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Comparative transcriptome analysis of *AP2/EREBP* gene family under normal and hormone treatments, and under two drought stresses in NILs setup by Aday Selection and IR64

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Abstract The *AP2/EREBP* genes play various roles in developmental processes and in stress-related responses in plants. Genome-wide microarrays based on the gene expression profiles of the *AP2/EREBP* family were analyzed under conditions of normal growth and drought stress. The preferential expression of fifteen genes was observed in specific tissues, suggesting that these genes may play important roles in vegetative and reproductive stages of growth. A large number of redundant genes were differentially expressed following phytohormone treatments (NAA, GA3, KT, SA, JA, and ABA). To investigate the gene expression responses in the root, leaf, and panicle of three rice genotypes, two drought stress conditions were applied using the fraction of transpirable soil water (FTSW) under severe (0.2 FTSW), mild (0.5 FTSW), and control (1.0 FTSW) conditions. Following treatment, transcriptomic analysis using a 44-K oligoarray from Agilent was performed on all the tissue samples. We identified common and specific genes in all tissues from two near-isogenic lines, IR77298-14-1-2-B-10 (drought tolerant) and IR77298-14-1-2-B-13 (drought susceptible), under drought stress conditions. The majority of the genes that were activated in the IR77298-14-1-2-B-10 line were members of the *AP2/EREBP* gene family. Non-redundant genes (sixteen) were found in the drought-tolerant line, and four genes were selected as candidate novel reference genes because of their higher expression levels in IR77298-14-1-2-B-10. Most of the genes in the AP2, B3, and B5 subgroups were involved in the panicle under severe stress conditions, but genes from the B1 and B2 subgroups were down-regulated in the root. Of the four subfamilies, RAV exhibited the highest number of up-regulated genes (80%) in the panicle under severe stress conditions in the drought-tolerant

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line compared to Minghui 63 under normal conditions, and the gene structures of the RAV subfamily may be involved in the response to drought stress in the flowering stage. These results provide a useful reference for the cloning of candidate genes from the specific subgroup for further functional analysis.

Abbreviations

NILs	**N**ear-isogenic lines
GA3	Gibberellic acid
KT	Kinetin
NAA	Naphthalene acetic acid
SA	Salicylic acid
JA	Jasmonic acid
ABA	Abscisic acid
RT-PCR	Reverse transcription polymerase chain reaction
QTL	Quantitative trait loci

Introduction

Under different adverse environmental conditions, genes show specific expression patterns in accordance with their biological and physiological functions. Hence, an important goal in developmental biology is to detect the expression of unique genes or groups of genes and to determine their expression patterns to identify the exact functional network of that gene(s) and whether it is linked to specific or multiple tissues. Transcription factors (TFs) are important for maintaining the expression of genes that encode functional proteins in the genome. In plants, the *AP2/EREBP* gene family encodes a large number of TF genes. TFs that are encoded by *AP2/EREBP* genes contain the highly conserved AP2/ERF DNA-binding domain (Riechmann and Meyerowitz 1998). Jofuku et al. (1994) first described this conserved DNA-binding domain in the homeotic gene APETALA2 (AP2) and in ethylene-responsive element binding proteins (EREBPs) in tobacco (Ohme-Takagi and Shinshi 1995). Based on the number of AP2/ERF domains and the gene structure, the *AP2/EREBP* gene family has been divided into four subfamilies, AP2, RAV, dehydration-responsive element binding protein (DREB), and ERF (ethylene-responsive element binding factor) (Sakuma et al. 2002; Sharoni et al. 2011). The RAV, DREB, and ERF subfamilies are of particular interest due to their involvement in plant responses to stress. The C-terminal domain of RAV subfamily members is similar to that of the ABI3 and VP1 transcription factors, which have two core motifs CAACA and CACCTG (Kagaya et al. 1999). TFs

encoded by genes in the DREB subfamily bind to both GCC and the DRE (dehydration-responsive element) *cis*-elements (Park et al. 2001; Sakuma et al. 2002). The ERF subfamily of genes encode a large number of ERFs (Fujimoto et al. 2000) that participate in plant responses to biotic stresses, such as pathogens, by recognizing the *cis*-acting element AGCCGCC, known as the GCC box (Hao et al. 1998). The ERF- and DREB-subfamily transcription factors have been discovered in several plant species including rice (Quan et al. 2010), *Arabidopsis* (Sakuma et al. 2006) and tobacco (Agarwal et al. 2010). Furthermore, the functions of the ERF and DREB transcription factors in the plant response to biotic and abiotic stress have been extensively documented (Agarwal et al. 2006, 2010). A combination of genetic engineering and molecular approaches have been used to characterize a series of regulatory genes of the *AP2/EREBP* gene family that are involved in various pathways, including genes related to drought and high salinity (Dubouzet et al. 2003), diseases (Gutterson and Reuber, 2004; Agarwal et al. 2006), cold (Qin et al. 2007a), and the control of flowering (Elliott et al. 1996). The overexpression of DREB1A (CBF3) or DREB1B (CBF1) in transgenic *Arabidopsis* induces the strong expression of target stress-inducible genes and results in increased tolerance to drought and high salt (Quan et al. 2010; Zhu et al. 2010). Similarly, the overexpression of a number of ERF genes enhances the resistance to biotic and abiotic stresses in tobacco (Park et al. 2001). In addition, TERF1 and TSRF1 exhibit drought and salinity tolerance in rice (Gao et al. 2008; Quan et al. 2010). Members of the RAV subfamily are involved in the ethylene response (Alonso et al. 2003), the brassinosteroid response (Hu et al. 2004), and biotic and abiotic stress responses in pepper (Sohn et al. 2006). A microarray analysis in *Arabidopsis* has shown that there are different pathways that independently respond to abiotic stress, and one such important pathway involves the DREB regulon (Fowler and Thomashow 2002).

Drought is a serious abiotic stress that imposes a constraint on rice production and severely reduces the grain yield potential of rice (Serraj et al. 2009). Among cereal crops, rice is very sensitive to soil water deficits and evaporative demands, and the greatest sensitivity is found in the lowland-adapted genotypes (Parent et al. 2010). Different mechanisms of the drought response can be measured at many different levels, from the whole plant down to the molecular level. In plants, knowledge of the molecular aspects of drought control or drought tolerance is limited. Therefore, a promising approach is to examine near-isogenic lines (NILs) that have a common genetic background but contrasting levels of tolerance to water deficits in a long-term drought stress situation that is similar to field conditions, using a dry-down method. Isogenic lines and NILs are invaluable tools for testing hypotheses in

physiological and genetic studies because there is no interference from variations in other traits (Lafitte et al. 2006). Therefore, the use of NILs reduces the noise of gene expression data by comparing it with the IR64 recurrent parent. Through selection in the drought-breeding program of IRRI (International Rice Research Institute), a series of advanced backcrossed rice plant lines was developed by backcrossing Aday Selection (Aday Sel), a drought-tolerant traditional variety, to IR64 (Khush et al. 2004). IR64 is resistant to blast, bacterial leaf blight, and brown planthopper, but it is susceptible to tungro (Khush et al. 2004) and drought stress (Guan et al. 2010). IR64 is the most widely grown high-yielding rice variety in the tropics and has important agronomic traits. Using the IR64 as a recurrent parent, the following pair of NILs with contrasting drought tolerances (severe and mild) was selected (Venuprasad et al. 2007) from the IR77298-14-1-2-B family: IR77298-14-1-2-B-10 (highly drought tolerant) and IR77298-14-1-2-B-13 (susceptible to drought). These advanced backcrossed lines are considered pre-NILs because they are sister lines that were derived from a single family that was segregated for drought-tolerant phenotypes. Venuprasad et al. (2011) reported that the two NILs of the IR77298-14-1-2-B family are 96.5% genetically similar using 491 SSR markers. The genome-wide identification of genes that are regulated by drought treatments has manifold significance (Degenkolbe et al. 2009). The identification of these genes enables a more detailed understanding of the transcriptional response to stress and provides a clue for further elucidating the role of single genes in the stress response, which will have a significant impact on crop engineering. The rice genome is thought to contain 163 *OsAP2/EREBP* genes (Sharoni et al. 2011); however, only a few of these genes have been characterized in this species. The characterization of genes from the *OsAP2/EREBP* family in rice facilitate the understanding of the molecular mechanisms of resistance to stress and thus, will aid in the development of drought-resistant rice varieties using transgenic technology. In this study, we used Affymetrix microarray studies to systematically characterize the expression of the entire *OsAP2/EREBP* gene family in the rice Minghui 63 genotype to gain a better understanding of the role each family/subfamily member or subgroup plays in several developmental stages under normal conditions and following phytohormone treatments. Furthermore, we used the Agilent 44 K oligoarray system to profile the transcriptomes of root, leaf, and panicle tissues from three rice genotypes (IR77298-14-1-2-B-10, IR77298-14-1-2-B-13, and IR64) grown under different drought stress conditions to identify the putative drought responsive genes in tolerant NIL by examining their different gene expression patterns and comparing them with the control. Initially, global gene expression analyses in root tissue from these NILs grown under drought stress

conditions were performed, and the gene expression data were analyzed using gene ontology profiling (Moumeni et al. 2011, personal communication). Although there were many more genes in this array, this study focuses only on *OsAP2/EREBP* gene family members to select the best candidate genes for further functional analysis. A number of specific subfamily genes or subgroup family genes were differentially expressed, and the data revealed novel information about their roles in the plant response to different stress conditions. The conserved *cis*-elements in the 2-kb upstream sequence of the promoter region of differentially expressed genes (DEGs) under different stress conditions were revealed. Introgressed regions were examined and Gene Ontology (GO) enrichment tests were performed. We used RT-PCR to confirm the expression of selected *OsAP2/EREBP* genes under stressed and non-stressed conditions. To our knowledge, this is the first report that focuses on *OsAP2/EREBP* genes to identify family-level expression patterns, and these results provide a solid basis for future functional genomic research of the *OsAP2/EREBP* gene family.

Materials and methods

Expression profile analysis (Minghui 63)

The expression profile data for *OsAP2/EREBP* genes in Minghui 63 and in different hormone-treated seedlings were extracted from the NCBI-GEO database (GSE 19024, GSE7567, GSE32634, and GSM26043; http://www.ncbi.nlm.nih.gov/). This database contains Affymetrix rice microarray data from 26 tissues that cover the entire life cycle of rice. As shown in Table 1, vegetative and reproductive tissues at different developmental stages covering the whole rice plant body were used for the *OsAP2/EREBP* gene expression profile analyses used in this study. After normalization, the average signal value of two biological replicates of each sample was used for analysis. To identify preferentially expressed genes, Student's t test was performed. A gene in a given tissue was defined as being differentially expressed only if the expression value of the gene in this tissue was more than twofold ($P \leq 0.05$) greater than that in the control (CK). In the phytohormone-treated samples, genes that were up- or down-regulated more than 1.5-fold ($P \leq 0.05$) compared with the control or an untreated sample were considered differentially expressed. Trefoil-stage seedlings were exposed to 100 mM concentrations of gibberellic acid (GA3), kinetin (KT), and naphthalene acetic acid (NAA) for 15, 30, and 60 min, and the samples were then mixed (Nuruzzaman et al. 2008). The different hormone treatments were performed in seedlings growing under normal agricultural conditions of temperature, light,

Table 1 Sample vegetative and reproductive developmental stage analyses in Minghui 63

Sample	Developmental tissues
CK	Seed: germination (72 h after imbibition)
1	Calli 1: 15 days after subculture
2	Calli 2: screening stage
3	Calli 3: 5 days after regeneration
4	Seedling 1: 3 days after sowing
5	Seedling 2: root and leaf at the three-leaf stage
6	Shoot: seedling with 2 tillers
7	Root : seedling with 2 tillers
8	Stem 1: 5 days before heading
9	Stem 2: heading stage
10	Flag leaf 1: 5 days before heading
11	Flag leaf 2: 14 days after heading
12	Leaf 1: young panicle at stage 3
13	Leaf 2: 4–5 cm young panicle
14	Sheath 1: young panicle at stage 3
15	Sheath 2: 4–5 cm young panicle
16	Panicle 1: young panicle at stage 3
17	Panicle 2: young panicle at stage 4
18	Panicle 3: young panicle at stage 5
19	Panicle 4: 4–5 cm young panicle
20	Panicle 5: heading stage
21	Stamen: just before heading
22	Spikelet: 3 days after pollination
23	Endosperm 1: 7 days after pollination
24	Endosperm 2: 14 days after pollination
25	Endosperm 3: 21 days after pollination

and humidity. Benzothiadiazole (BTH) is a functional analog of salicylic acid (SA) and protects plants from disease by activating the SA signaling pathway. The single-color method was used to compare four biological replicates of BTH-treated leaves with mock-treated leaves. Leaf disks (6 mm diameter) from seedlings were floated on jasmonic acid (JA, 500 μM) and incubated under light at 25°C. The samples were harvested at 6, 12, 24, and 48 h after the treatment. The *japonica* rice variety Nipponbare were grown for 3 weeks at 28°C in the dark and were treated with abscisic acid (ABA, 100 μM) for 5 h. Plants treated with H_2O for 5 h were used as controls. Whole plants were used for RNA extractions (Yazaki et al. 2003). The log_2 ratio values of the means were normalized, and up- or down-regulated genes with values >0.585 or <−0.585 were selected for the JA and ABA treatments.

Plant growth and drought-treatment conditions for rice

The two NILs and the IR64 plants used for this study were chosen because of their different rice yields under drought stress conditions (DSCs). The two NILs plants were derived from the IR77298-14-1-2 family and developed at IRRI. Under stress, IR77298-14-1-2-B-10 is a high-yielding (highly drought-tolerant) plant, whereas IR77298-14-1-2-B-13 is a low-yielding (drought-susceptible) plant. Both IR77298-14-1-2-B-10 and IR77298-14-1-2-B-13 NILs plants exhibit similar yield potentials under normal growth conditions (Venuprasad et al. 2011). Plants were grown in a PVC pipe filled with 10 kg of a soil and sand mixture (2:1) in which all the soil nutrients were controlled. The test was initially conducted in a green house, and the plants were moved outside to natural conditions before imposing drought stress. Before planting, the saturated soil in the pots was covered with white or transparent plastic covers with an opening in the center to facilitate planting. Feeder pipes were fixed to the pots to facilitate watering. In one pot, five germinated seeds were transplanted, and the plants were subsequently separated to one plant per pot when they reached the three-leaf stage. This test was carried out on eight replicate samples using a randomized complete block design. All of the pots were watered twice daily to keep the soil saturated. The day before beginning the progressive soil drying process, the soil in each pot was saturated with water. Stress was imposed by initiating a soil dry-down protocol starting 35 days after sowing. The dry-down process continued until the pot reached the target fraction of transpirable soil water (FTSW) (Sinclair and Ludlow 1986). The weight of each pot was determined every day during the dry-down process to estimate the transpiration of soil water. The DSCs were as follows: a control (1.0 FTSW) consisting of well-watered plants with soil that was kept saturated throughout the experiment, and two drought stresses, one severe (0.2 FTSW) and one mild (0.5 FTSW). No water was applied to the soil during the dry-down period. The target FTSW was maintained in all of the pots until the plants were harvested. The water-use efficiency in the different rice genotypes was calculated using the lysimeter system.

Rice oligo microarray (44 K) and data analysis

Cyanine-3 (Cy3) and cyanine-5 (Cy5)-labeled target complementary RNA (cRNA) samples were prepared from 850 ng total mRNA using a fluorescent linear amplification kit (Agilent Technologies) in accordance with the manufacturer's instructions. Transcriptome profiles specific to stressed plants were examined by the direct comparison of transcription activities between stressed and non-stressed plants on the same oligoarray. We hybridized 1 μg of fluorescent linear-amplified, Cy3- and Cy5-labeled cRNA (825 ng of each cRNA) to a customized rice oligo microarray. The fragmented cRNAs were added to the hybridization buffer, applied to the microarray, and hybridized for

17 h at 60°C. The slides were washed with SSC (10 min in $6 \times$ SSC and 0.005% Triton X-102 at room temperature, followed by 5 min in $0.1 \times$ SSC and 0.005% Triton X-102 at 4°C). The slides were dried and scanned using an Agilent Technologies G2565BA microarray scanner system. The scanned microarray images were analyzed using Feature Extraction 6.1.1 software (Agilent Technologies), and the dye-normalized, background-subtracted intensity and ratio data were exported to a text file. This software flags corrupted spots and detects a lack of differences between sample spots and the background. The slide images were scanned using a DNA microarray scanner (G2505B, Agilent Technologies).

The image files of the slides were processed using Feature Extraction version 9.5 (Agilent Technologies). The Cy3 and Cy5 signal intensities were normalized using rank-consistency filtering and the LOWESS method. The signal intensities of all the samples were transformed into $\log_2$-based numbers and normalized according to the quantile method for standardization among the array data using Expander version 5.0 (Shamir et al. 2005). A significantly DEG was defined as an expressed gene with a $\log_2$-based ratio (stressed sample and control sample) >1 or <−1, and significance in changes in gene expression between two plants was defined as $P \leq 0.05$ using a paired t test (permutations, all possible combinations, FDR correction, adjusted Bonferroni method). Data processing was performed using MeV version 4.5 (Saeed et al. 2003). The Michigan State University (Osa1, Ouyang et al. 2007) database contained at least 163 *AP2/EREBP* genes in the rice genome and contained corresponding locus IDs. We identified 140 *OsAP2/EREBP* genes from our 44 K microarray data collected under DSCs. The probe arrangement of the array data (platform number GPL7252) is available at NCBI-GEO (series number GSE30464, Barrett et al. 2007).

Transcript-level analysis

Total RNA was extracted from the root, leaf, and panicle of all of the rice genotypes using an RNeasy Maxi Kit (Qiagen) in accordance with the manufacturer's instructions. The concentration and quality of the RNA samples were determined using a spectrophotometer (Nanodrop ND-1000, Nanodrop Technologies) and a model 2100 bioanalyzer (Agilent Technologies). The RNA samples were pretreated with RNase-free DNase I (Invitrogen) to eliminate any contaminating genomic DNA. The first-strand cDNA was synthesized from 1 μg of total RNA in a 20-μl reaction volume using Superscript II Reverse Transcriptase (Invitrogen), and 2 μl of the reaction mixture was subsequently used for RT-PCR in a 50-μl reaction volume. The RT-PCR was performed using SuperScriptII Reverse Transcriptase in accordance with the manufacturer's

instructions to synthesize first-strand cDNA from the DNase I-treated total RNA. The same RNA samples were used for hybridization and RT-PCR experiments. Approximately 1/20 of the first-strand cDNA that was generated from 1 μg total RNA was used as a template for PCR (reaction volume 50 μl) using rTaq DNA polymerase (Takara). The RT-PCR procedures consisted of 25–35 cycles, depending on the linear range of the PCR amplification for each gene. Each PCR was performed (in triplicate) in an ABI 9700 thermocycler (Applied Biosystems). The program entailed incubation at 94°C for 45 s, at 55°C for 50 s, and at 72°C for 1 min. The rice *actin* gene (LOC_Os05g36290) was used as an internal control (Nuruzzaman et al. 2010; Sharoni et al. 2011), and the primers are listed in Supplementary Table 1. For convenience, all the "LOC_" prefixes in the Osa1 (Ouyang et al. 2007) locus ID are omitted in the remainder of the manuscript.

Results

Expression patterns of *OsAP2/EREBP* genes under normal growth conditions using Affymetrix arrays

The rice tissues from the vegetative and reproductive stages that were selected for microarray analysis cover the complete life cycle of the rice plant. Detailed information on the selected tissues is presented in Table 1. The expression pattern of the microarray data was confirmed to reveal the expression profiles of the various genes (Nuruzzaman et al. 2008; Ye et al. 2009). Probes for 116 of the 163 *OsAP2/EREBP* genes that were identified in the microarray and demonstrated differential expression during various stages of development when compared with their expression in the seed are summarized in Supplementary Table 2, and the P values and the fold-change values are included in this table. The *AP2/EREBP* genes were classified into four subfamilies and two subfamilies of those (ERF and DREB), and these were further divided into different subgroups, e.g., DREB-A1 to subgroup DREB-A6 and ERF-B1 to subgroup ERF-B7 (Sharoni et al. 2011). A number of up-regulated genes were found in the calli, shoot, leaf-2, and spikelet stages. In contrast, a higher number of down-regulated genes were observed in Minghui 63 in most of the stages of development (Fig. 1a). During the vegetative stages, 26 *OsAP2/EREBP* genes were up-regulated and 25 genes were down-regulated by two-fold or more in Minghui 63. These data show that the differentially regulated *OsAP2/EREBP* genes did not exhibit high variations, implying that their expression may be somewhat conservative during vegetative development. In contrast, of the 52 genes differentially regulated in reproductive development, 22 *OsAP2/EREBP* genes were up-regulated more than

Fig. 1 **a** Number of up- or down-regulated genes in different tissues under normal conditions in Minghui 63 are presented. **b** Tissue-specific up-regulated genes in Minghui 63: calli 2, *Os02g32040*; calli 3, *Os06g44750*; root, *Os04g44670*; stem 1, *Os02g51300* and *Os09g28440*; flag leaf 1, *Os08g45110*; leaf 1, *Os09g13940*; leaf 2, *Os08g42550*; panicle 2, *Os03g12950*, *Os05g25260*, and *Os08g36920*; panicle 5, *Os02g45450*; stamen, *Os09g11460*, and endosperm 1, *Os05g03040* and *Os08g41030*. The number of genes is shown on the *Y*-axis, and the different developmental stages are indicated on the *X*-axis. Tissue-specific up-regulated genes are shown in Supplemental Table 2
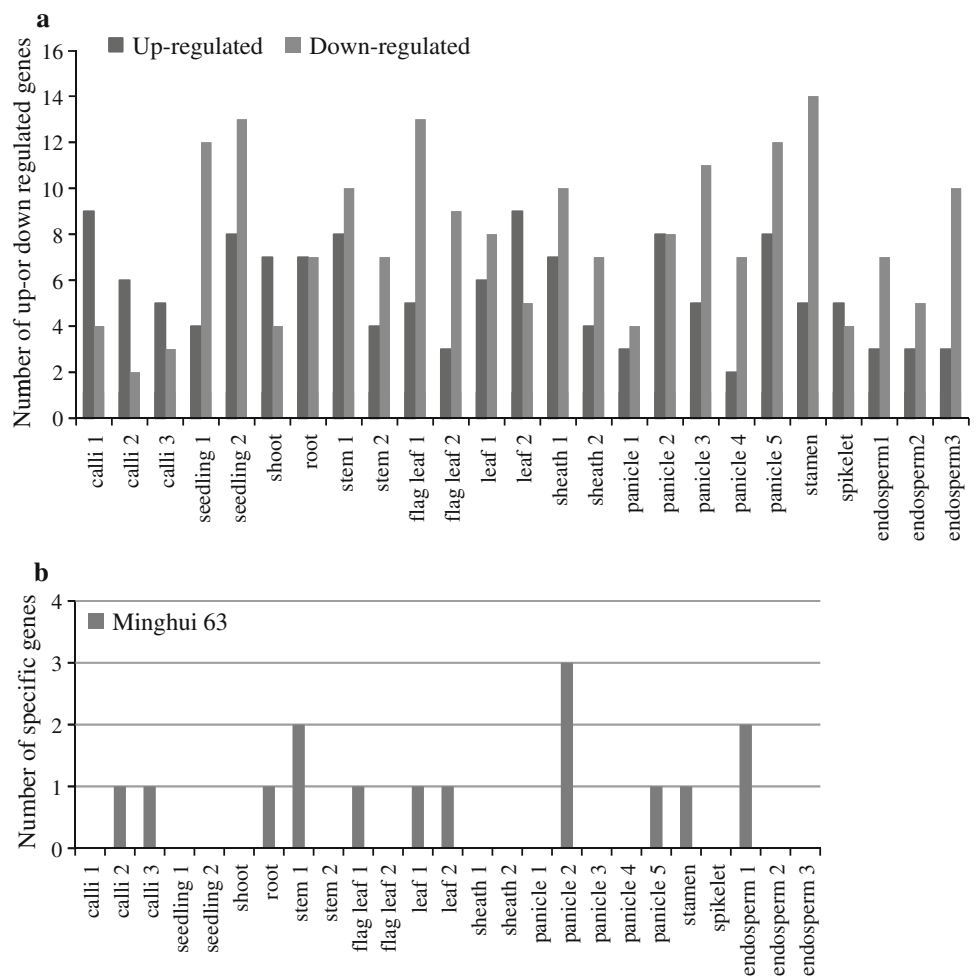
twofold and 30 were down-regulated. This observation suggests that the expression pattern of *OsAP2/EREBP* genes in reproductive development is complex and implies the activation of different genes at different developmental stages. A biphasic expression pattern was also observed for *OsAP2/EREBP* genes during reproductive development, nine genes (e.g., *Os06g11860*, *Os09g11480*, and *Os06g07030*) were up-regulated during several stages but were down-regulated during the other stages. Specific genes were up-regulated or down-regulated during vegetative and reproductive development, probably because of the prevalence of similar processes in these stages. *OsAP2/EREBP* genes, nine in total (e.g., *Os02g43940*, *Os03g09170*, and *Os08g31580*, subfamily DREB), exhibited up-regulated expression during both vegetative and reproductive development, whereas five genes were down-regulated (e.g., *Os02g34260*, *Os01g12440*, and *Os04g48350*, subfamily ERF). Overall, the DREB subfamily exhibited the highest number of up-regulated genes, followed by ERF, AP2, and RAV, whereas the AP2 subfamily demonstrated a higher number of down-regulated genes, followed by RAV, ERF, and DREB, respectively (Supplementary Table 2). Moreover, we found

15 up-regulated genes in specific tissues of plants grown under normal growth conditions (Fig. 1b; Supplementary Table 2). For example, three genes (*Os03g12950*, *Os05g25260*, and *Os08g36920*) were specifically expressed in the panicle-2 stage under non-stressed conditions (Fig. 1b). The number of differentially expressed genes under normal conditions was compared with the number of differentially expressed genes under different drought stresses, as described later. Taken together, these results provide a global overview of the *OsAP2/EREBP* transcriptome in relation to rice tissues and the developmental stages of the plant life cycle.

Responses of *OsAP2/EREBP* genes to GA3, KT, NAA, SA, JA, and ABA treatments

Phytohormones play a critical role in plant growth and development. To investigate the response of *OsAP2/EREBP* genes to phytohormone treatment, microarray analysis was performed. To understand the expression patterns of the *OsAP2/EREBP* gene family, data were extracted from the NCBI database as described previously. The

Minghui 63 rice cultivar was used for microarray expression analysis following three different hormonal treatments of plants at the seedling stage. We identified a total of 21 redundant *OsAP2/EREBP* genes out of 116 from Gene Chip that were differentially expressed following treatments with one or more of the phytohormone GA3, KT, and NAA in the Minghui 63 genotypes compared with the control (Fig. 2). The fold-change values of differentially expressed genes in the seedling stage with respect to the control (CK, untreated sample) are shown in Supplementary Table 3. Of these 116 genes, 18 genes were up-regulated, whereas three genes were down-regulated following different hormone treatments. Interestingly, two genes (*Os02g51300* and *Os05g03040*, subfamily AP2) were up-regulated following treatment with all three phytohormones. For instance, the *Os03g60430* gene of the AP2 subfamily was up-regulated specifically by NAA treatment of Minghui 63, and the *Os06g40150* gene of the B3 subgroup of the ERF subfamily was up-regulated by GA3 and NAA treatments in seedlings (Supplementary Table 3). Furthermore, 12 DEGs were found between the SA and ABA treatments, whereas 41, 54, 63, and 57 redundant DEGs were observed following JA treatment at four different time points (Supplementary Table 3). We observed 24 up- or down-regulated genes that were common to all the time points in JA-treated plants. The gene expression profiles of up- or down-regulated genes following treatment of Minghui 63 with different phytohormones were compared in the root, leaf, and panicle of the NILs under severe and mild drought stress conditions.

Gene expression patterns in different rice genotypes under different stress conditions

IR64 and two NILs, IR77298-14-1-2-B-10 and IR77298-14-1-2-B-13, were used in this study. In these NILs, gene identification and the determination of gene expression patterns are important to understand the molecular basis of plant responses and to improve drought stress tolerance in the progeny from a common genetic background using transgenic techniques. The three water regimes used were severe stress (0.2 FTSW), mild stress (0.5 FTSW), and control (1.0 FTSW), and gene expression in the root, leaf, and panicle tissues of the two NILs and IR64 at the reproductive stage were examined. Therefore, the goals of this study are to identify DEGs in IR77298-14-1-2-B-10 versus IR77298-14-1-2-B-13 and IR64, to determine the number of DEGs that are specifically induced in each NIL, to determine the number of DEGs that are common to both NILs under both DSCs in multiple tissues, and to identify putative drought responsive genes in the drought-tolerant line for further functional analysis. Altogether, the results of the expression patterns and analyses indicate
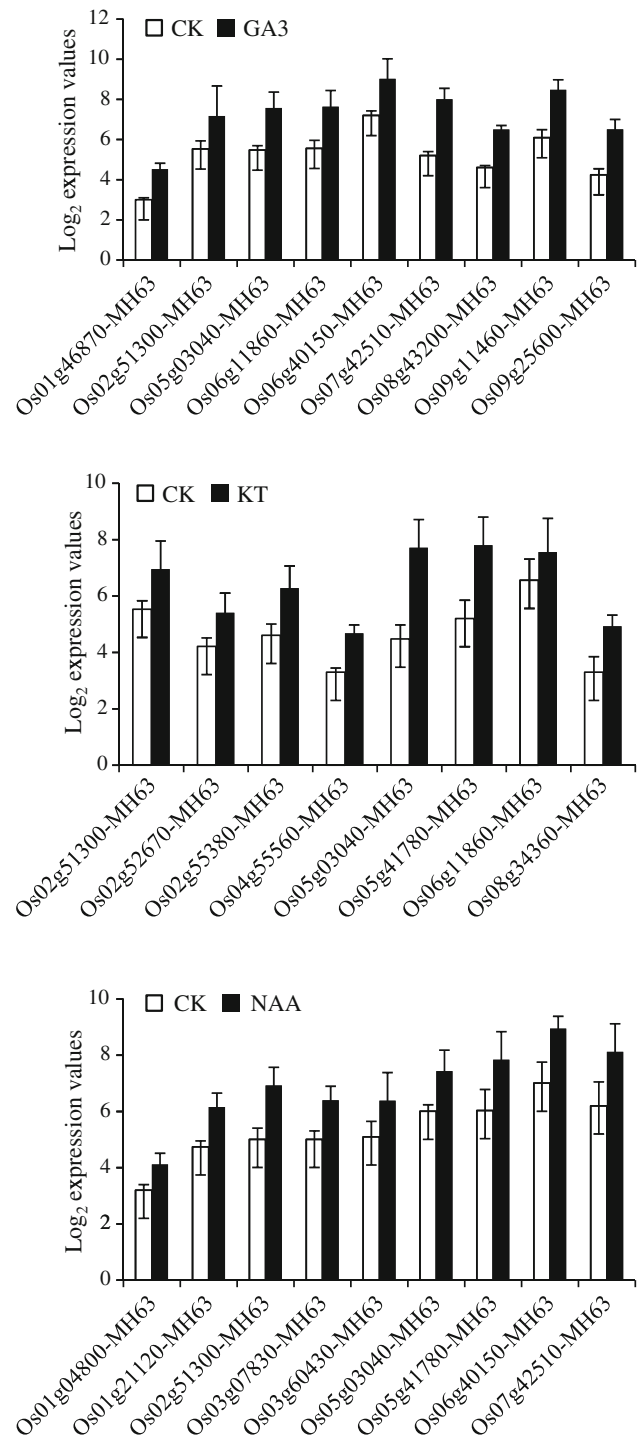

Fig. 2 Expression profiles of differentially expressed *OsAP2/EREBP* genes in 7-day-old seedlings subjected to three phytohormones (*GA3* gibberellic acid, *KT* kinetin, and *NAA* naphthalene acetic acid). *X*-axis represents differentially expressed genes. *Y*-axis represents $\log_2$ expression values. Each treatment is indicated at the top of each diagram. *CK* control

the involvement of this large gene family in a number of signaling pathways that are triggered by the drought response.

The physical appearance of the plants, such as plant height, transpiration, flowering, the total biomass (root, shoot, and panicle) at harvest, the spikelet fertility score and the grain yield were recorded for the IR77298-14-1-2-B family (Moumeni et al. 2011; Venuprasad et al. 2011). The drought-tolerant NIL in the IR77298-14-1-2-B family exhibited a significantly higher grain yield compared with the drought-susceptible NIL under drought-stress but not under non-stress conditions (Venuprasad et al. 2011). We found 162 out of a possible 163 *OsAP2/EREBP* genes in our 44 K array data. Among these genes, 140 were differentially expressed under both DSCs in different experimental tissues. A gene was defined as preferentially responsive if the signal values of the gene in this tissue increased more than twofold and had a P value less than 0.05 compared with the control (Supplementary Table 4). We compared gene expression profiles of three rice genotypes under two DSCs. Supplementary Table 5 shows the number of DEGs obtained from microarray experiments using tissues from the different rice genotypes under both DSCs. The differentiation of the expression patterns in the three tissues of the rice genotypes indicated that a higher number of DEGs was observed under severe stress compared with mild stress conditions. We observed that the tolerant line IR77298-14-1-2-B-10 exhibited the highest number of up-regulated genes, followed by IR77298-14-1-2-B-13 and IR64, in all tissues under both stresses, with the exception of the root and the leaf under severe and mild stress conditions. In response to severe stress, this tolerant line demonstrated the highest number of up-regulated genes (61) in the panicle. Therefore, despite their common genetic background as backcrossed progeny from Aday Sel x IR64, the two NILs showed distinctive differences in their gene expression patterns in response to drought. We further analyzed the DEGs in two NILs of the IR77298-14-1-2-B family and compared their expression profiles under normal conditions and after the hormone treatment of seedlings, as outlined below.

AP2/EREBP gene expression in the root common to both NILs under severe and mild stress conditions

In this study, gene expression profiles were compared between the drought-tolerant and drought-susceptible lines. It is believed that genotypes responding differentially to drought stress exhibit variations in gene expression and that these differences are strongly related to drought avoidance or to drought response. Spollen et al. (1993) reported that mild osmotic stress rapidly inhibits the growth of the leaves and stems, whereas the roots continue to elongate, and the root architecture is a key trait for dissecting genotypic differences in rice responses to DSCs. Thus, the analysis of the genes observed entirely in two NILs i.e. up-regulated

genes in both NILs and in individual is of interest to identify the most promising candidate genes among three tissues at procreative stage under both DSCs, which will provide a starting point for further elucidating role of single gene in stress response. In the root under severe stress, fourteen genes were shared common in IR77298-14-1-2-B-10 and in IR77298-14-1-2-B-13 (Fig. 3; Supplementary Table 6), while most of them were also up- or down-regulated in different tissues under both DSCs in two NILs and assigned to DREB subfamily (Supplementary Table 4). We considered a gene specifically expressed in one NIL if that gene was not differentially expressed and exhibited no change in expression levels or a low level of expression compared with susceptible NIL under severe or mild stress in a certain tissue. In IR77298-14-1-2-B-10, we identified two specific genes (*Os06g42990* and *Os11g06770*, DREB subfamily) that were expressed in the root under severe stress but were down-regulated or highly sensitive in the root under severe stress in IR77298-14-1-2-B-13. Similarly, in IR77298-14-1-2-B-13, four genes (*Os02g40070*, *Os04g46440*, *Os04g55970*, and *Os06g09390*, ERF and AP2 subfamilies) were specifically induced in the root under severe stress, and all of the genes were down-regulated, their expression levels did not change or these genes were highly sensitive in IR77298-14-1-2-B-10 under conditions of severe stress (Supplementary Table 6). *Os02g40070* was down-regulated in the root under non-stressed conditions. This study assumed that these genes are line and tissue specific under conditions of severe stress. In the root under mild stress conditions, 13 up-regulated genes were common to both NILs (Fig. 3; Supplementary Table 6). Out of these 13 genes, eight genes belong to the DREB subfamily. A total of six genes (*Os02g52670*, *Os03g08500*, *Os09g39850*, *Os02g32140*, *Os03g19900*, and *Os06g05340*) assigned to subfamilies DRED, ERF, and AP2 were specifically up-regulated in IR77298-14-1-2-B-10 under mild stress conditions, but they were down-regulated or exhibited no change in expression in IR77298-14-1-2-B-13 in the root. These genes were not up- or down-regulated following treatment with the different plant hormones, whereas *Os03g19900* of the AP2 subfamily was expressed with a sixfold increase in the drought-tolerant line compared with the drought-susceptible line in the root, and its expression was down-regulated in root tissue under normal growth conditions. In IR77298-14-1-2-B-13, four genes (*Os02g54160*, *Os05g32270*, *Os01g67410*, and *Os04g42570*, DREB and AP2 subfamilies) were up-regulated under mild stress conditions. *Os01g67410* and *Os05g32270* expression was induced in leaf-1 and panicle-1, respectively, under normal conditions, whereas *Os01g67410* was up-regulated and *Os05g32270* was down-regulated at all time points of the JA treatment. Interestingly, two genes of the AP2 subfamily were severely down-regulated in IR77298-14-1-2-B-10
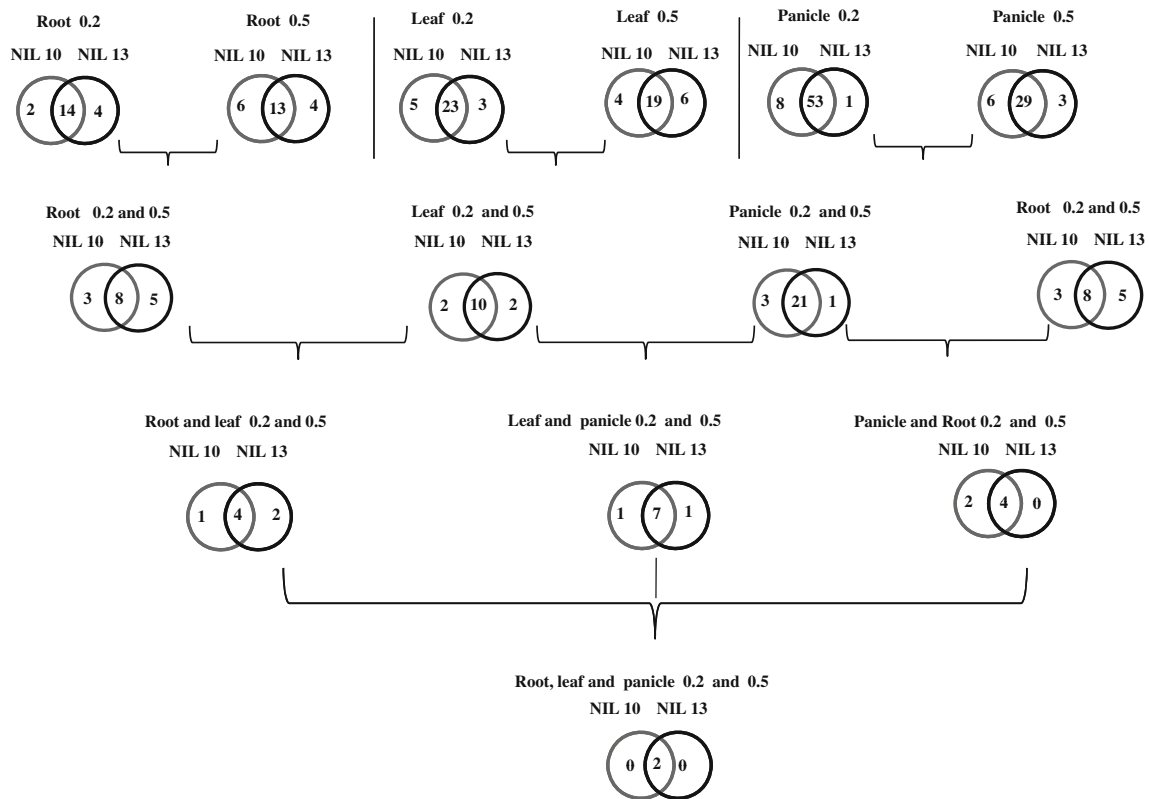
Fig. 3 Up-regulated overlapped and specific genes were detected in NIL10 and NIL13 lines under severe and mild stress conditions in the root, leaf, and panicle. NIL10 = R77298-14-1-2-B-10 and NIL13 = IR77298-14-1-2-B-13, 0.2 = severe stress and 0.5 = mild stress

under mild stress conditions. In the root, under both DSCs, the two NILs shared eight common genes (Fig. 3; Supplementary Table 6). Of these eight genes, *Os02g51300* is from the AP2 subfamily and was up-regulated following treatment of seedlings with GA3, KT, and NAA. Under both DSCs in the root, three genes were up-regulated specifically in IR77298-14-1-2-B-10. Among these three genes, *Os11g06770* (subgroup B4, ERF subfamily) was down-regulated under severe stress conditions, and its expression was induced in the root under mild stress conditions in IR77298-14-1-2-B-13. *Os02g52670* (A5 subgroup, DREB subfamily) and *Os03g19900* (AP2 subfamily) exhibited a low level of expression in the root in IR77298-14-1-2-B-13 under mild stress conditions, whereas *Os03g19900* was down-regulated under non-stress conditions in the same tissue. Similarly, five genes were commonly found in IR77298-14-1-2-B-13 under both stress conditions, although the majority of these genes exhibited high expression levels under either DSC in the drought-tolerant line compared with the drought-susceptible line. We speculate that expression responses of these genes are root tissue- and NILs-specific under both DSCs.

AP2/EREBP gene expression in the leaf common
to both NILs under severe and mild stress conditions

We found that 23 up-regulated genes in the leaf were common to both lines under severe stress conditions (Fig. 3; Supplementary Table 6). Of these 23 genes, 14 were not differentially expressed in the root under both DSCs (Supplementary Table 4). Under severe stress conditions, five genes (*Os08g31580*, *Os10g38000*, *Os03g08500*, *Os01g10370*, and *Os07g22770*, subfamilies DREB and ERF) were up-regulated in the leaf, specifically in IR77298-14-1-2-B-10. With the exception of *Os03g08500*, these genes were up-regulated at least once in the JA treatment time point. All of these genes were expressed in the leaf at a low level or was highly sensitive in IR77298-14-1-2-B-13 under severe stress conditions (Supplementary Table 6). In IR77298-14-1-2-B-13, three genes (*Os01g64790*, *Os04g57340*, and *Os11g03540*; AP2, DREB, and ERF subfamilies) were highly activated in the leaf under severe stress conditions and down-regulated or expressed at a low level in that tissue in IR77298-14-1-2-B-10 under severe stress conditions. These genes are specific to NILs and to leaf tissue under severe stress. Of the genes expressed at

high levels in the leaf under mild stress, 19 were common to both NILs (Fig. 3; Supplementary Table 6). In IR77298-14-1-2-B-10, four genes (*Os02g43940*, *Os09g11460*, *Os04g44670*, and *Os02g51300*, subfamilies AP2, DREB, and ERF) were highly responsive, specifically in IR77298-14-1-2-B-10, under mild conditions in the leaf and not differentially expressed or highly responsive in IR77298-14-1-2-B-13. Of these four genes, two (*Os09g11460* and *Os02g43940*) were up-regulated by JA treatment at the seedling stage (Supplementary Table 3), whereas *Os04g44670* was highly induced specifically in the root under normal conditions and was down- and up-regulated in seedlings following SA and JA treatments, respectively (Fig. 1b; Supplementary Tables 2 and 3). Similarly, six genes (*Os08g45110*, *Os06g07030*, *Os04g34970*, *Os06g09390*, *Os06g10780*, and *Os02g54050*, ERF and DREB subfamilies) were highly activated in the leaf, specifically in IR77298-14-1-2-B-13 under mild stress condition, but were not differentially expressed and did not exhibit lower intensities in IR77298-14-1-2-B-10. *Os04g34970* was activated in root tissue under normal conditions. During both stress conditions, ten genes were up-regulated in the leaf in both NILs (Fig. 3; Supplementary Table 6). All of these genes were expressed in the different experimental tissues under both DSCs (Supplementary Table 4). *Os08g31580* (DREB-A5) and *Os09g11460* (ERF-B2) were up-regulated in the leaf under both stress conditions in IR77298-14-1-2-B-10, but one of them (*Os09g11460*) was more highly expressed in IR77298-14-1-2-B-13. *Os09g11460* exhibited up-regulation specifically in the stamen under normal conditions and following GA3 and JA treatments (Fig. 1b; Supplementary Tables 2 and 3). On the other hand, *Os06g07030* and *Os04g34970* genes (subgroups A3 and A4 of the DREB subfamily) were expressed at high levels in IR77298-14-1-2-B-13 under both DSCs, whereas both genes were down-regulated in drought-tolerant NILs under mild stress conditions (Fig. 3; Supplementary Table 6). These *Os06g07030* and *Os04g34970* genes were up-regulated in the root and the leaf-1 tissue under normal conditions in Minghui 63, respectively.

AP2/EREBP panicle genes expression common in two NILs under severe and mild stress conditions

Plants are very susceptible to drought during the reproductive stage of development. Under severe stress conditions, a greater number up-regulated genes (53) common to both NILs were found in the panicle compared with the root and leaf (Fig. 3; Supplementary Table 6). Of these 53 genes, thirteen genes (e.g., *Os02g34270* and *Os04g46250*) were up-regulated in the panicle in both NILs under severe stress conditions, but they were not up-regulated in any other tissue (Supplementary Table 4). *Os02g34270* exhibited

down-regulation in the root under non-stressed conditions. Interestingly, eight genes (*Os05g27930*, *Os03g08500*, *Os06g08340*, *Os01g46870*, *Os11g06770*, *Os07g22770*, *Os12g39330*, and *Os06g05340*) belonged to the subfamilies AP2, DREB, and ERF and were up-regulated specifically in IR77298-14-1-2-B-10 in the panicle; however, they were not differentially expressed or only expressed at a low level in IR77298-14-1-2-B-13 under severe stress. Out of these eight genes, five belong to the ERF subfamily, and one gene (*Os01g46870*, ERF-B3) was up-regulated in the 3-leaf stage following treatment with GA3 (Supplementary Table 3). Similarly, *Os02g45420* (subfamily DREB-A1) was up-regulated specifically in the IR77298-14-1-2-B-13 panicle under severe stress (Fig. 3; Supplementary Table 6). This study detected 29 common genes in the panicles of IR77298-14-1-2-B-10 and IR77298-14-1-2-B-13 under mild stress conditions (Fig. 3; Supplementary Table 6). All of these genes were differentially expressed in at least one tissue in both NILs (Supplementary Table 4). In IR77298-14-1-2-B-10 under mild stress conditions, six genes (*Os04g55520*, *Os03g08500*, *Os05g49010*, *Os01g21120*, *Os10g25170*, and *Os09g28440*; subfamilies DREB and ERF) were up-regulated specifically in the panicle but were not differentially expressed or highly sensitive in IR77298-14-1-2-B-13. Of these six genes, four (*Os04g55520*, *Os01g21120*, *Os10g25170*, and *Os09g28440*) were up-regulated following JA treatment at all the time points, whereas *Os01g21120* was up-regulated following NAA treatment. In leaf-1 tissue, *Os04g55520* gene was down-regulated under normal conditions. Similarly, three genes (*Os03g22170*, *Os07g22730*, and *Os01g58420* from ERF) were activated specifically in the IR77298-14-1-2-B-13 panicle under mild stress conditions but exhibited low expression levels in IR77298-14-1-2-B-10 (Supplementary Table 6). In the panicle under both DSCs, both NILs shared 21 common genes, and these genes were expressed in at least one of the stress conditions in the root or leaf, as indicated above. The *Os03g08500* gene (subgroup A6, DREB subfamily) and the *Os01g21120* and *Os11g06770* (subgroups B1 and B4, ERF subfamily) genes were highly induced in the panicle under both DSCs in IR77298-14-1-2-B-10, whereas *Os03g08500* in the panicle was highly sensitive in IR77298-14-1-2-B-13 under both DSCs. In the drought-tolerant line, the expression intensity of the *Os01g21120* gene under severe stress conditions was higher than that under mild stress. Similarly, the *Os07g22730* (subgroup B3, ERF subfamily) was activated in the panicle in IR77298-14-1-2-B-13 under both DSCs but up-regulated approximately ninefold in IR77298-14-1-2-B-10 under severe stress conditions. The rapid change in the expression level of this gene under the drought stress conditions in the drought-tolerant line suggests the recruitment of genes that are normally expressed

at lower level for emergencies. In contrast, some genes exhibit ubiquitous expression under normal conditions and during phytohormone and drought stress treatments. A number of genes were up-regulated in the drought-tolerant line, and these genes may be required to maintain grain yield in response to severe drought. Functional analyses are needed to further explore these possibilities.

AP2/EREBP genes expression in both NILs
under both DSCs in root vs. all tissues and in leaf vs.
panicle and all tissues

The up-regulation of four genes was observed in both lines under the two DSCs in the root and the leaf (Fig. 3; Supplementary Table 6). *Os02g52670* (subfamily DREB-A5) was expressed in the root and in the leaf in both NILs under both stress conditions but was highly sensitive in the root in IR77298-14-1-2-B-13 under mild stress. Similarly, *Os02g54160* and *Os04g34970* (DREB subfamily) were activated in the root and leaf under severe and mild stress conditions in both lines but were not differentially expressed in the root and leaf under mild stress in IR77298-14-1-2-B-10. We observed that seven overlapping genes were up-regulated in both the leaf and panicle in both NILs under both DSCs (Fig. 3; Supplementary Table 6). The *Os08g31580* (DREB-A5) gene exhibited low expression levels in the leaf under severe stress in IR77298-14-1-2-B-13. *Os06g07030* (DREB-A3) was down-regulated in the leaf under mild stress in IR77298-14-1-2-B-10. The expression responses of both genes were higher in IR77298-14-1-2-B-10 than in IR77298-14-1-2-B-13 in all tissues (Fig. 3; Supplementary Table 6). The induction of four genes in both the panicle and the root were observed in the tolerant and susceptible lines under both DSCs (Fig. 3; Supplementary Table 6). The *Os05g49700* and *Os01g07120* (BREB subfamily) genes were uniformly expressed in all tissues in both lines, suggesting that these are housekeeping genes. The *Os05g49700* gene response was higher in the root and panicle under both DSCs in IR77298-14-1-2-B-10 when compared with IR77298-14-1-2-B-13 (Fig. 3; Supplementary Table 6). On a broad scale, our findings contribute to the understanding of gene responses to different abiotic stresses.

Identification of putative drought responsive genes,
subgroups, and subfamilies

The specific *AP2/EREBP* genes that were activated during the drought stress response in the drought-tolerant line are of particular interest. In total, sixteen non-redundant genes were up-regulated in IR77298-14-1-2-B-10, and these genes were expressed at a low level or were not differentially expressed in the drought-susceptible IR77298-14-1-2-B-13 line under

both DSCs in all tissues (Table 2). To identify putative genes that are responsible for drought avoidance in the tolerant NIL, this study focused on DEGs with high levels of expression in response to two DSCs in the tolerant line versus the susceptible line and IR64. In the tolerant line, two (*Os05g49700* and *Os06g42990*) out of four genes demonstrated higher expression levels in the root than in the leaf and panicle under severe stress and two of these genes (*Os01g66270* and *Os03g12950*) demonstrated higher expression levels in the panicle compared with the root and leaf under severe and mild stress (Fig. 4). *Os03g12950* is induced in the panicle and *Os05g49700* is down-regulated in the endosperm under normal conditions, whereas *Os01g66270* and *Os06g42990* are absent in the Affymetrix Gene Chip. Therefore, we suspected that these genes might play functional roles in the specific tissue under both stress conditions. However, further work is required to distinguish these possibilities. We found that most of the genes belonging to subgroups B3 and B5 and to the RAV subfamily were responsive in the panicle under severe stress, but the genes belonging to subgroups B and B2 of the ERF subfamily were down-regulated in the root. The genes belonging to subgroup A2 of the DREB subfamily were involved in the root and panicle, whereas other stress-responsive genes are randomly distributed in the other phylogenetic subgroups or subfamilies (Supplementary Table 7). Among the four subfamilies, we compared the DEGs of two rice genotypes, Minghui 63 and IR77298-14-1-2-B-10, in the root, leaf, and panicle tissues under normal and severe drought stress conditions. Table 3 shows that none of the RAV subfamily genes were differentially expressed in any tissue under normal conditions in Minghui 63, but this subfamily exhibited a large number of up-regulated genes (80%) in the panicle under severe drought stress condition in IR77298-14-1-2-B-10, followed by the ERF, DREB, and AP2 subfamilies. Our data suggest that the up-regulation of RAV subfamily genes may be tissue specific during severe drought stress conditions. Furthermore, the expression intensities of a number of up-regulated genes in IR77298-14-1-2-B-10 were higher than or equal to their expression intensities in IR77298-14-1-2-B-13 or IR64 in all examined tissues (Fig. 5). Taken together, these results enhance our knowledge of the involvement of *OsAP2/EREBP* TFs in plant resistance to drought and show that members of certain subgroups or subfamilies of the *OsAP2/EREBP* gene family are activated in response to drought stress.

Consensus *cis*-regulatory elements in tolerant NIL

The interaction between TFs and *cis*-regulatory DNA sequences controls gene expression and constitute the essential functional linkage of gene regulatory networks. We examined the *cis*-elements in the promoter sequences (2-kb upstream region) of genes that were specifically

Table 2 Specific up-regulated genes in the tolerant line IR77298-14-1-2-B-10 compared with the sister line IR77298-14-1-2-B-13 and control IR64

Root severe			
NIL10 specific	IR77298-14-1-2-B-10	IR77298-14-1-2-B-13	IR64
Os06g42990	1.293	0	0
Root mild			
NIL10 specific	IR77298-14-1-2-B-10	IR77298-14-1-2-B-13	IR64
Os09g39850	1.528	0	0
Os06g05340	1.12	0	0
Leaf severe			
NIL10 specific	IR77298-14-1-2-B-10	IR77298-14-1-2-B-13	IR64
Os10g38000	1.11	0	0
Os03g08500	1.007	0	0
Leaf mild			
NIL10 specific	IR77298-14-1-2-B-10	IR77298-14-1-2-B-13	IR64
Os02g43940	1.619	0	0
Os04g44670	1.271	0	0
Os02g51300	1.106	0	0
Panicle severe			
NIL10 specific	IR77298-14-1-2-B-10	IR77298-14-1-2-B-13	IR64
Os03g08500	1.424	0	0
Os06g08340	1.745	0	0
Os01g46870	1.283	0	0
Os07g22770	1.238	0	0
Os06g05340	1.297	0	0
Panicle mild			
NIL10 specific	IR77298-14-1-2-B-10	IR77298-14-1-2-B-13	IR64
Os04g55520	1.257	0	0
Os03g08500	1.036	0	0
Os05g49010	1.358	0	0
Os01g21120	1.274	0	0
Os10g25170	1.015	0	0
Os09g28440	1.229	0	0

0 = genes not differentially expressed in IR77298-14-1-2-B-13 and IR64

up-regulated in tolerant NIL using a Rice *Cis*-Element Searching tool (RiCES, Doi et al. 2008). We determined that in the tolerant NIL under both DSCs, the most important *cis*-elements contained *cis*-motifs matching the published ARF, ASF-1/2, Dof1, GT-1, RAV1, WRKY, W-box, and MYB/C motifs. The different types of *cis*-elements that are involved in the responses to abiotic and biotic stresses are listed in Supplementary Table 8. The majority of the *OsAP2/EREBP* up-regulated genes with tissue-specific expression patterns contained at least two of these *cis*-elements (Supplementary Table 8), whereas uncommon *cis*-elements were found in genes in the susceptible line (data not shown). With the help of bioinformational analysis, we predict that further analysis of a number of these TFs will contribute to a better understanding of gene regulatory networks in rice under different kinds of drought stress conditions.

Introgressed regions of the *OsAP2/EREBP* gene in the IR64 background

Advanced backcross quantitative trait loci (QTLs) analysis facilitates simultaneous identification and introgression of identified QTLs into a recipient genotype. We observed introgressed lines in our present study that can be used as an important genetic material to decipher the physiological and molecular basis of tolerance in individual genomic regions. In different rice genotypes, this study identified four *OsAP2/EREBP* genes that were introgressed (McNally et al. 2009) on chromosomes 3, 5, 7, and 10 in the IR64 background (Supplementary Fig. 1). From this genome-wide comparison, four regions showed differential hybridization; these regions were further genotyped using SSR markers to confirm polymorphism between the parental lines. The four regions mapped for QTL identification were at 6.8–7.3, 6.7–7.2,
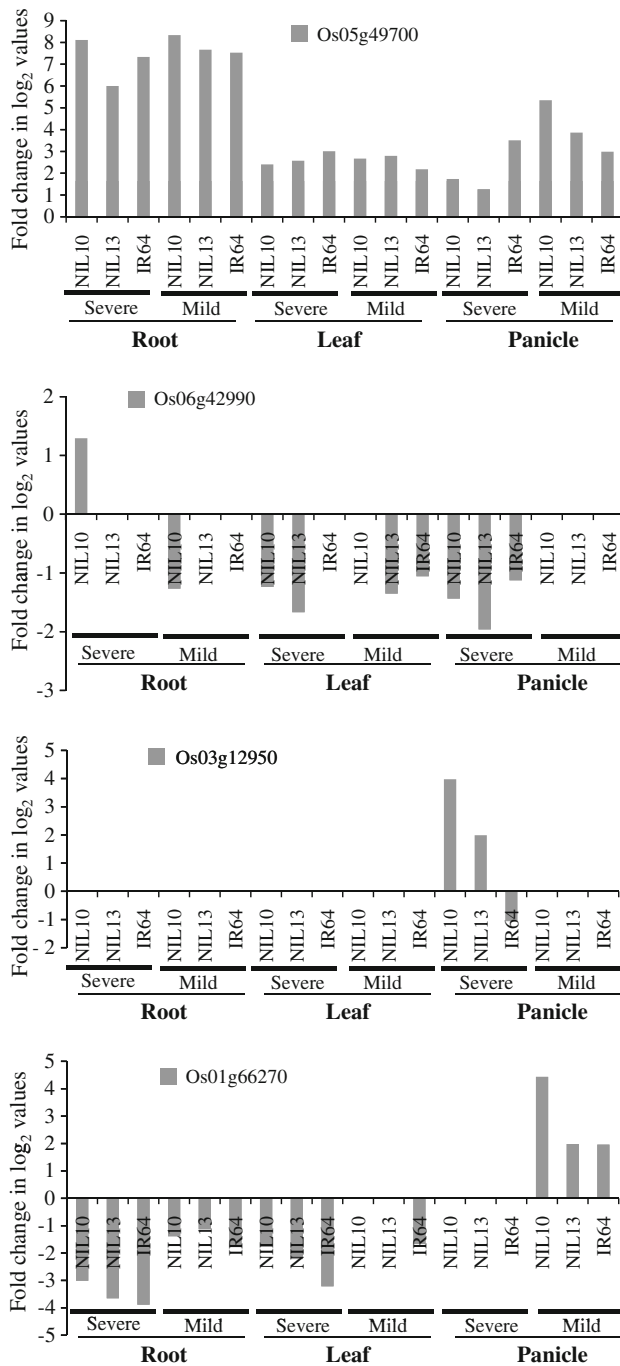
Fig. 4 Identification of the reference genes showing the highest expression in the tolerant NIL compared with its counterparts under severe and mild stress conditions in the root, leaf, and panicle. Fold changes in log₂ values are shown on the *Y*-axis, and the different genotypes are indicated on the *X*-axis. NIL10 = IR77298-14-1-2-B-10 and NIL13 = IR77298-14-1-2-B-13

14.6–16.5 and 18.6-19.3 Mb on chromosomes 2, 4, 9 and 10, respectively (personal communication, Arvind Kumar). Of these four genes, one (*Os07g23710*) was not differentially expressed under both DSCs in the tissues that were examined. The *Os03g08460* gene was down-regulated in the root

under both DSCs conditions in all genotypes, whereas the *Os09g30840* gene was down-regulated in the root and leaf under mild stress and in the panicle under severe stress in the drought-susceptible line. Only *Os05g29810* was up-regulated in the leaf and panicle under severe stress in all genotypes (Supplementary Table 4). The data show that the effect of the introgressed QTL of the *OsAP2/EREBP* genes on grain yield under drought conditions is unclear. The *Os09g25070*, *Os09g24490*, and *Os06g48950* genes are from WRKY, MYC, and ARF families, which are placed in the QTL region (personal communication, Arvind Kumar). We observed that the *cis*-element motif of *OsAP2/EREBP* genes is found in the different transcription factors mentioned above. *AP2/EREBP* may regulate target genes directly or indirectly thorough intermediary WRKY, MYB/C, and ARF transcription factors in tolerant NILs. Functional analysis is required to substantiate this suggestion. Bernier et al. (2007) have suggested that marker-assisted mapping and the introgression of major-effect QTLs for grain yield under drought conditions may provide a fast-track approach for developing drought-tolerant varieties, and Venuprasad et al. (2009) have reported several QTLs for yield and yield-related traits on different rice chromosomes under drought stress conditions.

Gene enrichment analysis for differentially expressed genes in NILs

The specifically expressed genes in tolerant NILs versus susceptible sister NILs during two drought stress treatments were examined for Gene Ontology (GO, Du et al. 2010) category enrichment using agriGO and KEGG orthology (KO, Kanehisa et al. 2008) analyses. The terms KEGG orthology (KO) and Gene Ontology (GO) are widely applied to understand the biological significance of microarray differential gene expression data (Du et al. 2010). Overall, a global view of the gene expression analysis demonstrated that approximately 85% of genes were differentially expressed in all rice tissues in response to drought stress treatment. The GO and KO enrichment tests and biological pathway analysis indicate that the up-regulated *OsAP2/EREBP* genes in the drought-tolerant NIL IR77298-14-1-2-B-10 are not involved in other pathways, such as secondary metabolism, amino acid metabolism, response to stimuli, defense response, transcription, photosynthesis, cell wall growth, and signal transduction, and the down-regulated genes were not involved in any biological pathway. An assumption of this analysis is that the *OsAP2/EREBP* genes that demonstrated up-regulated expression in tolerant NIL contribute indirectly to these categories of gene function; however, further research is required to support this hypothesis. Information like this would contribute to a deeper understanding of gene expression linked to regulatory networks under drought stress.

Table 3 The number of differentially expressed gene patterns in Minghui 63 under non-stress conditions and severe stress conditions in the drought-tolerant IR77298-14-1-2-B-10

Subfamily	No. of gene	Root		Leaf		Panicle	
		Up	Down	Up	Down	Up	Down
Minghui 63 under normal conditions							
AP2	20 (24)	2 (10%)	2	1	2	0	1
RAV	**5 (5)**	**0**	**0**	**0**	**0**	**0**	**0**
DREB	35 (57)	3 (8.5%)	1	2 (5.7%)	2	3 (8.5%)	0
ERF	56 (77)	2 (3.5%)	4	2 (3.5%)	8	0	3
	Total	7	7	5	12	3	4
IR77298-14-1-2-B-10 under stress conditions							
AP2	18 (24)	3 (16.6%)	5	1	3	**6 (33%)**	4
RAV	**5 (5)**	**0**	**3**	1	2	**4 (80%)**	**0**
DREB	49 (57)	**9 (18.3%)**	15 (30.5%)	13 (26.5%)	7 (14.2%)	**19 (38.7%)**	13 (26.5%)
ERF	68 (77)	4 (5.8%)	28 (41.1%)	13 (19.1%)	14 (20.5%)	**32 (47.1%)**	7 (10.2%)
	Total	16	31	28	26	**61**	24

The number of genes in each subfamily is indicated in parentheses, classification by Sharoni et al. (2011). Selected tissues of Minghui 63, Root = seedling with 2 tillers, Leaf 1 = young panicle at stage 3, Panicle 1 = young panicle at stage 3, Up = up-regulated genes, Down = down-regulated genes. Bold values indicate greater difference between normal and stressed conditions
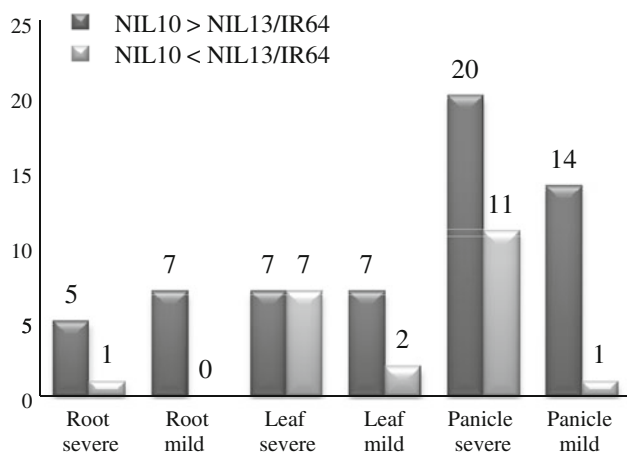

Fig. 5 The expression intensity of up-regulated genes was higher in NIL10 than in NIL13 or IR64 in all tissues except the leaf under severe stress conditions. The *X*-axis represents the selected tissues, and the *Y*-axis represents the number of up-regulated genes. The number of genes is provided at the top of each diagram. NIL10 = IR77298-14-1-2-B-10 and NIL13 = IR77298-14-1-2-B-13

Fig. 6 RT-PCR analysis of differential gene expression (up- and down-regulated) in all tissues under both drought treatments in all genotypes. **a** In the root under severe conditions. **b** In the leaf under severe conditions. **c** In the panicle under severe conditions. *Actin* gene expression was used as the internal control

RT-PCR analysis for confirmation of the transcriptome data

To validate the accuracy of our microarray data, we selected eight up- or down-regulated genes from the different tissues of all genotypes and both DSCs. We found that in most cases, the DEGs that were identified using the microarray were also detected using RT-PCR analysis (Fig. 6). The PCR results suggest that the DEGs observed through microarra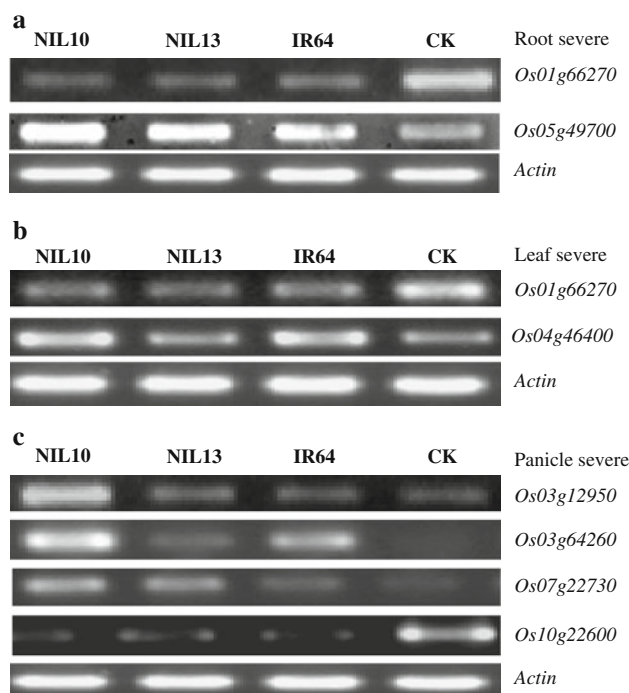y analysis represent real differences between drought-stressed and non-stressed rice genotypes. Furthermore, Moumeni et al. (2011) confirmed this microarray data using qRT-PCR analysis on 10 root tissue DEGs selected from different functional pathways.

Discussion

Expression patterns of *OsAP2/EREBP* genes under normal stress conditions

The objectives of this study were (1) to determine the expression patterns of members of this gene family under normal, hormone, and different drought stress conditions, (2) to assess the number of genes responding to normal conditions or different stress treatments (responses in the drought-tolerant line in particular is a novel contribution), (3) to identify the important subfamily based on gene expression profiles in specific tissues during normal or stress treatments, (4) to identify the drought response *cis*-element, which may assist the understanding of the function of these novel genes in network systems, and (5) to select the most appropriate candidate genes for further functional analysis. Our studies present genomic information on the *OsAP2/EREBP* gene family in rice. These results indicate that, to varying extents, normal treatment (no drought stress) induced different expression levels of a number of *OsAP2/EREBP* genes. The similarity of the expression patterns among the *OsAP2/EREBP* genes may reflect shared induction mechanisms. However, differences in the accumulation levels of transcripts during normal treatment indicate that *OsAP2/EREBP* genes may be specifically controlled by specific factors. For example, *Os09g11460* was highly expressed in the stamen before flowering, and we can speculate that it plays an important role in pollen tube growth at this stage. In addition, *Os03g12950*, *Os05g25260*, and *Os08g36920* are mainly transcribed in panicles, suggesting that they participate in the development of young panicles (Fig. 1b). Consequently, pollen is the major site of variations in the expression levels for many genes (Becker et al. 2003; Czechowski et al. 2005). Moreover, *Os08g41030* is preliminarily expressed in the endosperm, suggesting that it might be needed for endosperm development (Fig. 1b). *Os03g08460/OsEBP-89* and *Os02g43970/AP59* exhibited a low expression or down-regulation in Minghui 63, and these plants exhibited a lower grain yield under normal and drought stress conditions (Yang et al. 2002; Oh et al. 2009). A significant percentage of *OsAP2/EREBP* genes were identified as showing higher expression levels in the reproductive stage compared with the vegetative stage. The expression pattern of a gene can reflect its functional relevance, e.g., the wide expression patterns of some *OsAP2/EREBP* members suggest that these genes may play regulatory roles at multiple development stages, whereas the unique expression patterns of other genes may indicate that these genes participate in specific biological processes.

Responses of *OsAP2/EREBP* genes in different treatments

Hormones regulate plant growth and development through a complex set of interactions. *OsAP2/EREBP* gene expression under hormone treatment requires extensive cross talk between the response pathways, and there are likely substantial physiological connections between AP2/EREBP protein production and hormone treatments. A higher expression pattern of a particular gene under these circumstances suggests that the genes play an important protective role against stressful hormone treatments (Fig. 2). *OsAP2/EREBP* genes (e.g., *CRL5*, *crownrootless5* and *OsAP239/Os04g52090*) in plants are affected by auxin and cytokinin (Kitomi et al. 2011), ABA and GA3 (Yaish et al. 2010). Shen et al. (2002) reported that the levels of different types of proteins increase or decrease following GA3 treatment in rice leaf sheaths. Under adverse conditions, abscisic acid (ABA) plays an important role in adapting the plant's response and can stimulate root growth and increase the ability of the plant to uptake water from the soil during drought stress. In this study, a number of genes (e.g., *Os05g41780*, *Os05g51300*, and *Os09g11460*) were induced in the root, leaf, and panicle under both hormone and drought stresses. Therefore, these genes may be involved in the uptake of water when the soil is dehydrated. The expression of *CRL5* (*crownrootless5*), which encodes a member of the AP2/ERF transcriptional factor family, was observed in the stem region where crown root initiation occurs (Kitomi et al. 2011). ABA triggers the closing of the stomata when soil water is insufficient to keep up with transpiration. Drought-stressed leaves produce large amounts of ABA, which triggers potassium ions to be transported out of the guard cells, causes the stomata to close, and water is held in the leaf. On basis of our results, we speculate that *OsAP2/EREBP* genes function in ABA signaling pathways and in the defensive response against water deficit. The expression of *OsAP239/Os04g52090,* which was up-regulated in the leaf in both NILs under severe stress but was higher in IR77298-14-1-2-B-10, was shown to control the ABA/GA3 balance in rice to allow seed production (Yaish et al. 2010). Based on the expression data, it is difficult to configure the complex regulatory network of this gene family, but the data may provide valuable insights for understanding the responses to different hormones. A number of *OsAP2/EREBP* genes demonstrated a high or low expression under different hormone treatments at the seedling stage (Fig. 2).

AP2/EREBP gene expression profiles under severe stress and mild stress conditions in NILs

During drought stress, plants adapt to the new environment by changing their gene expression profiles after perceiving

stress signals (Hazen et al. 2005). Analyses of *AP2/EREBP* gene family have revealed a wealth of information about the gene expression in this family of genes with fundamental implications for understanding the biological processes underlying growth and development of the plant. Knowing the gene expression profile of *OsAP2/EREBP* genes may provide clues for selecting the most appropriate candidate genes for functional analyses. Our study focused on the identification and expression profiles of drought stress-responsive genes from rice genotypes at different developmental stages under different stresses, with the aim of identifying important components of the drought-stress response. The analysis of the *OsAP2/EREBP* genes showed high water-usage efficiency in the drought-tolerant genotype. In the whole rice genome, approximately 7% of the genes are induced by drought or salt stresses (Zhou et al. 2007), whereas in the *AP2/EREBP* family, at least 85% (140 of 163 rice genes) of the genes were differentially expressed (up- or down-regulated) in one or more tissues with two folds under both DSCs. We considered the DEGs in a tolerant line compared with the sister (susceptible) line and the control and focused on those genes that were exclusively up-regulated in IR77298-14-1-2-B-10. Although the common genetic background of rice NILs, which are the backcross progeny from Aday Se. x IR64, is approximately 97% for the IR77298-14-1-2-B family (Venuprasad et al. 2011), the tolerant NIL showed distinctive differences in its gene expression responses to different drought stress levels. Comparative gene expression profiling is an alternate and efficient way to identify pathways and genes regulating the stress response (Udupa et al. 1999).

Sharoni et al. (2011) identified 13 rice *AP2/EREBP* genes that were drought-inducible in the seedling stage at different time points; however, our study revealed some common and unique up-regulated genes in different tissues in both NILs under drought stress (Fig. 3) and during hormone treatments (Figs. 1, 2). Complex genetic networks function during the development of every organ in plants, and substantial gene expression overlaps exist between the developmental pathways and the stress-response pathways (Cooper et al. 2003; Shinozaki et al. 2003). More DEGs and higher gene expression intensities were observed in IR77298-14-1-2-B-10 compared with IR77298-14-1-2-B-13 and IR64, and 16 genes were up-regulated in the IR77298-14-1-2-B-10 tolerant line specifically that were not up-regulated in IR77298-14-1-2-B-13 or IR64 (Table 2). For example, *Os09g39850*, *Os02g43940*, and *Os06g08340* were up-regulated in IR77298-14-1-2-B-10 specifically in the root, leaf, and panicle with higher expression under both DSCs than in IR77298-14-1-2-B-13/IR64. *OsDRED2A/Os01g07120*, *OsDREB1A/Os09g35030*, and *AP37/Os01g58420* expression intensities were higher in the root, leaf, and panicle in IR77298-14-1-2-B-10 compared

with IR77298-14-1-2-B-13 and the control under severe or mild stress, and these genes are tolerant to drought, high salt, and cold stress in the transgenic rice plant (Dubouzet et al. 2003; Oh et al. 2009). TSRF1 improves the osmotic and drought tolerance of rice seedlings without growth retardation and increases the expression of MYB, MYC, and photosynthesis-related genes, probably by binding to dehydration-responsive element and GCC boxes in the promoter regions of the target genes (Quan et al. 2010). A number of genes that are common or overlapped among tissues, for example, *Os05g49700* and *Os01g07120* (DRED-A1 and DREB-A4 subgroups) were highly expressed in all three tissues under severe and mild DSCs in both NILs. A large fraction of the genes that are regulated by dehydration are also up-regulated by pollination (Lan et al. 2005). In our study, the expression of several *OsAP2/EREBP* genes (e.g., *Os03g08500* and *Os05g49010*) was highly induced at the pollination stage; thus, these genes may be involved in the drought response in IR77298-14-1-2-B-10 (Table 2), although further research is required to establish this possibility. Similar conclusions have been drawn from the analysis of drought-inducible *OsDREB2A/Os01g07120*, *AP37/Os01g58420*, and *OsDREB1A/Os09g35030* genes, which regulate plant tolerance in transgenic rice during conditions of drought stress (Dubouzet et al. 2003; Oh et al. 2009). Conversely, *Os03g08460/OsEBP-89* and *Os02g43970/AP59* genes are expressed at a low level in the root, leaf, panicle, and endosperm, and a decrease in the grain yield by 23 to 43% was observed under normal and drought stress conditions (Yang et al. 2002; Oh et al. 2009). A number of novel genes are candidate reference genes because of their specific/higher expression patterns (Fig. 4; Table 2). Differential gene expression is thought to reflect the drought response. Among the ERF subgroups, the expression of a number of genes from subgroups ERF-B3 and ERF-5 was induced in the panicle under both DSCs, whereas almost all the genes in subgroup DREB-A6 exhibited very little up-regulation under both DSCs in all tissues that were analyzed (Supplementary Table 7). The genes of the RAV subfamily are highly induced in panicle tissue under conditions of severe drought stress compared with normal growth conditions. The N-terminal regions of the RAV subfamily genes are homologous to the AP2 DNA-binding domain but their C-terminal regions are homologous to ABI3 and VP1 transcription factors and the AP-2 and B3-like domains of RAV bind autonomously to CAACA and CACCTG motifs (Kagaya et al. 1999). The protein product of the genus *Phaseolus vulgaris* ABI3-like factor (*PvAlf*) is similar to the ABI3 and VP1 proteins and functions to develop seed maturation and seed abscission (Bobb et al. 1995). CAACA and CACCTG motifs were found in the promoter region of RAV subfamily gene members and the genes were induced in the panicle under severe

stress conditions in the drought-tolerant NIL, which indicates that these genes may be involved in endosperm development (Table 3).

Defense system of *AP2/EREBP* genes in rice

We predicted that some gene-specific *cis*-elements might be important in the regulation of the target gene by other factors and that these *cis*-elements may influence the introgressed regions of drought QTLs. Transcription factors and *cis*-elements function in the promoter region of different stress-related genes, and overexpression of these genes may improve the plant's tolerance to abiotic or biotic stress. In response to drought, the AP2/EREBP TF regulates many target genes by binding to the *cis*-element (A/GCCGAC) in the promoter region of candidate genes, and this transcriptional regulatory system is known as a regulon. ABA is produced under drought stress conditions and plays a crucial role in plant tolerance to drought (Shinozaki et al. 2003). AP2 and other regulons, such as *OsDREB2*, respond to dehydration in rice (Dubouzet et al. 2003), and the dehydration-responsive element binding protein (DREB1 and DREB2)/C-repeat binding factor (CBF) regulons function in ABA-independent gene expression. Meanwhile, the ABA-responsive element (ABRE) binding protein (AREB)/ABRE binding factor (ABF) or the ABRE and coupling element (CE) regulon functions in ABA-dependent gene expression. The DREB1/2 homologous genes (OsDERB1A and OsREB2A) were isolated from grasses, such as rice and maize (Dubouzet et al. 2003; Qin et al. 2007b). ABA-activated OSRK1 protein kinases phosphorylate and activate AREB/ABF-type proteins in rice (Chae et al. 2007). ABA-independent and ABA-dependent signal transduction pathways convert the initial stress signal into cellular responses. The members of TF families that are involved in both ABA-independent (AP2/ERF, bHLH, and NAC) and ABA-dependent (MYB, bZIP, and MYC) pathways are up-regulated in rice. TFs belonging to these families interact with specific *cis*-elements and/or proteins, and their overexpression confers stress tolerance in heterologous systems (Kizis and Pages 2002; Zhu et al. 2003; Lee et al. 2004). It has been shown that *OsAP2-39/Os04g52090* is overexpressed in transgenic rice and up-regulates the ABA biosynthetic gene *OsNCED-1* (Yaish et al. 2010). Microarray analyses have shown that up-regulated genes in rice plants overexpressing *AP37/Os01g58420* were drought stress tolerant and exhibited increased seed setting (16–57% more than controls) in the field (Oh et al. 2009). The homologous genes, wheat *TaDREB1*, wheat *WDREB2*, and maize *ZmDREB2A* are also responsive to cold stress (Shen et al. 2003; Qin et al. 2007b). These results indicate that the AP2 regulon may have additional functions in rice. Therefore, comparative analysis of gene expression profiles

under both DSCs to determine the functional role of these genes in the growth of the plant and its response to stress, and the identification of target genes for TFs involved in stress responses are vital. In this study, the regulation of *OsAP2/EREBP* genes was precise in terms of the spatial and temporal distribution.

This study provides an overview of gene expression patterns of the *OsAP2/EREBP* gene family under normal and stress conditions. The application of a new comprehensive 44 K oligoarray platform together with rice NILs enabled us to determine gene expression patterns in different tissues of two NILs with contrasting yield performances under drought stress at the reproductive stage of growth. By comparing the gene expression patterns of NILs under drought stress and normal conditions, we identified several putative genes that may be responsible for the drought response in the drought-tolerant line. These genes should be considered novel reference genes, and further research is needed to prove their functions. A number of putative *cis*-elements were identified, which may help understand the function of these key genes. These data provide a useful reference and establish a starting point for determining the functions of the *OsAP2/EREBP* family of genes in rice at the reproductive stage of growth. The genes belonging to the ERF-B3 and ERF-B5 subgroups and the RAV subfamily gene members were activated in leaf and panicle tissues but not in the root. The specific subfamily (RAV) or subgroups of this gene family provide a new avenue for investigating appropriate candidate genes for functional analysis under different treatments. Overexpression and knockdown analyses of selected members of this gene family are underway in our laboratory to investigate optimal molecular breeding schemes for the *OsAP2/EREBP* gene family.

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