beta-amylase, putative, expressed	0.7213107	−1.386365	6.144898	0.000109051	0.000641986	8.652452	8.652452	−14.96014	3.59E-08	8.98E-07
Os.4174.1.S1_at	Os.4174.1.S1_at	LOC_Os08g02070	MADS	Os01g0192300	OsMADS26 – MADS-box family gene with MIKCc type-box, expressed	0.5838522	−1.712762	8.213053	9.35E-06	7.61E-05	6.534619	6.534619	−14.6532	4.38E-08	1.05E-06
Os.4653.1.S1_at	Os.4653.1.S1_at	LOC_Os01g03340		Os01g0192300	BBTI4 – Bowman-Birk type bran trypsin inhibitor precursor, expressed	0.7905561	−1.264932	4.820709	0.000701482	0.003265594	93.06326	93.06326	−58.78087	4.93E-14	9.78E-11
Os.487.1.S1_at	Os.487.1.S1_at	LOC_Os01g15340		Os01g0192300	flowering promoting factor-like 1, putative, expressed	0.6803307	−1.469873	3.693127	0.004155347	0.01544387	3.729641	3.729641	−16.16678	1.70E-08	5.12E-07
Os.49627.1.S1_at	Os.49627.1.S1_at	LOC_Os06g37150			L-ascorbate oxidase precursor, putative, expressed	0.6437845	−1.553315	13.38054	1.04E-07	1.73E-06	3.328604	3.328604	−8.155917	9.94E-06	9.06E-05

Os.51794.1.S1_at	Os.51794.1.S1_at	LOC_Os06g12370.1			OsFtsH6 FtsH protease, homologue of AtFtsH6, expressed	0.7416078	−1.348422	4.382306	0.001372449	0.005893766	13.06315	13.06315	−18.87482	3.78E-09	1.65E-07
Os.51651.1.A1_at		LOC_Os11g40160			expressed protein	0.1688724	−5.92163	16.5582	1.35E-08	3.28E-07	13.14387	13.14387	−24.71251	2.69E-10	2.49E-08
Os.55479.1.S1_at	Os.55479.1.S1_at	LOC_Os05g41490			circadian clock coupling factor ZGT, putative, expressed	0.5988968	−1.669737	3.566563	0.005124966	0.018493	21.72544	21.72544	−35.15437	8.23E-12	2.46E-09
Os.6120.1.S1_at	Os.6120.1.S1_at	LOC_Os02g37590		Os01g0192300	glycerophosphoryl diester phosphodiesterase family protein, putative, expressed	0.5523015	−1.810605	7.803636	1.46E-05	0.000112551	5.332777	5.332777	−14.50603	4.82E-08	1.14E-06
Os.623.1.S1_x_at	Os.623.1.S1_x_at	LOC_Os01g09640	MYB-related	Os01g0192300	Myb transcription factor, putative, expressed	0.5395522	−1.853389	8.228138	9.20E-06	7.51E-05	6.761892	6.761892	−15.05377	3.38E-08	8.60E-07
	Os.623.2.S1_x_at	LOC_Os01g09640	MYB-related	Os01g0192300	Myb transcription factor, putative, expressed	0.7027872	−1.422906	6.482703	7.05E-05	0.0004389	7.180647	7.180647	−16.97568	1.06E-08	3.55E-07
Os.623.3.S1_x_at	Os.623.3.S1_x_at	LOC_Os01g09640	MYB-related	Os01g0192300	Myb transcription factor, putative, expressed	0.5496835	−1.819229	13.13476	1.24E-07	2.00E-06	7.351117	7.351117	−17.34807	8.58E-09	3.02E-07
Os.7831.1.S1_at	Os.7831.1.S1_at	LOC_Os06g11980		Os01g0192300	DUF581 domain containing protein, expressed	0.8791218	−1.137499	3.05436	0.01216092	0.03895092	13.07566	13.07566	−25.85482	1.72E-10	1.89E-08
Os.8681.1.S1_at	Os.8681.1.S1_at	LOC_Os04g49670.1			DUF581 domain containing protein, expressed	0.8616227	−1.160601	4.477436	0.00118371	0.005176624	4.574592	4.574592	−14.1418	6.15E-08	1.38E-06
OsAffx.27219.1.S1_at	OsAffx.27219.1.S1_at	LOC_Os05g38290			protein phosphatase 2C, putative, expressed	0.821906	−1.216684	3.419453	0.006553445	0.02284866	14.51497	14.51497	−29.90708	4.09E-11	7.18E-09
OsAffx.32170.1.S1_at	OsAffx.32170.1.S1_at	LOC_Os12g43450		Os01g0192300	thaumatin family domain containing protein, expressed	0.6486837	−1.541583	9.066523	3.87E-06	3.56E-05	58.4978	58.4978	−35.30543	7.88E-12	2.41E-09
OsAffx.5702.1.S1_s_at	OsAffx.5702.1.S1_s_at	LOC_Os08g04560			decarboxylase, putative, expressed	0.7791576	−1.283437	6.979298	3.81E-05	0.000256453	6.545578	6.545578	−18.69646	4.14E-09	1.77E-07

phatase 2C, putative, expressed), LOC_ Os09g36680 (ribonuclease T2 family domain containing protein, expressed), and LOC_Os10g32810 (beta-amylase, putative, expressed) within the determined drought-stress-related QTLs, greatly suggested their potential roles in the drought tolerance. This, thereupon, may trigger interest in looking for their functional roles in the drought tolerance and for manipulating such a natural phenomenon in rice.

Identification of genes encoding the transcription factor

Advances in genomic studies have indicated that regulatory elements like transcription factors also regulate many other downstream stress-responsive genes associated with stress tolerance (Kudo et al., 2016). It is remarkable that during the analytical process, 15 probe sets for transcription factors were found to be significantly upregulated in a drought-tolerant rice genotype, while relatively downregulated in a drought-sensitive genotype. Fifteen probe sets showed similarity to the transcription factors belonging to various families including NAM, ATAF, and CUC transcription factors (NAC), myeloblastosis (MYB), myeloblastosis-related (MYB-related), WRKY (The name derived from amino acid residues W, R, K, and Y in the conserved WRKY domain), minichromosome maintenance1 agamous deficiens serum response factor (MADS), basic leucine zipper family (bZIP), heat shock factor (HSF), FAR-RED IMPAIRED RESPONSE1 (FAR1 I), cysteine-rich polycomb-like protein (CPP), and Cys3His (C3H), the details of which are presented in Table 2. In this study transcription factors were considered notable and will be discussed further.

As shown in Table 2, LOC_OS02G36880.3 (putative OSNAC1 protein) is a member of the NAC family. There are 140 members of the NAC transcription factor family in *O. sativa* (OsNAC) or OsNAC-like genes (Fang et al., 2008). Previously, a comprehensive overview has been presented on the role and spatial distribution of the rice NAC family members under environmental (biotic and abiotic) stresses (Nuruzzaman et al., 2010). Along with such analyses, several surveys have been conducted to functionally characterize the *OsNAC* genes such as *OsNAC5* (Takasaki et al., 2010), *OsNAC6* (Nakashima et al., 2007; Ohnishi et al., 2005), *OsNAC52* (Gao et al., 2010), *OsNAC9*, and *OsNAC10* (Redillas et al., 2012; Jeong et al., 2010) associated with drought response and about their potential use in developing drought-tolerant rice cultivars. Drought tolerance in rice, owing to increased stomatal closure and sensitivity to abscisic acid (ABA) in the guard cells, is caused by a strong expression of a stress-induced transcription factor NAC1 (*OsNAC1*). In addition, more than 80 genes were upregulated in the *OsNAC1*-overexpressing transgenic rice plants (Hu et al., 2006). These upregulated genes are involved in the detoxification and redox homeostasis, the protection of osmolytes, macromolecules, as well as in increasing the stomatal closure in transgenic leaves (Hu et al., 2006).

It is noteworthy that LOC_Os05g41540.1 encoding the bZIP transcription factor domain containing protein, which was found to be a drought-responsive genotype from this study, has not been reported before. As far as rice is concerned, there are approximately 100 members of bZIP TF family (Guo et al., 2005; Nijhawan et al., 2008). Genome-wide expression analyses of rice bZIP family revealed that 33 genes were engaged in drought responses, among which 9 genes were downregulated while 24 were detected as upregulated (Hadiarto and Tran, 2011). OsbZIP23 gene expression level was substantially and quickly increased by salt, drought, polyethylene glycol (PEG), and ABA treatments. However, it remained unaffected by cold. OsbZIP23-overexpressing transgenic rice plants, on the other hand, displayed a significantly enhanced tolerance to drought and salinity stresses, as well as sensitivity to ABA (Xiang et al., 2008).

LOC_Os05g46020.1 encoding WRKY transcription factor 7 was also found uniquely upregulated in a tolerant rice genotype in this study. Furthermore, a comprehensive transcriptional profiling of the WRKY family in rice has identified 103 WRKY TF encoding genes, among which 4 were upregulated under drought stress (Ramamoorthy et al., 2008). According to the report published by Yu and coworkers (2010), the OsWRKY72 was upregulated by NaCl, ABA, PEG, and high temperature.

Despite the fact that the MYB TF family is large, with 183 members in rice, its role in the drought tolerance was not well documented (Xiong et al., 2014). At the same time, overexpression of an R1R2R3 MYB gene (*OsMYB3R-2*) was reported to enhance the tolerance to abiotic environmental stresses such as salt, drought, and freezing in transgenic Arabidopsis (Dai et al., 2007). Moreover, *OsMYB48-1*, a novel MYB-related TF, functions in drought and salinity tolerance by regulating

Table 2. Lists of genes encoding transcription factors that uniquely significantly up-regulated in tolerant genotype

Affymerix probesets	MSU	Gene annotation	TF family	Sensitive genotype			Tolerant genotype		
				fold change	*P*-value	adjusted *P*-value	fold change	*P*-value	adjusted *P*-value
Os.9492.1.S1_a_at	LOC_Os02g36880.3	Putative OsNAC1 protein	NAC	0.6559201	0.0014369	0.006131459	3.480441	6.92E-05	0.00047772
Os.52643.1.S1_at	LOC_Os12g38400.2	MYB family transcription factor, putative, expressed	MYB	0.2563135	4.41E-10	2.39E-08	6.,338256	5.54E-06	5.55E-05
Os.623.1.S1_x_at	LOC_Os01g09640.1	Myb transcription factor, putative, expressed	MYB-related	0.5395522	9.20E-06	7.51E-05	6.761892	3.38E-08	8.60E-07
Os.623.2.S1_x_at	LOC_Os01g09640.1	Myb transcription factor, putative, expressed	MYB-related	0.7027872	7.05E-05	0.0004389	7.180647	1.06E-08	3.55E-07
Os.623.3.S1_x_at	LOC_Os01g09640.1	Myb transcription factor, putative, expressed	MYB-related	0.5496835	1.24E-07	2.00E-06	7.351117	8.58E-09	3.02E-07
Os.8961.1.S1_s_at	LOC_Os05g46020.1	OsWRKY7 – superfamily of TFs having WRKY and zinc finger domains, expressed	WRKY	0.6691555	0.0018955	0.007818159	3.085346	7.93E-06	7.50E-05
Os.4174.1.S1_at	LOC_Os08g02070.1	OsMADS26 – MADS-box family gene with MIKCc type-box, expressed	MADS	0.5838522	9.35E-06	7.61E-05	6.534619	4.38E-08	1.05E-06
Os.26821.1.S1_at	LOC_Os05g41540.1	bZIP transcription factor domain containing protein, expressed	bZIP	0.8394835	0.015175	0.04704493	3.834626	1.18E-07	2.36E-06
Os.11250.1.S1_at	LOC_Os02g13800.1	HSF-type DNA-binding domain containing protein, expressed	HSF	0.8258015	0.0017388	0.007250845	4.245346	5.24E-08	1.22E-06
Os.10899.1.S1_at	LOC_Os03g62660.2	transposon protein, putative, unclassified, expressed	FAR1	0.6724967	0.002143	0.008697159	3.176152	9.87E-06	9.01E-05
Os.24970.1.A1_at	LOC_Os10g34884.1	RIPER7 – ripening-related family protein precursor, expressed	FAR1	0.3639177	3.14E-05	0.000216875	3.354372	5.89E-08	1.34E-06
Os.7693.1.S1_s_at	LOC_Os08g28214.6	tesmin/TSO1-like CXC domain containing protein, expressed	CPP	0.7462462	0.0029773	0.01157467	3.225367	2.67E-05	0.000210518
OsAffx.29395.1.S1_at	LOC_Os08g28214. 2	tesmin/TSO1-like CXC domain containing protein, expressed	CPP	0.5124125	1.34E-05	0.000104101	8.532405	2.83E-10	2.56E-08
Os.49042.1.A1_s_at	LOC_Os01g09620	zinc finger/CCCH transcription factor, putative, expressed	C3H	0.6493396	1.21E-05	9.55E-05	4.884521	3.32E-08	8.47E-07
Os.14762.3.S1_at	LOC_Os11g47920.1	GRAS family transcription factor containing protein	GRAS						

the expression of ABA synthesis genes (Xiong et al., 2014). The present study showed that 2 loci (LOC_Os12g38400.2 and LOC_Os01g09640.1) belonging to MYB and MYB-related family were also uniquely upregulated in a tolerant rice genotype.

The expression of another gene, LOC_Os08g02070.1, encoding OsMADS26 transcription factor, was repressed in the sensitive rice genotype and induced in the tolerant one. Interestingly, there have been reports that a few MADS-box genes in plants were associated with the environmental response regulation. A study conducted by Lee and coworkers (2008) resulted in a finding that the genes inducible by ethylene, jasmonate, and reactive oxygen species were upregulated in *OsMADS26*-overexpressing plants of both rice and Arabidopsis, suggesting that *OsMADS26* has induced numerous responses to different types of stress.

LOC_Os02g13800.1, encoding the heat stress transcription factor C-2a (*HsfC2a*), was another gene that has been found to be uniquely upregulated in the tolerant rice genotype from this analytical survey. Heat shock factors (Hsfs) are the transcriptional activators of heat shock proteins (Hsps) (Hu et al., 2009) in plants and they act by specific recognition and binding to the highly conserved heat shock element (HSE, a *palindromic motif of nGAAn*) (Miller and Mittler, 2006). HSFs play a pivotal role in provoking responses to various abiotic stresses via regulating the expression of stress-responsive genes, such as Hsps (Guo et al., 2016). *HsfC2a,* for instance, was reported to be induced by heat, drought, and salt (Hu et al., 2009). It has been reported previously that *HsfC2a* was upregulated under various abiotic stresses (dehydration, salt, cold, and heat) both in root and shoot tissues in rice (Chauhan et al., 2011). Consistent with these concepts, according to the microarray analysis, *HsfC2a* was upregulated in the tolerant rice genotype while downregulated in the sensitive one.

The role of several members of FAR-RED impaired response 1 family (FAR1, downregulated after exposure to far-red light) under environmental stresses has also been reported. It is known that a FAR1 domain containing protein (NM_001057341) is one of 84 drought-responsive genes detected exclusively in roots of the tolerant rice genotype (Rabello et al., 2008). According to Tang and coworkers (2013), FAR1 is a positive regulator of ABA signaling whose expression is induced by various abiotic stresses such as drought, osmosis, and salt. Upregulation of ABA signaling network enables plants to better adapt to environmental stresses (Tang et al., 2013). These examples support the notion that 2 identified transcription factors belonging to this family, namely LOC_Os03g62660.2 and LOC_Os10g34884.1, might play roles in rice drought tolerance.

LOC_Os08g28214, encoding tesmin/TSO1-like CXC domain containing protein, is another transcription factor that was found to be uniquely upregulated in the tolerant rice genotype in this study. As shown in Table 2, it is a member of the CPP family. It has been observed that *TSO1*, belonging to CPP family adjusts cell expansion and cytokinesis in *Arabidopsis thaliana* (Hauser et al., 2000). Andersen and coworkers reported that *TSO1* is required for the fertility in both male and female reproductive organs in *A. thaliana* (Andersen et al., 2007). Genome-wide analyses of the maize CPP-like gene family and expression profiling data has revealed that these genes were expressed differentially in leaves under abiotic stress, compared to the control conditions (Song et al., 2016).

Zinc finger/CCCH transcription factor encoded by LOC_Os01g09620 is another transcription factor belonging to C3H family that was found to be uniquely upregulated in the tolerant rice genotype. This further supports the role of C3H proteins, a large family containing zinc finger C3H-type motifs, which are most probably RNA-binding proteins functioning in the RNA processing (Thompson et al., 1996; Li et al., 2001; Delaney et al., 2006). Given the gene expression profiling of *OsC3H33*, *OsC3H50*, and *OsC3H37,* it was found that the genes were differentially expressed under various salt concentrations (Muhamman et al., 2010). *OsC3H12* confers the resistance against rice bacterial blight disease and its function is probably related to the JA-dependent pathway (Deng et al., 2012).

The GRAS proteins belong to an important plant-specific gene family of putative transcription factors whose name is derived from the first 3 members isolated, namely, gibberellic acid insensitive (GAI), repressor of GA1 (RGA), and scarecrow (SCR) (Bolle, 2004). The present study has revealed that LOC_Os11g47920.1, encoding the GRAS family transcription factor containing protein, was found to be uniquely upregulated in the tolerant rice genotype. GRAS proteins were suggested to function in a meristem and root development, gibberellin signal transduction, light signaling, as well as biotic and abiotic stress responses. At least 57 GRAS

encoding genes in rice were identified via a genome-wide analysis of the GRAS gene family in rice (Tian et al., 2004). Some of the rice GRAS genes such as *MOC1* (MONOCULM 1, a gene that is important in the control of rice tillering) (Li et al., 2003), *SLR1* (the gene associated with the slender phenotype and its product is an intermediate of the GA signaling pathway) (Ikeda et al., 2001), *SCR* (SCARECROW, an essential gene for the asymmetric division of the cortex/endodermis progenitor cell in the root) (Kamiya et al., 2003), *DLT* (dwarf and low-tillering gene) (Tong et al., 2009), and *OsGRAS19* (Chen et al., 2013), have been well characterized, while the functions of other rice GRAS genes are largely unknown (Xu et al., 2015). According to the report provided by Xu and coworkers (2015), *OsGRAS23*, a stress-responsive GRAS transcription factor, positively regulates the drought tolerance in rice through the induction of several stress-responsive genes.

When a probe set is precisely linked to a gene, its intensity is interpreted as a gene expression (Yu et al., 2007). The probe set annotation at a transcript level presented in Table 2 yielded 3 probe sets for LOC_ Os01g09640 and 2 probe sets for LOC_Os08g28214.

There is no report on alternative splicing events in LOC_Os01g09640 (MYB transcription factor, putative, expressed), yet 6 alternatively spliced forms of LOC_ Os08g28214 (tesmin/TSO1-like CXC domain containing protein, expressed) have been reported. As presented in Table 2, OsAffx.29395.1.S1_at and Os.7693.1.S1_s_at were upregulated with fold changes of 8.53- and 3.22-fold, respectively. These probe sets were annotated to 2 alternative transcripts, namely, LOC_Os08g28214.2 and LOC_Os08g28214.6. The overexpression, when put in a comparative paradigm, of 2 out of 6 alternative transcripts of this transcription factor, substantiated the biological roles of alternative splicing in rice gene expression reacting to the drought stress. This finding supports, more reliably, the idea of the presence of mutual relations between the splicing process and various molecular machineries participating in the gene expression regulation (Gracz, 2016).

Given that these transcription factors were uniquely upregulated in the tolerant rice genotype, and based on previous knowledge about the roles of several identified transcription factors, one might hypothesize that they may be involved in the drought tolerance. Some of the identified transcription factors in this study have not been reported before and may thus be used as a source of novel genes for improving the drought tolerance. Further experimental investigations on gene function will be needed to investigate the role of these transcription factors in rice.

Conclusions

The transcriptome analysis of 2 contrasting genotypes pinpointed several candidate genes, which are valuable in contributing to a better understanding of the mechanisms underlying the drought tolerance in rice. Additionally, drought-responsive transcription factors have been identified. The biclustering analysis has revealed 2 subsets of co- and upregulated genes harboring 3 transcription factors, namely Os01g0192300 (Myb transcription factor, putative, expressed), LOC_ Os02g 13800 (heat stress transcription factor C-2a), and LOC_ Os08g02070 (OsMADS26 – MADS-box family gene with MIKCc type-box, expressed), which were likely to play a significant role in the drought tolerance of *O. sativa*. The analysis also included the identification of genes in subsets that contained the binding site motif for Os01g 0192300 (Myb transcription factor, putative, expressed) in their promoter regions. These genes that were uniquely upregulated in a tolerant genotype with fold change value equal to or greater than 3 could be considered as the significant candidates for contributing to the drought tolerance. Likewise, several reverse genetics approaches such as insertional mutagenesis, homologous recombination, and RNAi suppression of the gene of interest (An et al., 2005) could be exploited to validate the roles of unknown genes in the rice drought tolerance responses. The cloning and functional validation of stress tolerance genes deepened our understanding of signaling networks in a response to stresses in rice varieties, and it may eventually lead to the development of more stress-tolerant crops (Liang et al., 2014). All in all, these results suggest some novel candidate genes and about their potential for engineering drought-tolerant rice cultivars; those genes may serve as potential targets for engineering the stress-tolerant rice.

This investigation was aimed at providing a framework toward a more focused understanding of the genome-wide transcriptional changes in contrasting genotypes under drought stress conditions. It also raises expectations that new avenues of similar studies in other crops will open up.

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