

Global genome expression analysis of rice in response to drought and high-salinity stresses in shoot, flag leaf, and panicle

Junli Zhou · Xiangfeng Wang · Yuling Jiao · Yonghua Qin · Xigang Liu ·
Kun He · Chen Chen · Ligeng Ma · Jian Wang · Lizhong Xiong ·
Qifa Zhang · Liumin Fan · Xing Wang Deng

Received: 16 September 2006 / Accepted: 3 November 2006 / Published online: 16 January 2007
© Retained by Author 2007

Abstract To elucidate genome-level responses to drought and high-salinity stress in rice, a 70mer oligo-mer microarray covering 36,926 unique genes or gene

Electronic supplementary material The online version of this article (doi:10.1007/s11103-006-9111-1) contains supplementary material, which is available to authorized users.

Junli Zhou, Xiangfeng Wang contributed equally to this work.

J. Zhou · X. Wang · X. Liu · K. He · L. Ma ·
L. Fan · X. W. Deng
Peking-Yale Joint Center of Plant Molecular Genetics and
Agrobiotechnology, College of Life Sciences, Peking
University, Beijing 100871, People's Republic of China

J. Zhou · X. Wang · X. Liu · K. He · L. Ma ·
L. Fan · X. W. Deng
National Institute of Biological Sciences, 7 Science Park
Road, Zhongguancun Life Science Park, Beijing 102206,
People's Republic of China

J. Zhou · Y. Jiao · K. He · X. W. Deng (✉)
Department of Molecular, Cellular, and Developmental
Biology, Yale University, New Haven, CT 06520-8104, USA
e-mail: xingwang.deng@yale.edu

Y. Qin · L. Xiong · Q. Zhang
National Key Laboratory of Crop Genetic Improvement,
Huazhong Agricultural University, Wuhan, Hubei Province,
People's Republic of China

X. Wang · C. Chen · J. Wang
Beijing Institute of Genomics, Chinese Academy of
Sciences, Beijing Genomics Institute, Beijing 101300,
People's Republic of China

L. Fan
The National Laboratory of Protein Engineering and Plant
Genetic Engineering, College of Life Sciences, Peking
University, Beijing 100871, People's Republic of China

models was used to profile genome expression changes in rice shoot, flag leaf and panicle under drought or high-salinity conditions. While patterns of gene expression in response to drought or high-salinity stress within a particular organ type showed significant overlap, comparison of expression profiles among different organs showed largely organ-specific patterns of regulation. Moreover, both stresses appear to alter the expression patterns of a significant number of genes involved in transcription and cell signaling in a largely organ-specific manner. The promoter regions of genes induced by both stresses or induced by one stress in more than one organ types possess relative enrichment of two *cis*-elements (ABRE core and DRE core) known to be associated with water stress. An initial computational analysis indicated that novel promoter motifs are present in the promoters of genes involved in rehydration after drought. This analysis suggested that rice might possess a mechanism that actively detects rehydration and facilitates rapid recovery. Overall, our data supports a notion that organ-specific gene regulation in response to the two abiotic stresses may primarily be mediated by organ-specific transcription responses.

Keywords Rice · Drought · Salinity · Rehydration · Promoter · *cis*-element

Introduction

Drought and highly saline soils are among the most serious challenges to crop production in the world today. This is particularly the case in developing countries, where these abiotic stresses severely limit crop

growth and productivity. Both traditional breeding and genetic engineering of crop plants have been utilized to improve drought and high-salinity tolerance or resistance with the goal of increasing agricultural productivity in affected regions. Understanding plant responses to abiotic stresses at the genomic level provides an essential foundation for future breeding and genetic engineering efforts.

Recent research on drought and high-salinity responses in *Arabidopsis* implied that a large proportion of the genome is involved in drought (Shinozaki et al., 2000, 2003) or high-salinity stress responses (Xiong et al., 2002; Zhu, 2001, 2002). In several cases, it has been shown that alteration of individual gene expression level can significantly impact responses to drought (Garg et al., 2002; Haake et al., 2002) or high-salinity stresses in plants (Kasuga et al., 1999; Shi et al., 2003; Xu et al., 1996; Zhang et al., 2004). Genome-wide identification of genes regulated by drought or high-salinity conditions has manifold significance. First, it provides a more comprehensive understanding of the transcriptional responses to those stresses. Second, it provides a starting point for further elucidating the role of individual genes in stress responses, which will be of great value in crop engineering. Third, it aids in the identification of stress responsive promoters and responsible *cis*-elements within them that are important both for basic study and crop engineering applications.

DNA microarrays provide a high throughput means of analyzing genome expression, which has been used to study patterns of gene expression in response to drought or high-salinity stresses in several plant species (Seki et al., 2003, 2004). Initially, a microarray containing ~1,300 full-length cDNA clones from *Arabidopsis* was used to study gene expression under drought and cold stresses. This study resulted in the identification of 44 and 19 cDNA clones as drought and cold-inducible genes, respectively (Seki et al., 2001). Other studies employed an improved microarray containing around 7,000 *Arabidopsis* full-length cDNA clones to profile gene expression in response to abscisic acid (ABA) treatment (Seki et al., 2002a) as well as cold, drought, and high-salinity stresses (Seki et al., 2002b). Another study employed an Affymetrix GeneChip covering approximately 8,100 genes from *Arabidopsis* to monitor changes in gene expression under salt, osmotic, and cold stresses. This study revealed that resulting expression changes varied significantly between root and leaf, with only minor overlap (Kreps et al., 2002). Similar studies have also been performed

in barley to assess the drought and high-salinity gene expression responses using a microarray containing 1,463 DNA elements (Ozturk et al., 2002).

Rice (*Oryza sativa*) is a model plant for cereal crops and has perhaps the richest set of resources available for plant genomic studies (Feng et al., 2002; Goff et al., 2002; RCSC, 2003; Sasaki et al., 2002; Yu et al., 2002). In rice, the high-salinity stress response has been analyzed with a microarray containing 1,728 cDNA clones from a root cDNA library of salt-tolerant rice (var Pokkali) (Kawasaki et al., 2001). Another study with a microarray containing 8,987 DNA elements detected 509 possible abscisic acid (ABA) or gibberellin-responsive genes (Yazaki et al., 2003). Later, the same group used a 22,000 rice cDNA based oligonucleotide array to identify ABA and GA responsive genes, made comparison of ABA-responsive genes and their putative responsible promoter elements between rice and *Arabidopsis* (Yazaki et al., 2004). This analysis gave an initial global view of the ABA- and GA-responsive genes in rice. A total of 73 genes induced by cold, drought, high-salinity and ABA treatment were further identified using a microarray containing 1,700 full-length cDNA clones (Rabbani et al., 2003). Recently, an Affymetrix rice genome array containing 55,515 probe sets was used to profile the transcriptomes of rice strains with salt-tolerant and salt-sensitive genotypes, revealing genome-wide differential transcription under salinity treatments during vegetative growth (Walia et al., 2005). Taken together, previous studies in rice have identified various genes regulated by different stress conditions. However, a systematic comparison of whole-genome expression responses to drought and high-salinity stresses in various organs has not yet been performed.

The availability of the complete genome sequences of two rice sub-species (Yu et al., 2005) makes the construction of a whole-genome microarray possible. A whole-genome 70mer oligomer microarray for rice was developed and successfully employed to obtain genome expression profiles (Ma et al., 2005b; Jiao et al., 2005). Here, we use this whole-genome microarray to monitor expression changes for a total of 36,926 genes in response to drought and high-salinity stresses in shoots at the four-tiller stage and in flag leaves and panicles at 1 week prior to heading. This analysis revealed the extent of reprogramming and alteration of cellular pathways in response to drought and high salinity stresses. The possible underlying mechanism for the observed reprogramming of the genome expression was examined.

Materials and methods

Plant material and stress treatments

Plant material used in this study was *O. Sativa* L. ssp. *indica* (cv. Minghui 63). Shoot samples were selected at the four-tiller stage (vegetative stage) and flag leaf and panicle samples were selected at one-week-before-heading (reproductive stage). The plants used for shoot samples were grown in hydroponic half-strength Hoagland solution up to the four-tiller stage, and then plants allowed to reach the reproductive stages were grown in soil.

For drought treatment samples, plants were taken out from the Hoagland solution directly and their roots were put on filter paper for drought treatment. Under this condition, the phenotype of shoot was observed. Materials at the following stages were collected: Stage 1 (D1), when leaves were slightly rolled and leaf relative water content (RWC) was around 90–95%; Stage 2 (D2), when leaves were half-rolled and leaf RWC was around 80–85%; Stage 3 (D3), when leaves were completely rolled and leaf RWC was around 70–75%. For most of the plants, usually it took half to 1 h to reach stage 1, and then after another 2 h or 4 h, they could reach stage 2 or stage 3 respectively. Rehydration samples were prepared by first subjecting the rice plants to drought treatment until leaves were completely rolled (D3), then supplying enough water and samples were collected after 48 h. For each treatment stage, three independent replicates were collected. For sample collection under high-salinity treatment (200 mM NaCl), rice plants were collected when plants reached stages 1, 2, or 3 as outlined above for drought treatment. Rice plants were taken out from Hoagland solution and were directly put into Hoagland solution with 200 mM NaCl. For most of plants, usually it took 5 h to reach stage 1, and then after another 12 and 24 h, they could reach stage 2 and stage 3 respectively. For each stage, three independent replicates were collected. Samples from normal grown plants corresponding to each drought and high-salinity stages and rehydration stage were also collected at the same time as respective controls. Upon collection, samples were frozen immediately in liquid nitrogen and stored in the -80°C freezer. The estimation of RWC in rice plants was prior reported method (Bare and Weatherley, 1962).

RNA isolation, probe labeling, and hybridization

RNA preparation, fluorescent labeling of probes, slide hybridization, washing and scanning were performed as described previously (Ma et al., 2005b). Total RNA

was prepared from frozen samples using the RNAswiz reagent (Ambion). Each RNA preparation was used to generate a labeled cDNA probe for hybridization. There were at least three high-quality replicate data sets for each experiment, with each data set obtained from an independent biological sample.

The cDNA synthesis, probe labeling, and microarray hybridization, microarray slide washing, and array scanning were performed following previously described protocols (Jiao et al., 2005). Hybridized microarray slides were scanned with an Axon GenePix 4000B scanner, and independent TIFF images for both Cy3 and Cy5 channels were used for subsequent analysis.

Microarray and initial data analysis

The microarray construction was described previously (Ma et al., 2005b, Jiao et al., 2005). Microarray data were preprocessed with Limma R Package (Smyth, 2005). We first performed Loess normalization to balance the dye-bias, and to remove the print-tip effect within each array. Quantile normalization was performed to remove the experimental variances across replicate arrays. Based on the distribution of negative control oligos within each slide, we determined an experimental threshold for minimum non-specific hybridization intensity, and the gene oligos above 90-percentile of negative oligos were set as to be detected in each experiment.

To identify the genes that respond to drought and salinity stress, we used the Limma R implementation to perform hypothesis tests by fitting a linear model to the expression data. During pair-wise comparison between each treated sample versus control, a moderated *t*-statistics were computed, by using an empirical Bayes method to shrink the gene-wise sample variances towards a common value (Smyth, 2004), and the differential level was represented by a log (2) of intensity ratio. The *P* value adjustment used in false discovery rate control for multiple testing is Benjamini and Hochberg method (Benjamini and Hochberg, 2000; Reiner et al., 2003). The genes with significantly differential expression were selected by the adjusted *P* values less than 0.05.

RT-PCR analysis of genes

The expression profiles were further quantified by RT-PCR and compared to results obtained by chip hybridization. The first strand of cDNA was generated from 1 μg of total RNA isolated independently from each sample in a 100 μl volume and 1 μl was used as template in each PCR reaction (25 cycles of 1 min at 94°C , 1 min

at 58°C, 1 min at 72°C). A total of eight drought-induced genes were selected for RT-PCR analysis (the primers of these genes are listed in Table S18). The *Actin1* gene of rice was used as a control for RT-PCR experiments (forward primer, 5'-cgcagtccaagagggtatc-3'; reverse primer, 5'-tcttggtcatagtcagggc-3').

Functional classification

GO terms used in rice gene functional annotations were downloaded from the BGI-RIS database at <http://rise.genomics.org.cn> (Zhao et al., 2004). For biochemical pathway analysis, we classified genes by associated biochemical pathway(s) using the AraCyc database (<http://www.arabidopsis.org/tools/aracyc>) for *Arabidopsis*, which is based on MetaCyc pathway collections (Mueller et al., 2003). A rice gene was considered to be associated with a biochemical pathway if it had an *Arabidopsis* homolog in that pathway. To compare the expression profiles of conserved genes between rice and *Arabidopsis*, we selected reciprocal best-matched gene pairs between rice and *Arabidopsis*, which are based on a TBLASTN search of both rice and *Arabidopsis* gene sequences. This method used to identify highly homologous genes between rice and *Arabidopsis* has been described in previous reports (Jiao et al., 2005; Ma et al., 2005b). For the diagrams in Fig. S3, the expression ratios of the selected rice genes at D3 and S3 stage only were used.

Analysis of *cis*-acting regulatory elements

Differentially expressed genes supported by available full-length cDNA sequences were used to further elucidate regulatory *cis*-elements in the putative promoter regions. After mapping the FL-cDNAs on *indica* rice 12 pseudo-molecules, 2 kb DNA sequences upstream from the 5' end of the available cDNA sequences were extracted and searched against a plant *cis*-regulatory database (Higo et al., 1999) (<http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html>). The abundance of known ABRE core and DRE core elements in each query set were counted.

Identification of novel *cis*-regulatory elements was performed using the Improbizer tool (*cis*-Site Seeker) by Jim Kent (<http://www.cse.ucsc.edu/~kent/improbizer/>), which is based on ab-initio prediction of consensus binding sites among each co-regulated gene set. The upstream 2 kb regions of co-regulated FL-cDNA genes were used for common motif searching. To evaluate the significance for each putative novel motif, we generated an equal number of 2 kb contrast sequences in each query set by randomly arranging the four

nucleotides. We performed the standard Student's *t*-test on motif score tables, comparing query set versus contrast set. The known ABRE motif predicted by Improbizer has a pretty high confidence level (above 0.999), and the other two novel motifs were calculated to above a 0.95 confidence level. Motif consensus sequences were displayed by WEBLOGO program at <http://weblogo.berkeley.edu/> by Crooks et al. (2004).

Gel shift assays

Nuclear protein extracts and gel mobility shifts were performed as described previously (Gupta et al., 1998). Oligomers were synthesized using the following sequences: OsJRFA070715: GCAGATACTGCAGCCA ACCTCTCT, OsJRFA070715M: GCAGATACTGA AGCCAACCTCTCT. The complement sequence for each oligo was also synthesized. After denaturing and annealing, double-stranded probes were used for labeling.

Clustering and chromosomal location analysis of genes involved in stress responses

Unsupervised classification of stress-response genes was based on log-transformed ratios of all up- or down-regulated genes detected in at least one stage of drought treatment. We performed hierarchical clustering analysis to display the expression pattern and tree diagram at different stress stages by employing Cluster and TreeView (Eisen et al., 1998). Classification of rehydration-induced genes was based on the log-transformed ratios of 48-h rehydration and three stages of drought treatment in the three organs, and 807 genes in flag leaf, 281 genes in shoot, and 224 genes in panicle were grouped with the above method.

To analyze the genome-wide distribution of rehydration-induced genes in the three organs and drought and high-salinity induced genes in shoot, as well as identify potential clusters of co-regulated genes, we mapped these genes to the *indica* 12 chromosomes using the BLAT program. The expression profiles in Fig. 9 were drawn using a customized Perl script plus a scalable vector graphics (SVG) bundle.

Results

Identification of drought and high-salinity stress responsive genes using a rice whole genome oligomer microarray

A previously reported rice genome microarray containing 70mer oligomer probes for 36,926 unique genes

or gene models (Ma et al., 2005b; Jiao et al., 2005) was used for genome-wide profiling of gene expression responses to drought and high-salinity stresses. Total RNAs were isolated from four-tiller stage shoots and from flag leaves and panicles (as described in Experimental procedures) at three different time points (or sampling stages) after initiation of drought or high-salinity stress treatments (Fig. S1). The sampling stages for both drought and high-salinity treatments were based on morphological phenotypes (leaf rolling state) and physiological states (relative water content, RWC, see Bare and Weatherley, 1962). Stage one is defined as exhibiting slightly rolled leaves with 90–95% RWC. Stage two is defined as exhibiting halfway rolled leaves with 80–85% RWC. Stage three is defined as exhibiting completely rolled leaves and with 70–75% RWC. For drought treatment, the three stages were designated as D1, D2, and D3, while the three stages for the high-salinity treatment were designated as S1, S2, and S3 (see Fig. S1). The cDNA probes produced from the stress-treated and corresponding untreated control sample pairs were hybridized to microarray slides. For each sample point, three replicates were performed with dye-swap to correct for uneven dye effects.

The microarray data processing includes LOESS normalization within slide and quantile normalization between slides to remove the systematic errors (see Experimental procedures for details). To identify the differentially expressed genes, Limma R implementation was used to perform pair-wise comparison, and a moderated *t*-statistics was computed for each gene of treated sample versus control sample. Genes with FDR-adjusted *P* value less than 0.05 were considered to indicate significant differences (see Experimental procedures for details). Numbers of genes up- or down-regulated by drought or by high-salinity stress at each stage in each organ were shown in Fig. 1. In total, 582, 1,257 and 614 drought up-regulated genes and 795, 646 and 1,305 drought down-regulated genes were identified in flag leaf, shoot and panicle, respectively; and 1,676, 817 and 1,310 high-salinity up-regulated genes and 1,270, 1,323 and 2,284 high-salinity down-regulated genes were identified in flag leaf, shoot and panicle, respectively (Fig. 2A, B). The complete lists of these genes are available in the supplemental data (Tables S2–S13).

Most of the previously known genes responsive to both drought and high-salinity stress have been recovered from our microarray analysis. Those include the LEA protein (OsJRFA063984), aquaporin (OsIRUA001311), OsNAC1 (OsJRFA108080), dehydrin rab 16b (OsIFCC035025) and DREB1 (OsJRFA067313). Many other responsive genes have been identified for the first time in this study (Table S1C).

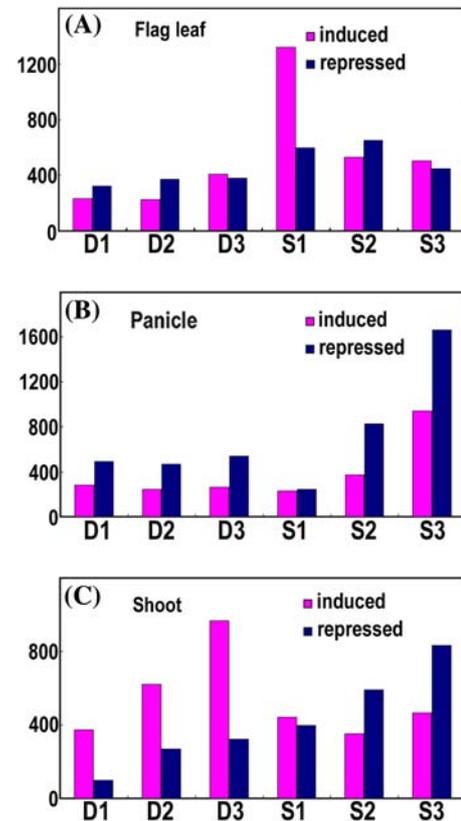


Fig. 1 The number of differentially expressed genes at each stage of drought and high-salinity treatments in three organs. Differentially expressed genes (both induced or repressed) are defined as those with $P < 0.05$. D1, D2 and D3: three stages of drought stress, S1, S2, and S3: three stages of high-salinity stress. y-axis shows the gene number. (A) Number of genes induced or repressed in flag leaf. (B) Number of genes induced or repressed in panicle. (C) Number of genes induced or repressed in shoot.

Among those newly revealed genes, some are expected to be involved in general cell function or known to be involved in other stress responses, while others have putative or unknown function. The annotation for some of those genes suggested that they include transcription factors from multiple families, heat shock proteins, various stress (drought, high-salinity, disease, cold, and ABA) responsive genes, protein kinases, transporters, photosynthesis enzymes, and other metabolic pathways (Table S1C). The diversity of affected processes suggests a high level of complexity in regulation.

There is a significant overlap between genome expression profiles in response to drought and high-salinity stress

To compare the gene expression profiles of the drought and high-salinity stress responses, genes commonly regulated by both stresses at each organ type were

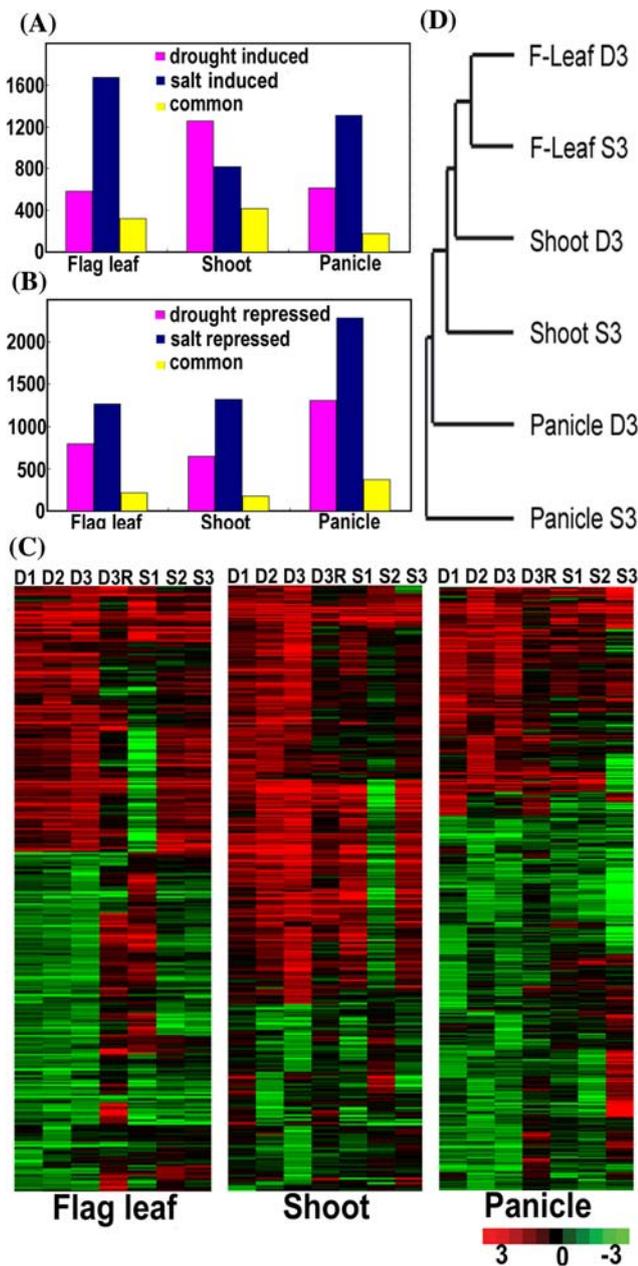


Fig. 2 Comparison of drought and high-salinity genome expression responses in the three rice organs. **(A)** Total number of drought and high-salinity stress inducible genes and the number of genes induced in response to both stresses in each of the three rice organs. Genes induced at least at one stage under drought or high-salinity treatments are included in the analysis. y-axis shows the gene number. **(B)** The total number of drought and high-salinity stress repressed genes and the number of genes repressed in response to both stresses in each of the three rice organs. Genes repressed at least at one stage under drought or high-salinity treatments are included in the analysis. y-axis shows the gene number. **(C)** Clustering analysis of all drought and high salinity responsive genes in each of the three rice organs. The differentially expressed genes (both induced or repressed) with $P < 0.05$ at least at one stage of drought treatment are included. The median ratio (treated/untreated sample) is log₂ transformed and subject to complete linkage hierarchical clustering. D1, D2, and D3: three stages of drought treatment; D3R: 48-h water recovery after drought; S1, S2, and S3: three stages of high-salinity treatment. In total, 1,377, 1,903, and 1,919 genes from flag leaf, shoot, and panicle, respectively, were included in this analysis. **(D)** Relatedness of the genome expression patterns across selected stress-treated rice organs. A complete-linkage hierarchical clustering analysis of overall relatedness for expression ratios from selected organs at the stage 3 of both abiotic stress treatments. The abbreviations for different sample types are the same as in panel C

examined. A total of 322, 415, and 174 genes were up-regulated and 215, 173, and 372 genes were down-regulated by both stresses in flag leaves, shoots and panicles, respectively (Fig. 2A, B). The common genes represent 55, 33 and 28% (induced) and 27, 27 and 29% (repressed) of all drought-responsive genes in flag leaf, shoot and panicle, respectively. These results indicate that about one-third, even half in flag leaf, of the drought responsive genes in each organ are also regulated by high salinity stress.

To further compare gene expression under the two abiotic stresses, all drought regulated genes were

selected for cluster analysis (see Experimental procedures). As shown in Fig. 2C, up to half of the genes up- or down-regulated by drought stress also exhibit a similar expression pattern under high-salinity stress. The remainder of the drought responsive genes exhibits minimal expression changes or distinct expression patterns in response to high-salinity stress.

Among genes specifically induced by drought stress, some have known functions while others were previously predicted to have functions related to drought stress or the ABA response. For example, our experiments showed that genes with putative functions including NAM, HLH, G-box binding, Zn-finger, AP2 transcription factors, and protein kinases (including MAPK family genes) were all affected by drought stress. Quite a few known drought-responsive genes such as DREB1A, LEA protein, WSI76 protein, MAP65 (microtubule associated protein), and ubiquitin were also included. These genes were specifically induced by drought and may function exclusively in the response to drought stress in rice. To gain an overall view of the effects of drought and high-salinity stress on various functional gene groups, gene ontology (GO) categories for drought and high-salinity stress induced genes in three organs were examined (Fig. S2). For the majority of these functional categories, the relative numbers of genes responsive to the two stresses were similar.

We also examined the effects of drought and high-salinity on rice genes coding enzymes in known metabolic pathways using shoot as a model (Fig. S3). Some pathways are similarly regulated by the two stresses, including the Calvin cycle, TCA cycle variation I, brassinosteroid biosynthesis II, gibberellin biosynthesis, and IAA biosynthesis I, and the de novo biosynthesis of pyrimidine ribnucleotides/pyrimidine deoxyribonucleotides/purine nucleotides (Fig. S3 and data not shown). While other pathways respond differentially to drought or high-salinity stresses, the representative pathways include sterol biosynthesis, sugars and polysaccharides, ABA biosynthesis, lignin biosynthesis, octane oxidation, cutin biosynthesis, and starch degradation (Fig. S3). For example, some steps in these pathways were repressed or invariable under high-salinity stress, but were induced by drought stress. It is noticeable that one step in the ABA-biosynthesis pathway (corresponding to *Arabidopsis* NCED3) showed slightly inhibited in gene expression under high-salinity stress (only at stage 3), but was induced under drought treatment (Fig. S3c). Our informatic analysis suggested that OsJRFA107649 is the closest *Arabidopsis* NCED3 homolog, with 66% identity ($E = 1.8E-195$). From our analysis, OsJRFA107649 expression ratios (\log_2 transformed) were 0.278 (D1), 3.461 (D2) and 2.53 (D3) under drought treatment and 3.626 (S1), 2.283 (S2) and -1.958 (S3) under high salinity treatment. The expression ratios of rice gene at D3 and S3 stage were used for the diagram in Fig. S3. It is interesting to note that under high salinity this gene was initially induced and then inhibited at stage 3 (S3 ratio: -1.958), while under drought treatment this gene was induced starting at stage 2 and maintained after stage 3 (D3 ratio: 2.53). This example illustrated a distinct response kinetics for different responsive genes in response to drought and high-salinity stresses.

By comparing transcriptomes under drought and high-salinity stresses in three distinct organs, we found that drought responsive genome expression in flag leaf is closer to that for high salinity stress than to the drought expression response observed in any other organ (Fig. 2C, D). In general, there seems to be a closer relationship between the transcriptome-level responses to the two abiotic stresses in the same organ than between or among transcriptomes in distinct organs in response to the same stress. However, the extent of overlap in the responses to drought or high-salinity stresses varies for the different rice organs. For example, it is more divergent in the responses to the two abiotic stresses in panicle, while flag leaves show the greatest overlap.

Distinct groups of genes are induced during rehydration after drought stress

In its natural environment, a plant's ability to respond to rehydration after drought stress is important for its survival, although little is known about the gene expression changes that occur during rehydration. Our experiments showed that after a period of drought (third stage), all the rice leaves rolled and turned yellow, and the RWC dropped to 70–75%. When we applied water to those severely stressed plants, rice leaves started to unroll after 5 h, and exhibited normal flatness within 24–48 hours of rehydration. We collected tissue samples 48 h after rehydration and extracted total RNA for gene expression analysis (Fig. 2C).

Comparing to samples from normal grown rice plants, in flag leaf, shoot and panicle, 807, 281 and 224 genes were up-regulated after 48 h of rehydration following drought stress, respectively (Fig. 3, Table S14–

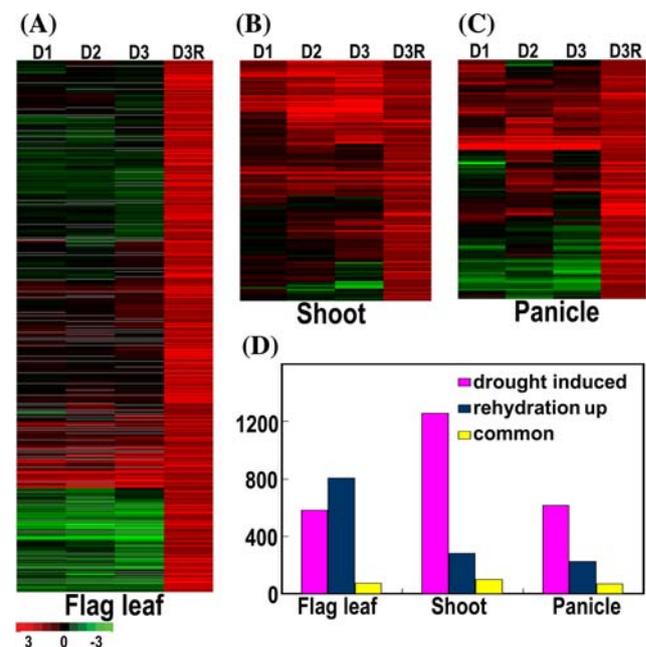


Fig. 3 Cluster analysis of genes exhibiting elevated expression after 48-h rehydration following drought. (A) (B) and (C) Cluster analysis of the genes exhibiting significant elevation of expression (with $P < 0.05$ as threshold) after 48 h of rehydration treatment for flag leaf, shoot, and panicle. The median ratio is \log_2 transformed and subject to complete linkage hierarchical clustering. A total of 807 genes in flag leaf (A), 281 genes in shoot (B), and 224 genes in panicle (C) are included in this analysis. D1, D2, and D3 represent three stages of drought treatment, and D3R represents 48-h rehydration of the D3 stage. (D) The histogram shows the number of genes in each of the three rice organs induced under drought treatment at least at one drought stage, after 48-h rehydration following the D3 stage, and the number of genes with overlapping expression in various pairs of organs. y-axis shows the gene number

S16). We further compared gene expression profiles during drought treatment with those after rehydration (Fig. 3A–C). The expression patterns of those genes seem to belong to three groups, with flag leaf exhibiting the greatest number of gene expression changes following rehydration. Group I, which included 71, 98 and 68 genes from flag leaf, shoot and panicle, respectively, was induced during both drought treatment and rehydration. Group II, which included 71, 8 and 21 genes in flag leaf, shoot and panicle, respectively, was repressed during drought stress but induced by rehydration (for shoot and panicle, see Table 1). Group III, which included all remaining 665 genes in flag leaf, 175 genes in shoot, and 135 genes in panicle, showed no significant expression changes under drought stress but were up-regulated during rehydration. We suspected that group I genes must be induced by drought stress, but have yet to return to normal levels after 48-h rehydration. Those genes in groups II and III were specifically induced by rehydration.

The down-regulation of group II genes in response to drought led us to speculate that they may play an

important role in conferring drought stress tolerance, whereas the up-regulation of group II and group III genes during rehydration could be important for recovery. In both group II and III genes, we found that two classes of genes were over-represented among the rehydration-inducible genes in shoot and panicle: transporter genes and photosynthesis-related genes. These transporter genes included: OsJRFA065471 (folate/biopterin transporter), OsJRFA066919 (putative potassium transporter), OsJRFA067899 and OsIFCC019970 (ABC transporter, putative), OsJRFA068003 and OsJRFA106202 (Transmembrane amino acid transporter protein), OsJRFA068765 (H-ATPase), OsIFCC040482 (phosphate:H⁺ symporter), OsJRFA072183 (Sodium:sulfate symporter transmembrane region), OsJRFA102086 (putative lipid transfer protein) and OsJRFA103807 (aquaporin). The photosynthesis related genes cover most of the gene components of the two photosystems, such as genes for putative chlorophyll *a/b*-binding protein, Photosystem I or II reaction center subunits, and plastocyanin. These photosystem genes and transporter genes

Table 1 Genes induced by 48-h rehydration and inhibited by drought stress in shoot and panicle

Gene name	Putative functions	D1	D2	D3	D3R
<i>Shoot</i>					
OsIFCC006273	Unknown	0.596	-1.611	-3.497	1.920
OsIFCC021002	Glycosyl hydrolases family 17	0.440	-1.105	-1.775	2.470
OsJRFA107373	Unknown	-1.521	-0.487	-4.348	2.470
OsJRFA101949	AP2 domain	0.216	-0.166	-2.456	2.026
OsJRFA106016	Kelch motif	-0.315	-0.582	-2.604	1.865
OsJRFA070715	Peroxidase	-0.189	-0.499	-2.456	2.215
OsJRFA065471	Folate/biopterin transporter	0.331	-0.533	-2.595	3.095
OsIFCC041459	Chitinase	-0.460	-0.136	-2.125	1.972
<i>Panicle</i>					
OsIFCC023423	Photosystem I reaction center subunit IV	-0.067	-0.293	-2.610	2.206
OsIFCC032344	Photosystem I reaction center subunit n	-0.141	-0.626	-1.700	2.390
OsIFCC001915	Photosystem II polypeptide	-0.119	-0.934	-1.774	1.787
OsJRFA061606	Pathogenesis-related protein PR-10a	-1.811	-0.007	-1.841	4.117
OsJRFA107373	Unknown	-3.693	0.395	-1.978	5.605
OsJRFA109398	Chlorophyll <i>a/b</i> binding protein	-1.867	-1.348	-1.899	1.905
OsIFCC025509	Photosystem II 10 kDa polypeptide PsbR	-1.077	-0.966	-1.735	1.831
OsIFCC038501	Chloroplast precursor	-1.087	-0.966	-1.805	1.837
OsIFCC022062	Photosystem I reaction center subunit VI	-0.540	-0.206	-1.996	1.811
OsIFCC024081	Chlorophyll <i>a/b</i> -binding protein	-0.595	-0.685	-1.814	2.298
OsIFCC014461	Unknown	-0.794	-0.660	-1.722	1.790
OsIFCC017469	Unknown	-0.879	-1.619	-1.764	2.417
OsIFCC012276	Catalase	-1.567	-0.594	-1.893	2.592
OsJRFA060135	Unknown	-0.922	-0.491	-2.540	1.793
OsIFCC033400	Pathogenesis-related protein PR-10a	-0.115	-0.032	-2.550	2.008
OsJRFA059435	Magnesium-protoporphyrin IX monomethyl esteraerobic oxidative cyclase	-0.344	-0.348	-1.837	2.851
OsIFCC022709	Protease inhibitor/seed storage/LTP family	-0.091	-0.300	-1.684	1.842
OsJRFA061968	Geranylreductase	-0.334	-0.244	-1.804	2.001
OsJRFA071762	Aluminium-induced protein	0.217	-0.594	-1.849	1.752
OsIFCC029079	Blight-associated protein p12 precursor	-1.110	0.379	-1.848	2.980
OsJRFA106991	Unknown	-0.899	-0.142	-1.874	1.715

Log₂ transformed ratios of all genes at three stages of drought stress and rehydration stage were listed

represent a large portion of all genes induced by rehydration, and their physiological roles in plants fit well with a potential contribution toward plant recovery from drought stress.

Limited overlap of stress responsive genes among rice organs

We also examined the degree of overlap in expression of stress responsive genes in two or more rice organs under drought or high-salinity stress. Venn diagrams revealed that only a small portion of genes was shared between each pair of organs (Fig. 4). The greatest overlap occurred between shoot and flag leaf, which shared more inducible genes than shoot and panicle or flag leaf and panicle under both drought and high-salinity conditions. Under drought stress, one-third of the genes up-regulated in flag leaf (197/582) were also induced in shoot (Fig. 4A), while under high salinity stress, over half of the induced genes (494/817) in shoot (mostly young leaves) were also up-regulated in flag leaf (Fig. 4B). Only 21 and 23% of genes up-regulated in panicle were also induced in flag leaf under drought

and high salinity stresses respectively. The percentage of genes shared between shoot and panicle is similar to that between flag leaf and panicle (Fig. 4A, B).

Interestingly, only a small fraction of stress responsive genes were expressed in all three organs examined. For example, 42 and 151 genes were induced in all three organs under drought and high-salinity stress respectively. In general, most of those genes exhibited high levels of expression as well as strong inducibility. Among them, 27 genes were induced by both drought and high-salinity stress in all three organs. This group of 27 genes includes a protein kinase (OsJRFA058518), chlorophyll *a/b* binding protein (OsJRFA062972), ABA-responsive protein (OsJRFA063578, OsIFCC018156), LEA protein (OsJRFA063984), dehydrin (OsIFCC035028), CHY zinc finger protein (OsIFCC003263), and homeobox protein (OsIFCC018343) (Table 2).

Analysis of stress down-regulated genes (Fig. 4C, D) revealed a similar small overlap among the three organs. Only 68 and 129 out of 795 total genes down-regulated in flag leaf under drought stress were also inhibited in shoot and panicle, respectively. For high-salinity stress, 135 and 214 out of 1,270 down-regulated genes in flag leaf were also repressed in shoot and panicle respectively. Only 16 and 38 genes were down-regulated in all three organs under drought and high salinity stress respectively, while only two genes were inhibited in all three organs under both drought and high salinity stress (Table 2).

The shoot samples we used consisted largely of young leaves and were thus physiologically closer to flag leaf than panicle. Indeed, shoot and flag leaf generally exhibited similar patterns of gene up-regulation in response to drought and high-salinity stress. However, this does not appear to be the case for repressed genes, where overlap between shoot and flag leaf gene expression is minimal.

To validate the microarray data, RT-PCR analysis was used to verify the transcription response of representative genes from microarray results. For this purpose, we picked a group of eight representative genes and designed primers for RT-PCR analysis (Table S18). The cDNA templates were synthesized from total RNA samples prepared from shoot, flag leaf and panicle under drought and unstressed controls. The semi-quantitative RT-PCR results for the eight representative genes were shown in Fig. 5. The expression patterns of all eight genes reflected changes observed by microarray analysis fairly and accurately for all three organs examined, except in three cases where RT-PCR failed to confirm the microarray results (Fig. 5), indicating that our microarray data is reliable.

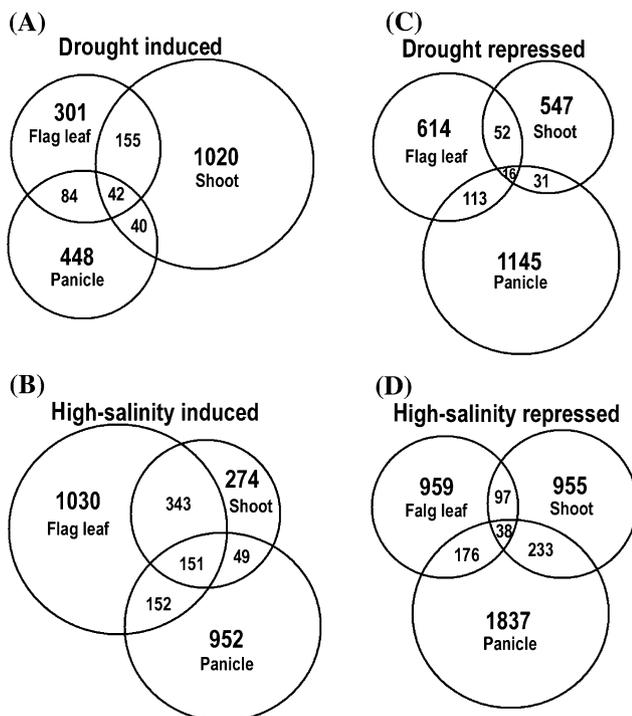


Fig. 4 Comparison of gene expression patterns among the three rice organs in response to drought and high-salinity stresses. **(A)** and **(B)**: Venn diagram of drought **(A)** and high-salinity **(B)** induced genes among the three rice organs. **(C)** and **(D)**: Venn diagram of drought **(C)** and high-salinity **(D)** repressed genes among the three rice organs

Table 2 Genes up- or down-regulated by both drought and high-salinity stresses in all three organs

Gene name	Putative functions	Flag leaf		Shoot		Panicle	
		D(h)	S(h)	D(h)	S(h)	D(h)	S(h)
<i>Up-regulated</i>							
OsJRFA058518	Protein kinase domain	2.591	1.939	3.939	3.443	3.134	3.553
OsJRFA058851	Unknown	5.430	3.900	5.894	4.600	2.042	3.878
OsJRFA062356	Unknown	4.959	2.477	4.699	4.660	3.314	5.727
OsJRFA062972	Chlorophyll <i>a/b</i> binding protein	4.758	2.542	1.895	2.931	2.291	2.204
OsJRFA063156	Unknown	1.837	1.743	4.405	3.921	2.606	2.866
OsJRFA063334	Unknown	3.208	2.140	4.521	3.188	1.809	2.167
OsJRFA063578	ABA-responsive protein	2.743	3.352	4.169	3.733	1.859	2.748
OsJRFA063889	Unknown	3.340	3.263	2.186	3.344	2.061	3.712
OsJRFA063984	LEA protein	5.513	4.730	3.763	4.629	3.971	6.773
OsJRFA068381	Unknown	2.648	1.830	3.630	2.833	1.820	1.786
OsJRFA070577	Unknown	3.036	2.877	3.065	2.899	1.883	2.372
OsJRFA070872	Unknown	8.485	7.733	3.446	6.346	3.963	4.107
OsJRFA071812	Unknown	2.748	2.268	3.015	2.244	1.767	2.559
OsJRFA072568	Unknown	3.001	3.121	2.411	3.326	1.714	2.005
OsJRFA106307	Unknown	4.284	3.493	4.399	2.511	2.316	2.960
OsJRFA106562	Unknown	3.797	4.009	2.544	3.467	2.010	3.978
OsJRFA107065	Unknown	2.770	1.751	4.859	3.648	2.984	2.292
OsJRFA108083	Unknown	2.274	2.597	4.853	4.241	3.077	4.631
OsIFCC031279	Alpha-galactosidase	3.407	3.087	3.819	3.538	2.389	2.206
OsIFCC036408	Unknown	3.137	3.987	3.038	3.111	4.742	4.192
OsIFCC035028	Dehydrin	7.974	7.689	5.148	6.767	8.045	6.382
OsIFCC018156	Abcisic acid-induced protein	5.167	7.224	3.243	4.911	2.329	2.327
OsIFCC003263	CHY zinc finger	1.709	2.147	1.994	2.282	3.791	1.956
OsIFCC018343	Homeobox domain	4.290	3.955	3.432	3.959	2.683	3.017
<i>Down-regulated</i>							
OsIFCC033098	Similar to <i>Arabidopsis</i> F16L1.3 protein	-2.676	-4.719	-2.542	-2.346	-1.701	-2.519
OsIFCC015113	Phosphoribulokinase /Uridine kinase family	-2.456	-2.689	-1.863	-1.717	-1.853	-1.899

The highest ratio of each gene at three stages under drought (D(h)) or high-salinity (S(h)) were log₂ transformed and listed

Enrichment of ABRE and DRE *cis*-regulatory elements in abiotic stress-induced gene promoters

Previous reports have suggested that several well-characterized drought-, high-salinity and cold inducible gene promoters contain two common *cis*-regulatory elements, the ABA-responsive element (ABRE) and the dehydration-responsive element (DRE) (Seki et al., 2001, 2002a, b; Yazaki et al., 2003; Rabbani et al., 2003; Dubouzet et al., 2003; Nakabayashi et al., 2005; Maruyama et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2005). ABRE and DRE confer ABA-dependent and ABA-independent gene expression in response to water stress. It has been reported that a single copy of ABRE in the promoter region only induces relatively minor elevation of expression level, while multiple copies of ABRE strongly activate expression (Shen and Ho, 1995; Nakabayashi et al., 2005).

To elucidate the relationship between copy numbers of DRE and ABRE *cis*-regulatory elements in gene promoter regions and abiotic stress-induced gene transcription, we divided the genes into groups based on their expression patterns. Genes induced specifi-

cally by drought or high-salinity stress in each of the three organs defined six groups, while genes induced by both drought and high-salinity stress in each organ defined another three groups. Common inducible genes shared by at least two organs under drought or high-salinity stress defined another nine groups. We only selected those genes among all those groups with full-length cDNA sequences available for further promoter analysis. In brief, the 2 kb upstream regions (-1 to -2,000 bp) preceding the ATG start codon of genes were selected for promoter motif analysis (see Experimental procedures). A similar number of rice genes with full length cDNA available that lack stress responsive expression were used as control. Copy number of ABRE and DRE core motifs on each promoter region were counted. The percentages of genes in each group with 1 to 6 copies of ABRE core or DRE core elements were then calculated.

Compared to control genes, copy numbers of the ABRE and DRE core motifs in the promoter regions of genes with organ-specific expression in response to drought or high-salinity stress were not markedly different (Fig. 6A, D). The promoter regions of genes

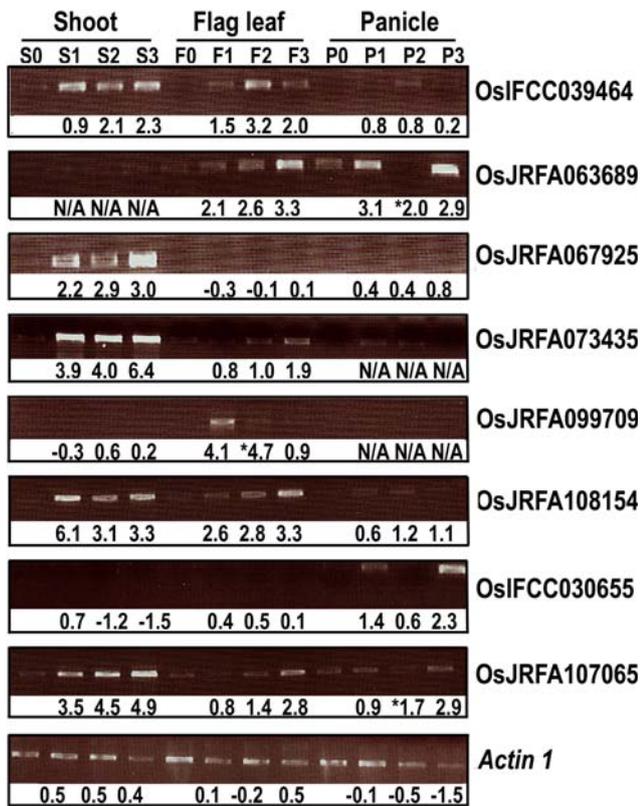


Fig. 5 RT-PCR analysis of the representative drought-induced genes among the three rice organs. Total RNA samples were prepared from the shoot (S1, S2, and S3), flag leaf (F1, F2, and F3), and panicle (P1, P2, and P3) and taken from plants at three stages of drought treatment and the untreated control plants (S0, F0, and P0). The corresponding log₂ transformed median ratio of microarray data is shown at the bottom of each mRNA blot line. N/A: no expression. The * corresponds to cases where the microarray data was not confirmed by the mRNA blot results

responsive to both drought and high-salinity stress in each organ were enriched for ABRE and DRE core motifs compared to the promoters of control genes (Fig. 6B, E). For example, only 25% of control genes have over four copies of ABRE core motif in their promoter regions, but 48, 44 and 48% of genes induced by both drought and high-salinity stresses in flag leaf, panicle and shoot respectively have over four copies of the ABRE core motif in their promoter regions (Fig. 6B).

When genes expressed in at least two organs under drought or high-salinity stress were compared to control genes, the ABRE and DRE core motif copy numbers also showed significant differences (Fig. 6C, F). For example, 50% of these genes contain over four copies of the ABRE core motif in their promoter regions, compared with 25% for the control genes (Fig. 6C). A similar pattern was found for the DRE core element (Fig. 6F).

Genes induced in an organ-specific manner by drought or high-salinity stresses did not contain a significantly different number of copies of ABRE or DRE core motifs compared to control genes (Fig. 6A, D). Thus it is plausible that the stress-induced expression in this fraction of genes may rely less on transcriptional activation mediated by ABRE and DRE motifs. Unidentified organ-specific *cis*-regulatory elements may exist in the promoter regions of these genes and play a more important role in transcription activation under drought or high-salinity stress.

Identification of two novel *cis*-regulatory elements that respond to rehydration after drought stress

As shown in Table 1, there are 8 and 21 genes with full-length cDNA sequences repressed by drought stress but induced by rehydration in shoot and panicle, respectively. An analysis of the upstream region of these eight shoot genes identified a novel motif (motif-SP, GGCAGCCG) located near to the translation start codon (-73 to -250) in five of them (Fig. 7A). To investigate the potential functional role of this novel motif, we performed a gel shift mobility assay which involved incubating a 24 bp probe containing motif-SP from one of those five genes (OsJRFA070715) and a control containing a single base mutation at an invariable position with nuclear extracts (see Experimental procedures). Only nuclear extracts from drought-treated shoot (at the third stage), but not from unstressed shoot or shoot under rehydration, showed a sequence specific binding activity with the probe (Fig. 7C). A single base mutation at an invariable position in the motif-SP core of the probe completely abolished protein binding activity, suggesting high specificity of interaction with the core motif included in this 24 bp sequence. This specific binding activity was only observed in drought-treated plants, suggesting that it is involved in transcriptional repression under drought conditions.

We also searched promoter regions of 21 genes inhibited by drought stress but induced by rehydration in panicle. Three conserved motifs were found through this blind search in 11 of the 21 genes: ABRE motif, the above-mentioned motif-SP, and another novel element, motif-P (Fig. 8). The presence of the ABRE motif suggests that a gene may respond to drought stress by employing an ABA-dependent pathway, while motif-SP may play an important role in drought-stress repression and subsequent activation during rehydration. We failed to detect specific binding activity using similar nuclear extracts (data not shown) and thus the role of motif-P remains to be defined.

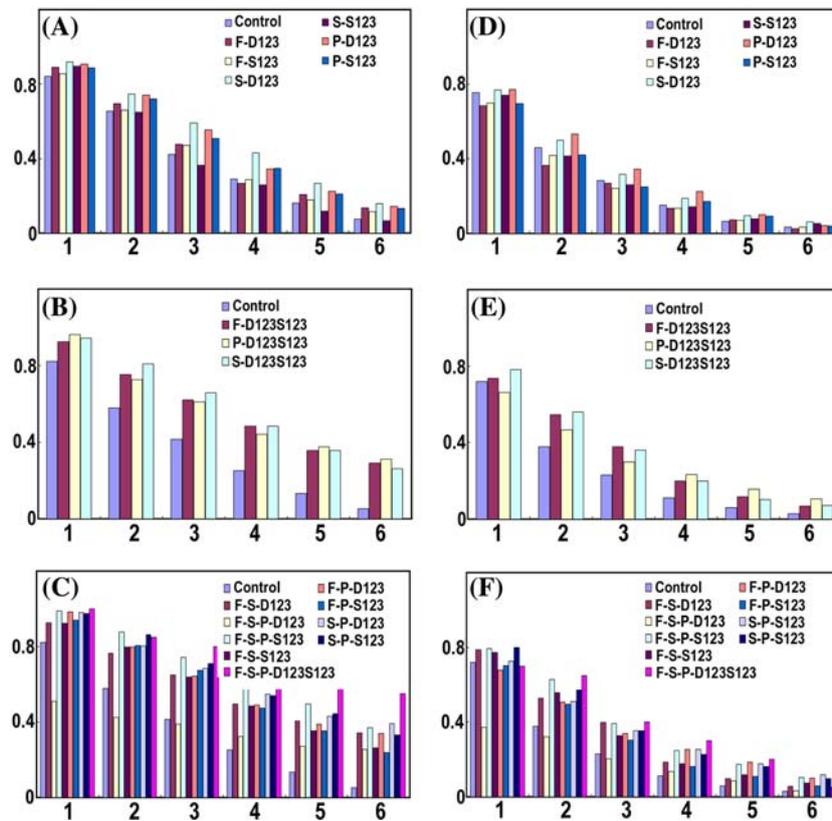


Fig. 6 Distribution of the ABRE and DRE core motif sequences among different groups of drought and high-salinity stress responsive genes. In all panels, the *x*-axis shows the copy number of ABRE or DRE core motifs and the *y*-axis shows the relative number of genes containing different copy numbers of ABRE or DRE core motifs. **(A)** ABRE core motif distribution among promoters of the genes induced only by drought or only by high-salinity stress in each single rice organ. **(B)** ABRE core motif distribution among promoters of genes induced by both drought and high-salinity stress in each rice organ. **(C)** ABRE core motif distribution among promoters of genes induced in two or three organs. **(D)** DRE core motif distribution among promoters of genes induced only by drought or only by high-salinity stress in each rice organ. **(E)** DRE core motif distribution among promoters of genes induced by both drought and high-salinity stress in each rice organ. **(F)** DRE core motif distribution among promoters of genes induced in two or three organs or by both stresses in more than one organ. The following 18 gene groups (with number of genes with full-length cDNA gene number) were analyzed. (1) Genes induced by drought stress only in flag leaf (F-D123, 82). (2) Genes induced by high-salinity

stress only in flag leaf (F-S123, 433). (3) Genes induced by drought stress only in shoot (S-D123, 272). (4) Genes induced by high-salinity stress only in shoot (S-S123, 77). (5) Genes induced by drought stress only in panicle (P-D123, 139). (6) Genes induced by high-salinity stress only in panicle (P-S123, 420). (7) Genes induced by both stresses in flag leaf (F-D123S123, 190). (8) Genes induced by both stresses in shoot (S-D123S123, 252). (9) Genes induced by both stresses in panicle (P-D123S123, 77). (10) Genes induced by drought stress in both flag leaf and shoot (F-S-D123, 123). (11) Genes induced by drought stress in all three organs (F-S-P-D123, 59). (12) Genes induced by high-salinity stress in all three organs (F-S-P-S123, 97). (13) Genes induced by high-salinity stress in both flag leaf and shoot (F-S-S123, 309). (14) Genes induced by drought stress in both flag leaf and panicle (F-P-D123, 59). (15) Genes induced by high-salinity stress in both flag leaf and panicle (F-P-S123, 184). (16) Genes induced by drought stress in both shoot and panicle (S-P-D123, 51). (17) Genes induced by high-salinity stress in both shoot and panicle (S-P-S123, 124). (18) Genes induced by both stresses in all three organs (F-S-P-D123S123, 20)

Transcription factor genes under drought and high salinity stress are expressed in a largely organ specific manner

We further examined transcription factor genes whose expression are regulated by drought and high-salinity stresses. Among all genes induced by drought or high-salinity treatment in the three organs, a total

of 186 genes (Table S17) were predicted to be transcription factors with a DNA-binding domain. These transcription factors belong to various families, including AP2, bHLH, MYB, HB, NAC, zinc finger, MADS, bZIP, WRKY and HSF families. These transcription factors could also be divided into several groups depending on their expression patterns. Among the 186 transcription factor genes, 12 genes

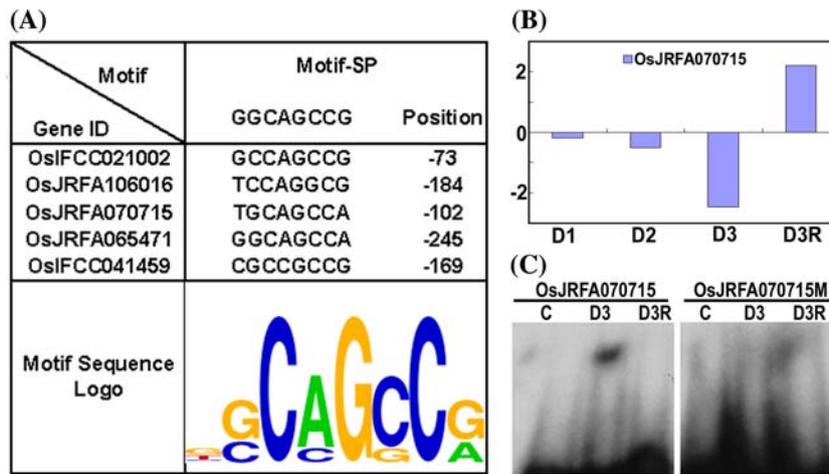


Fig. 7 A novel promoter motif associated with genes repressed by drought but induced after rehydration in rice shoot. (A) The core sequence of the motif and the position of the motif in each promoter. (B) The expression pattern of a representative gene containing motif-SP. The histogram shows the log2 transformed ratio at three drought stress stages (D1, D2, D3) and 48-hour rehydration (D3R). (C) Gel shift assay of motif-SP. Nuclear

proteins were extracted from shoots of untreated plants (C), plants at stage 3 of drought treatment (D3), and plants after 48-h rehydration following stage 3 (D3R). Core sequences of the probe OsJRFA070715: TGCAGCCA, and core sequence of the probe with a single base point mutation OsJRFA070715M: TGAAGCCA

Fig. 8 Three motifs associated with genes repressed during drought and induced by rehydration in panicle. The core sequence logos and the position of the motifs in each of the 11 promoters are listed

Oligo ID	ABRE		Motif-P		Motif-SP	
	GCCACGTGGCG	Position	GGGAGTA	Position	TTGGCAGC	Position
OsJRFA109398	GCCACGTGGCA	-167	GGGAGTA	-959	TTGGCAGC	-779
OsIFCC038501	GCCACGAGGTC	-533	GGGAGGC	-1453	ATGGCTGC	-832
OsIFCC024081	GCCACGCGGAG	-108	GGGAGTC	-1497	TTGGCAGT	-549
OsIFCC029079	TCCACGGGCAA	-941	GGGAGTC	-869	GTGGCTGC	-432
OsIFCC033400	GCCATGTGATG	-98	GGGAGTA	-244	GGGGCAGA	-1148
OsJRFA061606	GCCATGTGATC	-194	GGGAGTA	-1780	AGGGCTGC	-800
OsIFCC022062	GCCACGCGGCG	-183	GGGAGTA	-826	TGGGCTGC	-1012
OsIFCC023423	GCCACGCGGCG	-177	GGGAGTA	-1324	ATGGCAGC	-245
OsIFCC025509	GCCACGTGGCG	-202	GGGAGTA	-870	TTGGCAGA	-658
OsIFCC032344	TCCACGTGGCA	-228	GGGAGAC	-1693	ATGGCAGC	-176
OsIFCC001915	GCCACGTGCAC	-197	GGGAGGC	-153	GTGGCAGC	-992

were induced in all three organs in at least one stage following either drought or high-salinity stress, whereas the remaining 174 genes were mainly up regulated in one or two organs. Transcription factors induced only in one individual organ were listed in Table 3. It is interesting to note that over half of the transcription factors were expressed in at least two organs, while other transcription factors were activated in an organ-specific manner. This suggests that the expression of different transcription factors may play a key role in common or organ-specific gene expression in response to drought or high-salinity conditions.

Minimal co-regulation of neighboring genes in drought or high-salinity stress responses

Co-regulation of adjacent genes has been observed in several organisms (Hurst et al., 2004), including human (Caron et al., 2001; Lercher et al., 2002), *Drosophila* (Spellman and Rubin, 2002), *Arabidopsis* (Birnbaum et al., 2003, Ma et al., 2005a), and yeast (Cohen et al., 2000). All these studies identified co-regulation of neighboring gene clusters on chromosomes (chromatin domains). In rice, analysis of organ-specific gene expression revealed that about 10% of the genome belongs to chromatin domains

Table 3 Transcription factor genes induced in each organ by drought or high-salinity stress

Gene name	Putative function	S(h)	D(h)
Panicle: induced by high-salinity only			
OsIFCC018668	bHLH transcription factor	1.673	-0.850
OsIFCC029156	Helix-loop-helix DNA-binding domain	1.720	-0.599
OsJRFA110611	No apical meristem (NAM) protein	2.234	-1.500
OsJRFA105079	CCAAT-box binding factor HAP5 homolog	3.521	-0.850
OsJRFA070817	RING zinc finger protein	1.708	-0.685
OsIFCC039583	Zinc finger, C3HC4 type (RING finger)	2.278	0.530
OsJRFA108605	Helix-loop-helix DNA-binding domain	4.713	0.240
OsJRFA108208	AP2 domain	2.429	N/A
OsJRFA101136	C3HC4-type zinc finger	5.559	-0.753
OsIFCC008718	No apical meristem (NAM) protein	2.542	N/A
OsJRFA066984	Dof domain, zinc finger	1.846	0.168
OsJRFA110661	Zinc finger C-x8-C-x5-C-x3-H type	1.732	N/A
OsJRFA106969	Myb-like DNA-binding domain	2.010	N/A
OsIFCC016263	Zinc finger, C2H2 type	2.636	N/A
Panicle: induced by drought only			
OsIFCC042866	AP2 domain	N/A	3.050
Panicle: induced by both drought and high-salinity			
OsJRFA107283	NAM-like protein	7.773	2.080
Shoot: induced by drought only			
OsJRFA105599	DRE-binding protein 1A	N/A	1.784
OsIFCC031932	WRKY DNA -binding domain	-2.746	2.186
OsIFCC031182	Myb factor	N/A	2.565
OsIFCC042758	Helix-loop-helix DNA-binding domain	-0.024	2.367
OsJRFA100208	Helix-loop-helix DNA-binding domain	-0.836	3.132
OsJRFA107524	Dof domain, zinc finger	N/A	2.294
OsJRFA106333	Helix-loop-helix DNA-binding domain	0.430	1.762
OsJRFA106282	WRKY DNA -binding domain	N/A	2.026
OsIFCC043271	Helix-loop-helix DNA-binding domain	0.245	2.259
OsIFCC000715	Myb-like DNA-binding domain	0.949	2.617
OsJRFA110587	Similar to DNA-binding protein WRKY3	0.905	4.075
OsJRFA107146	AP2 domain	0.625	2.746
OsIFCC038336	Zinc finger transcription factor ZF1	0.529	2.407
Shoot: induced by both drought and high-salinity			
OsJRFA072192	Zinc-finger protein	2.074	2.059
OsIFCC000984	WRKY DNA -binding domain	1.744	2.153
Shoot: induced by high-salinity only			
OsIFCC017057	B3 DNA binding domain	2.026	N/A
OsJRFA067496	TRAF-type zinc finger	2.199	0.801
Flag leaf: induced by drought only			
OsIFCC029554	Zinc finger, C2H2 type	-0.553	2.032
Flag leaf: induced by high-salinity only			
OsIFCC001054	Zinc finger C-x8-C-x5-C-x3-H type	1.705	0.582

The highest ratio of each gene among three stages of drought (D(h)) or high-salinity (S(h)) were log₂ transformed and listed

that exhibit this co-expression pattern (Ma et al., 2005b). However, similar analysis of light-regulated genes failed to reveal significant co-regulation (Jiao et al., 2005).

To investigate possible co-regulation of neighboring genes in the drought and high-salinity responses, we mapped rehydration induced genes in three organs and drought and high-salinity induced genes in shoot to all 12 *indica* chromosomes (Figs. 9, S4). This analysis indicated that significant gene clustering among rehydration, drought, and high-salinity induced genes did

not occur at the chromosomal level, similar to the pattern characteristic of light-regulated genes (Jiao et al., 2005). Among the 12 rice chromosomes, only three regions showed significant enrichment of 10 or more co-regulated genes within any given 2 Mb domain. For example, the 33–35 Mb region on chromosome 2, the 11–13 Mb region in chromosome 3, and the 31–33 Mb region in chromosome 6 (Fig. 9B) contain significant enrichment of rehydration regulated genes in all three organs, although even in those cases genes are not directly adjacent.

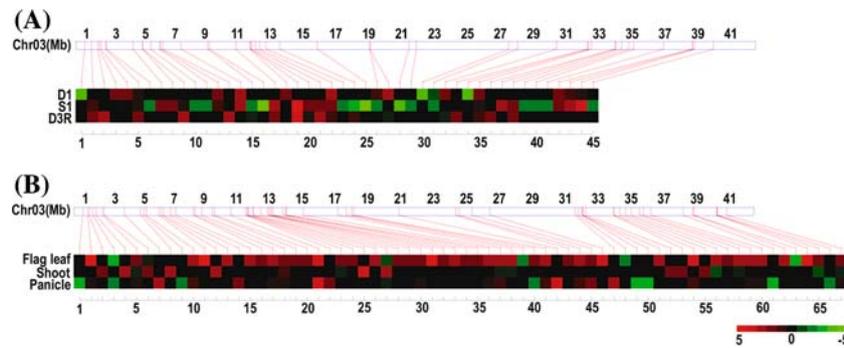


Fig. 9 Distribution of drought, high salinity and rehydration regulated genes in a representative rice chromosome. **(A)** Map of chromosome 3 genes differentially expressed at the first stage of drought, first stage of high-salinity stress, and 48 h after

rehydration in shoot. **(B)** Map of chromosome 3 genes differentially expressed at 48 h after rehydration in the three rice organs

Discussion

This study provides new insight into the rice response to drought and high salinity stresses at the whole genome level. Using a whole genome microarray, we monitored the expression of 36,926 unique or known rice genes or gene models in three different organs under drought and high salinity stress. Our work thus offers the first comprehensive picture of genome expression modulation in response to drought and high salinity stress in three distinct rice organs.

Genome expression reprogramming showed significant overlap between drought and high salinity responsive genes

Using the criteria outlined above, a total of 2,957 and 2,090 rice genes showed significant up- or down-regulation in response to high salinity stress and drought stress in at least one of the three organs. Our analysis suggested that 927 out of 2,090 (44%) genes induced by drought were also up regulated by high-salinity stress in the same organs. This number is consistent with previous studies reported for *Arabidopsis* (Seki et al., 2002a, b; Shinozaki et al., 2003). In rice, an even higher percentage of drought induced genes were also up-regulated under high-salinity stress, as reported previously on a smaller scale (Rabbani et al., 2003), which covered only cDNAs from plants under drought, cold and high-salinity treatment. Thus our analysis should be more representative of the overall rice genome response to drought and high salinity stresses without such bias. However, drought and salt stress conditions in the field may be more subtle and less regular as in our experimental conditions, thus our results are only meant to illustrate some particular situations rice genome expression responses to stresses.

It is interesting to note an overlap of up to about half of the genes induced or inhibited by drought and high-salinity stress in rice. This observation is consistent with current understanding that these two stresses affect plants in overlapping but not identical ways. At a physiological level, both stresses cause water depletion in the above-ground portion of the plants and induce similar morphological responses (Fig. S1). At the molecular level, half of the transcriptional factor genes identified in our results were shared by both stresses in each organ, which is consistent with the observation that about half of the genes that respond to the two stresses were shared at the whole genome level.

Reprogramming of genome expression in response to drought and high-salinity stresses is largely organ specific

In this study, we examined whole genome expression profiles under drought and high-salinity conditions in three organs: four-tiller stage shoot, filling stage flag leaf and panicle. Rice plants at these two growth stages, particularly the late one, are sensitive to drought and high-salinity stress. It is well known that drought or high-salinity stress at the heading and early panicle stages can severely compromise rice growth and development and reduce crop yield even with late rehydration. It is evident that the rice genome is subject to significant reprogramming with regard to which portion of genome is expressed under drought or high salinity stress.

Our results showed that only a limited number of drought and high salinity responsive genes were shared between any two organs. We found that only 13.5 and 20.5% of drought-induced genes in panicle were shared with those induced in shoot and flag leaf respectively, and 33.8% of drought-induced genes in shoot were also

activated in flag leaf (Fig. 4). The percentages of genes shared between any two organs under high-salinity conditions were similar to those under drought stress, suggesting that responses to drought or high-salinity stress in different organs were independently regulated. This observation is similar to the observed small overlap of cold responsive gene expression between root and leaf in rice (Kreps et al., 2002).

Genome expression reprogramming under either drought or high salinity stress entails a large number of genes involved in many aspects of cellular function. A GO analysis of stress responsive genes (Fig. S2) indicates that, in most categories, three organs activated similar scale (number) of genes in response to drought or high salinity stress, while only small percentage of responsive genes were overlapped between or among organs (Fig. 4). This suggests that responsive genes under the same category are largely distinct individuals under distinct organ types. It is thus possible that homologous or functionally similar gene family members are responsive to the same stresses in each organ. This is consistent with our observation of organ-specific transcription factor gene expression in response to both drought and high salinity stresses (Table 3). The fact that genes specifically induced in each organ do not exhibit enrichment of DRE and ABRE motifs (Fig. 6) suggests that organ-specific transcriptional factor gene expression may be responsible for activating organ-specific downstream genes in a secondary transcriptional response to stress. For these genes, it is likely that certain organ-specific promoter elements mediate this response pathway. According to the GO analysis, under high salinity stress, about 10% storage proteins were up-regulated in panicle, while in flag leaf and shoot only less than 1% storage protein genes were induced (Fig. S2). This kind of difference among organs may play a role in organ specific response to two stresses; further functional analysis of these differentially regulated genes among organs are needed.

Rice may possess specific mechanisms to facilitate plant recovery during rehydration after drought

According to our microarray analysis, rice genes induced by 48-h rehydration were divided into three groups according to their expression patterns (Fig. 3), somewhat similar to a previous report for *Arabidopsis* (Oono et al., 2003). This observation suggested that monocotyledonous and dicotyledonous plants share similar gene expression responses to rehydration after drought. Analysis of the genome-wide distribution of rehydration-regulated genes in the three organs

showed no significant co-regulation of neighboring genes at the chromosomal level. This observation is similar to the case of light-regulated genes (Jiao et al., 2005) but different from general gene transcription from organ samples (Ma et al., 2005b). This distinction in the modulation of genome expression may reflect different mechanisms employed in response to different developmental or environmental signals.

Up to now only one possible *cis*-element has been reported to be involved in the rehydration process after dehydration in *Arabidopsis* (Satoh et al., 2002; Oono et al., 2003). In rice, at the whole genome level we identified 807, 281 and 224 genes induced by rehydration in flag leaf, shoot and panicle separately (Fig. 3). These genes provided us an important starting point to study rehydration mechanisms and to search for novel *cis*-regulatory promoter elements associated with dehydration or rehydration. Two novel *cis*-regulatory elements (motif-SP and motif-P) were identified (Fig. 8). Motif-SP was also found in shoot-specific genes with similar expression patterns in response to drought and rehydration. Gel shift assays provided evidence that motif-SP may function as a *cis*-element to mediate the drought-induced repression and late de-repression (activation) during rehydration in rice. Further functional analysis of the promoter elements may substantiate the role of those novel promoter motifs.

Previous studies reported that drought stress suppressed plant photosynthesis systems and significantly modulated the activity of some membrane transporters (Rizhsky et al., 2002; Ramachandra et al., 2004; Johansson et al., 2004; Becker et al., 2003). Here microarray analysis revealed that genes involved in photosynthesis and genes encoding transporters were repressed or maintained at low levels of expression under drought but were then strongly activated after rehydration. The repression of metabolic genes during drought stress allows the plant to conserve energy and subsist on less water, conferring better drought tolerance. When supplied plenty of water upon rehydration, activation of these genes could aid in the recovery of full photosynthesis activity and transmembrane solute/water exchange, thus helping plant resume its normal growth and development quickly.

Acknowledgements We thank Yale DNA microarray laboratory of the Keck Biological Resource Center for the printing of the rice 70-mer oligo array slides used in this study (http://info.med.yale.edu/wmkeck/dna_arrays.htm). This research was supported by a grant from the Rockefeller Foundation (to XWD), The 863 Program of the Ministry of Science and Technology of China, and the National Basic Research Program of China (2006CB100100).

References

- Barr HD, Weatherley PE (1962) A re-examination of the relative turgidity technique for estimating water deficit in leaves. *Aust J Biol Sci* 15:413–428
- Becker D, Hoth S, Ache P, Wenkel S, Roelfsema MR, Meyerhoff O, Hartung W, Hedrich R (2003) Regulation of the ABA-sensitive *Arabidopsis* potassium channel gene GORK in response to water stress. *FEBS Lett* 554:119–126
- Benjamini Y, Hochberg Y (2000) The adaptive control of the false discovery rate in multiple hypotheses testing. *J Behav Educ Statist* 25:60–83
- Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003) A gene expression map of the *Arabidopsis* root. *Science* 302:1956–1960
- Caron H, van Schaik B, van der Mee M, Baas F, Riggins G, van Sluis P, Hermus M, van Asperen R, Boon K, Voûte PA et al (2001) The human transcriptome map: clustering of highly expressed genes in chromosomal domains. *Science* 291:1289–1292
- Crooks GE, Hon G, Chandonia JM, Brenner S (2004) WebLogo: a sequence logo generator. *Genome Res* 14:1188–1190
- Cohen BA, Mitra RD, Hughes JD, Church GM (2000) A computational analysis of whole-genome expression data reveals chromosomal domains of gene expression. *Nat Genet* 26:183–186
- Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J* 33:751–763
- Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 95:14863–14868
- Feng Q et al (2002) Sequence and analysis of rice chromosome (4). *Nature* 420:316–320
- Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc Natl Acad Sci USA* 99:15898–15903
- Goff SA et al (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. japonica). *Science* 296:92–100
- Gupta S, Chattopadhyay MK, Chatterjee P, Ghosh B, SenGupta DN (1998) Expression of abscisic acid-responsive element-binding protein in salt-tolerant indica rice (*Oryza sativa* L. cv. Pokkali). *Plant Mol Biol* 37:629–637
- Haake V, Cook D, Riechmann JL, Pineda O, Thomashow MF, Zhang JZ (2002) Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. *Plant Physiol* 130:639–648
- Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-regulatory regulatory DNA elements (PLACE) database. *Nucleic Acids Res* 27:297–300
- Hurst LD, Pal C, Lercher MJ (2004) The dynamics of eukaryotic gene order. *Nat Rev Genet* 5:299–310
- Jiao Y, Ma L, Strickland E, Deng XW (2005) Conservation and divergence of light-regulated genome expression patterns during seedling development in rice and *Arabidopsis*. *Plant Cell* 17:3239–3256
- Johansson I, Karlsson M, Johanson U, Larsson C, Kjellbom P (2000) The role of aquaporins in cellular and whole plant water balance. *Biochim Biophys Acta* 1465:324–342
- Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat Biotechnol* 17:287–291
- Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Bohnert HJ (2001) Gene expression profiles during the initial phase of salt stress in rice. *Plant Cell* 13:889–905
- Kim JK (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiol* 138:341–351
- Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiol* 130:2129–2141
- Lercher MJ, Urrutia AO, Hurst LD (2002) Clustering of housekeeping genes provides a unified model of gene order in the human genome. *Nat Genet* 31:180–183
- Ma L, Sun N, Liu X, Jiao Y, Zhao H, Deng XW (2005a) Organ-specific expression of *Arabidopsis* genome during development. *Plant Physiol* 138:80–91
- Ma LG et al (2005b) A microarray analysis of the rice transcriptome and its comparison to *Arabidopsis*. *Genome Res* 15:1274–1283
- Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K (2004) Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems. *Plant J* 38:982–993
- Mueller LA, Zhang P, Rhee SY (2003) AraCyc: a biochemical pathway database for *Arabidopsis*. *Plant Physiol* 132:453–460
- Nakabayashi K, Okamoto M, Koshiha T, Kamiya Y, Nambara E (2005) Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. *Plant J* 41:697–709
- Oono Y et al (2003) Monitoring expression profiles of *Arabidopsis* gene expression during rehydration process after dehydration using ca. 7000 full-length cDNA microarray. *Plant J* 34:868–887
- Ozturk ZN, Talame V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Mol Biol* 48:551–573
- Rabbani MA et al (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant Physiol* 133:1755–1767
- Ramachandra RA, Chaitanya KV, Vivekanandan M (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *J Plant Physiol* 161:1189–1202
- RCSC (The rice chromosome 10 sequencing consortium) (2003) In-depth view of structure, activity, and evolution of rice chromosome (10). *Science* 300:1566–1569
- Reiner A, Yekutieli D, Benjamini Y (2003) Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* 19:368–375
- Rizhsky L, Liang H, Mittler R (2002) The combined effect of drought stress and heat shock on gene expression in tobacco. *Plant Physiol* 130:1143–1151
- Sasaki T et al (2002) The genome sequence and structure of rice chromosome 1. *Nature* 420:312–316
- Satoh R, Nakashima K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2002) ACTCAT, a novel cis-regulatory element for proline- and hypoosmolarity-responsive expression of the ProDH gene encoding proline dehydrogenase in *Arabidopsis*. *Plant Physiol* 130:709–719
- Seki M et al (2002a) Monitoring the expression pattern of around 7,000 *Arabidopsis* genes under ABA treatments using a full-length cDNA microarray. *Funct Integr Genomics* 2:282–291

- Seki M, Kamei A, Yamaguchi-Shinozaki K, Shinozaki K (2003) Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Curr Opin Biotechnol* 14:194–199
- Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K (2001) Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* 13:61–72
- Seki M et al (2002b) Monitoring the expression profiles of 7,000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J* 31:279–292
- Seki M, Satou M, Sakurai T, Akiyama K, Iida K, Ishida J, Nakajima M, Enju A (2004) RIKEN *Arabidopsis* full-length (RAFL) cDNA and its applications for expression profiling under abiotic stress conditions. *J Exp Bot* 55:213–223
- Shen Q, Ho TH (1995) Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel cis-regulatory element. *Plant Cell* 7:295–307
- Shi H, Lee BH, Wu SJ, Zhu JK (2003) Overexpression of a plasma membrane Na⁺/H⁺ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. *Nat Biotechnol* 21:81–85
- Shinozaki K, Yamaguchi-Shinozaki K (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr Opin Plant Biol* 3:217–223
- Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr Opin Plant Biol* 6:410–417
- Smyth G (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3: Article 3
- Smyth GK (2005) Limma: linear models for microarray data. In: *Bioinformatics and computational biology solutions using R and bioconductor*. Springer, New York, pp. 397–420
- Spellman PT, Rubin GM (2002) Evidence for large domains of similarly expressed genes in the *Drosophila* genome. *J Biol* 1:5
- Walia H et al (2005) Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. *Plant Physiol* 139:822–835
- Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. *Plant Cell* 14i:S165–S183
- Xu D, Duan X, Wang B, Hong B, Ho T, Wu R (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol* 110:249–257
- Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-regulatory regulatory elements in osmotic- and cold-stress-responsive promoters. *Trends Plant Sci* 10:88–94
- Yazaki J et al (2003) Genomics approach to abscisic acid- and gibberellin-responsive genes in rice. *DNA Res* 10:249–261
- Yazaki J et al (2004) Transcriptional profiling of genes responsive to abscisic acid and gibberellin in rice: phenotyping and comparative analysis between rice and *Arabidopsis*. *Physiol Genom* 17:87–100
- Yu J et al (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). *Science* 296:79–92
- Yu J et al (2005) The Genomes of *Oryza sativa*: a history of duplications. *PLOS Biol* 3:e38
- Zhang JZ, Creelman RA, Zhu JK (2004) From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiol* 135:615–621
- Zhao W et al (2004) BGI-RIS: an integrated information resource and comparative analysis workbench for rice genomics. *Nucleic Acids Res* 32:D377–D382
- Zhu JK (2001) Cell signaling under salt, water and cold stresses. *Curr Opin Plant Biol* 4:401–406
- Zhu JK (2002) Salt and drought stress signal transduction in plants. *Annu Rev Plant Biol* 53:247–273