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A Comprehensive Transcriptional Profiling of the *WRKY* Gene Family in Rice Under Various Abiotic and Phytohormone Treatments

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WRKY transcription factors play important roles in the regulation of various biological processes. We have analyzed the publicly available rice genome sequence databases and predicted 103 genes encoding WRKY transcription factors. Among them, the majority of rice *WRKY* genes (77.7%) were located in duplicated regions; 45.6% of *WRKY* genes were fragmentally duplicated and 35% of them were tandemly duplicated. These results suggested that genome duplications might be regarded as a major mechanism for expansion of this family in the rice genome. Subsequently, we analyzed their expression profiles under normal and abiotic stress, as well as various hormone treatments. Under normal growth conditions, 65 *WRKY* genes were expressed differentially either in their transcript abundance or in their expression patterns. Under abiotic (cold, drought and salinity) stresses and various phytohormone treatments, 54 *WRKY* genes exhibited significant differences in their transcript abundance; among them three genes were expressed only in stressed conditions. Among the stress-inducible genes, 13 genes were regulated only by abiotic stresses, another set of 13 genes were responsive to only phytohormone treatments and the remaining 28 genes were regulated by both factors, suggesting an interaction between abiotic stress and hormone signaling. On the other hand, we have also surveyed the expression divergence of duplicated genes under normal or stressed conditions, and the results showed that high expression divergence has occurred not only among fragmentally but also among tandemly duplicated genes. These results suggested that the high expression divergence could be one of the mechanisms for the retention of these duplicated *WRKY* genes.

Keywords: Expression profile — *Oryza sativa* cv Nipponbare — Real-time PCR.

Abbreviations: GA_3, gibberellic acid; MeJA, methyl jasmonate; PEG, polyethylene glycol; RT–PCR, reverse transcription–PCR; SA, salicylic acid; TF, transcription factor.

Introduction

Transcription factors (TFs) are proteins that can activate or repress the transcription of downstream target genes by binding directly to promoters of target genes in a sequence-specific manner. By genome-wide identification, the Arabidopsis genome was found to encode 1,510–1,581 TF genes (Riechmann et al. 2000, Gong et al. 2004, Guo et al. 2005, Iida et al. 2005). Accordingly, the rice genome has been predicted to contain 1,611 TF genes (Xiong et al. 2005). Among them, the MYB superfamily constitutes the largest TF gene family in plants, consisting of 198 and 183 members in Arabidopsis and rice genomes, respectively (Chen et al. 2006). The *WRKY* gene family also encodes a large group of TFs. Proteins of this family contain one or two highly conserved WRKY domains and a zinc finger motif in the C-terminal region (Eulgem et al. 2000). The WRKY domain can bind to the W box or SURE (sugar-responsive *cis*-element) found in promoters of target genes and regulates its transcription (Rushton et al. 1995, Sun et al. 2003). Since identification of the first WRKY protein, SPF1, from sweet potato (*Ipomoea batatas*) (Ishiguro et al. 1994), large numbers of *WRKY* genes have been cloned from various plant species including wild oats (*Avena fatua*) (Rushton et al. 1995), orchardgrass (*Dactylis glomerata*) (Alexandrova et al. 2002), barley (*Hordeum vulgare*) (Sun et al. 2003, Xie et al. 2007), cocoa (*Theobroma cacao*) (Borrone et al. 2007), chickpea (*Cicer arietinum*) (Mantri et al. 2007), Japanese goldthread (*Coptis japonica*) (Kato et al. 2007), grape (*Vitis vinifera*) (Marchive et al. 2007), tobacco (*Nicotiana tabacum*) (Wang et al. 1998, Chen and Chen 2000, Hara et al. 2000, Yoda et al. 2002), camomile (*Matricaria chamomilla*) (Ashida et al. 2002), parsley (*Petroselinum crispum*) (Rushton et al. 1996, Cormack et al. 2002), a desert legume (*Retama raetam*) (Pnueli et al. 2002), sugarcane (*Saccharum* hybrid cultivar) (Lambais et al. 2001), bittersweet nightshade (*Solanum dulcamara*) (Huang et al. 2002), potato (*Solanum tuberosum*) (Dellagi et al. 2000, Beyer et al. 2001), wheat (*Triticum aestivum*) (Sun et al. 2003), etc. In Arabidopsis, 70 *WRKY* genes were identified by a sequence similarity search

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(Riechmann et al. 2000). In the rice genome, >100 WRKY members were predicted by means of a bioinformatics approach (Wu et al. 2005, Zhang and Wang 2005). On the other hand, WRKY proteins were previously regarded as plant-specific TFs; however, *WRKY* genes were also detected in non-plant species including *Giardia lamblia* and *Dictyostelium discoideum*, suggesting an earlier origin in lower eukaryotes (Zhang and Wang 2005).

Despite identification or prediction of many *WRKY* genes from different species, only a small number of them have been functionally characterized. Generally, WRKY proteins are regarded as TFs that are involved in various biological processes under normal or stressed conditions (Eulgem et al. 2000, Singh et al. 2002, Ulker and Somssich 2004, Eulgem and Somssich 2007), and some WRKY-interacting proteins have also been identified (Asai et al. 2002, Robatzek and Somssich 2002, Ishida et al. 2007) that are crucial to uncover their biological functions. Under normal growth conditions, some members of WRKY proteins may play regulatory roles in morphogenesis of trichomes (Johnson et al. 2002, Ishida et al. 2007) and embryos (Alexandrova and Conger 2002, Lagace and Matton 2004), senescence (Hinderhofer and Zentgraf 2001, Robatzek and Somssich 2001, Chen et al. 2002, Robatzek and Somssich 2002, Ulker et al. 2007), dormancy (Pnueli et al. 2002), plant growth (Chen and Chen 2002) and metabolic pathways (Rushton et al. 1995, Willmott et al. 1998, Johnson et al. 2002, Sun et al. 2003). On the other hand, accumulating data show that transcription of *WRKY* genes is strongly regulated in response to various biotic and abiotic stresses. Increased/decreased levels of WRKY mRNA, protein or DNA-binding activities have been reported to be induced/repressed by various biotic stresses including viruses (Yoda et al. 2002, Park et al. 2006), bacterial pathogens (Journot-Catalino et al. 2006, Oh et al. 2006, Xu et al. 2006, Lippok et al. 2007, Zheng et al. 2007), fungi (Zheng et al. 2006), oomycetes (Beyer et al. 2001, Kalde et al. 2003, Knoth et al. 2007) and some signaling substances including salicylic acid (SA)/benzothiadiazole (Chen and Chen 2002, Liu et al. 2007, Ishida et al. 2007), jasmonic acid (Xu et al. 2004, McGrath et al. 2005, Mao et al. 2007, Qiu et al. 2007), gibberellin (Zhang et al. 2004) and ABA (Xie et al. 2006). Besides biotic stresses, *WRKY* genes are also implicated in response to various abiotic stresses including wounding, cold, heat, phosphate, drought or salinity (Hara et al. 2000, Cheong et al. 2002, Rizhsky et al. 2002, Mare et al. 2004, Hwang et al. 2005, Devaiah et al. 2007, Wei et al. 2008).

Since *WRKY* genes are frequently reported to be involved in various stress responses, comprehensive expression analyses were carried out by reverse transcription–PCR (RT–PCR), cDNA or oligo microarray, or cDNA real-time PCR at the genome-wide level under certain stresses. Most of these analyses were carried out in Arabidopsis and, among 72 *WRKY* genes analyzed, 49 genes were differentially regulated in the plants infected by an avirulent strain of the bacterial pathogen *Pseudomonas syringae* or treated by SA (Dong et al. 2003). Microarray and real-time PCR data showed that some *WRKY* genes were induced in O_3-treated Arabidopsis plants (Tosti et al. 2006). In addition to biotic stresses, data also showed that some *WRKY* genes were responsive to several abiotic stresses including drought, cold and salinity (Seki et al. 2002, Jiang and Deyholos 2006). In rice, genome-wide expression analyses were carried out to analyze transcript profiles following SA and jasmonic acid treatments, or pathogen infection (Ryu et al. 2006). This study shows that a large number of *WRKY* genes were induced or suppressed under these stresses and suggested that many WRKY proteins might be involved in the rice defense response. In this study, we report the identification of 103 rice genes encoding WRKY domain-containing proteins based on the complete genome sequence analysis. We also performed phylogenetic analysis using their domain amino acid sequences to classify these WRKY proteins. Subsequently, we analyzed and compared their expression profiles under normal or various stressed conditions using RT–PCR and real-time PCRs, and then attempted to assign possible functions to a large group of uncharacterized genes based on their expression patterns.

Results

The japonica rice genome contains 103 WRKY transcription factor genes

By multiple cycles of BLAST searches as described in Materials and Methods, we have identified 103 putative rice genes encoding the WRKY domain. The previous nomenclature of *WRKY* genes used in rice was confusing; for example, *OsWRKY57* is located on chromosome 12 and is designated with the locus number *LOC_Os12g01180* (the TIGR prefix *LOC_Os* is omitted hereafter for convenience) in the reference (Zhang et al. 2004); however, this nomenclature was used to refer to a gene mapped on chromosome 5 by Wu et al. (2005). Therefore, these predicted genes in this study are named by their corresponding locus numbers presented in the TIGR database as listed in Fig. 1.

Non-random distributions of WRKY genes in rice chromosomes and family expansion

The physical positions of *WRKY* genes were obtained from the TIGR database and were used to map these genes onto the corresponding rice chromosomes (Fig. 1). The result showed that the chromosomal distributions of *WRKY* genes were non-random, although they were located in all 12 chromosomes. Chromosome 1 had the highest

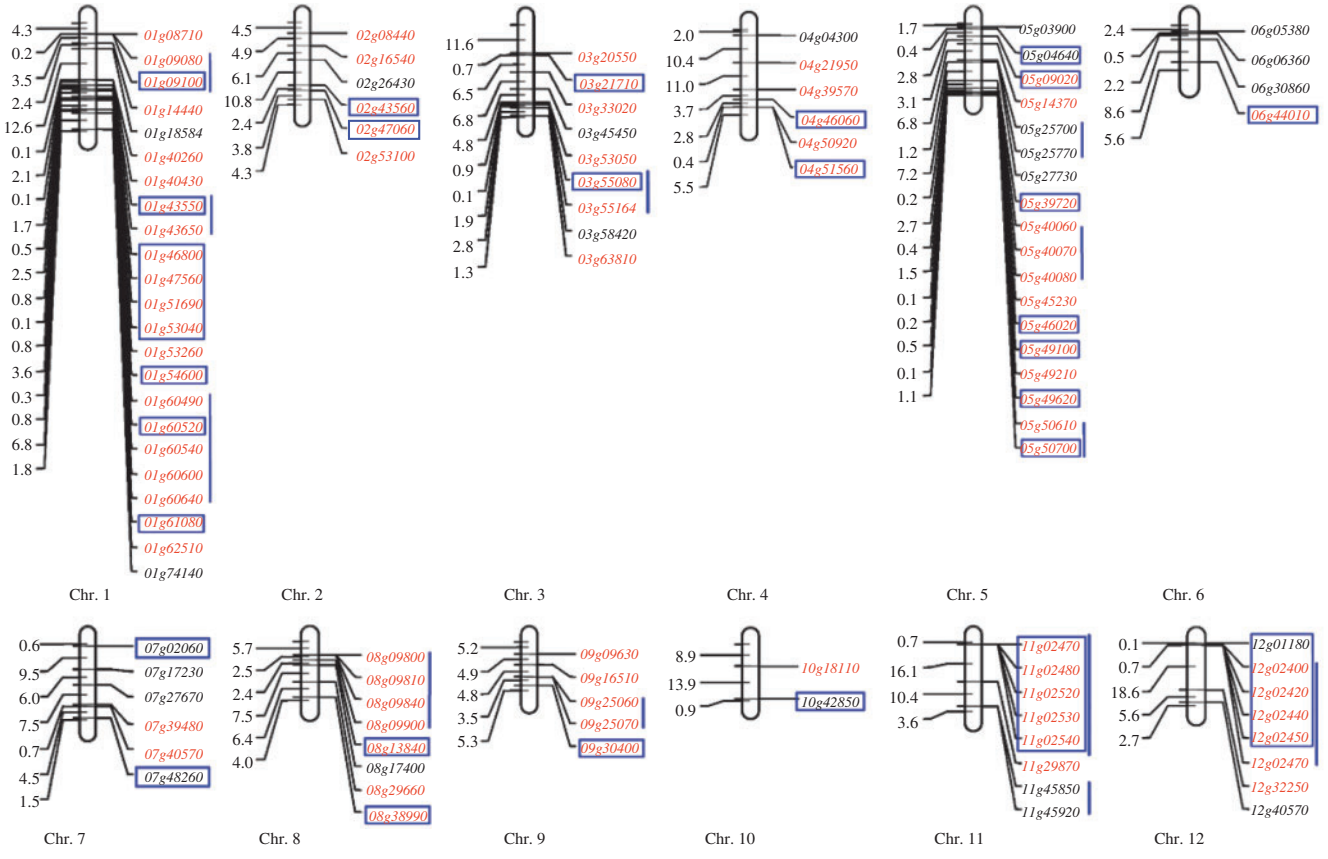


Fig. 1 Distribution of *WRKY* genes and detection of duplicated genes in rice chromosomes. Genes located in whole genome duplicated regions are highlighted in red. Blue outlined boxes represent fragmentally duplicated genes. Tandemly duplicated genes are indicated with vertical blue lines. The number on the left of each chromosome represents the physical position (Mb) of mapped genes. The penultimate number shows the physical distance to the last gene. The rest may be reduced by analogy.

density of *WRKY* genes with 23 members, while chromosome 10 had the least numbers with only two members. Based on their physical positions, 77.7% (80 of 103) of *WRKY* genes were detected on duplicated regions (Fig. 1). These data indicated that genome-scale duplication significantly contributed to the expansion of this family. On the other hand, 45.6% (47 of 103) of *WRKY* genes were detected to have their corresponding coordinates generated by segmental duplications (Fig. 1, Table 1). Furthermore, 35.0% (36 of 103) of *WRKY* genes were clustered together with a maximum of 10 extra genes between them and were regarded as tandemly duplicated genes (Fig. 1, Table 2). Taken together, our analyses suggested that genome-scale duplication is the main mechanism for expansion of this *WRKY* gene family accompanied by segmental and tandem duplications.

Expression profile of WRKY genes under normal growth conditions

To examine if these predicted genes were expressed in rice and to explore their expression patterns, total RNA samples from various rice tissues were subjected to RT–PCR analysis. In total, we have analyzed the expression of all 103 predicted genes in six different tissues including young and mature leaves, panicles and roots. The results showed that not all predicted genes were expressed in plants grown under normal growth conditions. Among 103 predicted genes, 65 genes (63.1%) were expressed in at least one of the six tissues (Fig. 2). Among these 65 genes, some of them were expressed in all tested tissues with similar or different expression intensities. Eighteen genes showed such expression patterns: *01g09100*, *01g46800*, *03g20550*, *03g53050*, *03g55080*, *03g63810*, *04g21950*, *05g09020*, *05g27730*, *07g02060*, *08g17400*, *09g09630*, *09g25060*, *11g02480*, *11g02530*, *11g45850*, *12g32250* and *12g40570*. Some of the *WRKY* genes revealed a lower expression level among all tested tissues. These genes were *01g14440*, *02g08440*, *02g43560*, *03g21710*, *04g46060*, *05g04640*, *05g49620*, *08g29660*, *10g42850*, *09g30400*, *11g02520* and *12g02450*. On the other hand, at least six *WRKY* genes showed root-specific expression: *01g09080*, *01g51690*, *01g54600*, *01g60640*, *02g16540* and *08g09810*;

Table 1 Expression divergence among fragmentally duplicated *WRKY* genes

Coordinate1	Tissue/stress[a]	Coordinator2	Tissue/stress[a]	Coordinator3	Tissue/stress[a]	Coordinator4	Tissue/stress[a]
01g09100	+/+(6)	*05g09020*	+/+(4)				
01g43550	**+/+(3)**	***05g50700***	**–/–**				
01g46800	**+/–**	***05g49620***	**+/+(2)**				
01g47560	**–/–**	***05g49100***	**+/+(4)**				
01g51690	+/+(3)	*05g46020*	+/+(2)				
01g53040	**+/–**	***01g54600***	**+/+(2)**				
01g60520	**–/–**	***12g02400***	**–/+(3)**				
01g61080	+/+(3)	*05g39720*	+/+(2)				
02g08440	**+/–**	***06g44010***	**+/+(3)**				
02g43560	**+/+(3)**	***04g46060***	**+/–**				
02g47060	**+/+(1)**	***10g42850***	**+/+(1)**	***04g50920***	**–/–**		
04g51560	**+/+(1)**	***08g13840***	**–/–**				
05g04640	**+/+(2)**	***01g18600***[b]					
07g02060	**–/–**	***03g55080***	**+/+(2)**				
07g48260	**+/+(1)**	***03g21710***	**+/+(3)**	***05g40060***	**–/–**	***01g60640***	**+/+(1)**
08g38990	**–/–**	***09g30400***	**+/–**				
11g02470	**–/–**	***12g02400***	**–/+(3)**	***05g40070***	**–/–**	***01g60540***	**–/–**
11g02480	**+/+(3)**	***12g02420***	**+/+(2)**	***01g60490***	**–/–**		
11g02520	+/+(3)	*12g02440*	+/+(5)				
11g02530	+/+(2)	*12g02450*	+/+(3)				
12g01180	**–/–**	***11g01180***[b]					

[a]The expression profiles of the corresponding genes. '+' or '–' before the solidus (/) indicates that the gene was expressed or not expressed in the tested tissues including young and mature leaves, panicles and roots, respectively. '+' or '–' after the solidus indicate that the gene was regulated or not regulated by various stresses, respectively. Values in parentheses indicate the numbers of tested stress elements significantly regulating the expression of the corresponding genes. Genes with bold fonts indicated fragmentally duplicates with significant divergence in their expression patterns.
[b]These coordinators have evolved into non-WRKY family members.

Table 2 Expression divergence among tandemly duplicated *WRKY* genes

Cluster	Locus No.	Tissue/stress[a]	Cluster	Locus No.	Tissue/stress[a]
1	*01g09080*	+/+(1)	**8**	***08g09800***	**–/–**
	01g09100	+/+(6)		***08g09810***	**+/–**
2	***01g43550***	**+/+(3)**		***08g09840***	**+/+(4)**
	01g43650	**+/–**		***08g09900***	**+/–**
3	***01g60490***	**–/–**	**9**	***09g25060***	**+/+(3)**
	01g60520	**–/–**		***09g25070***	**+/+(4)**
	01g60540	**–/–**	**10**	***11g02470***	**–/–**
	01g60600	**–/–**		***11g02480***	**+/+(3)**
	01g60640	**+/+(1)**		***11g02520***	**+/+(3)**
4	***03g55080***	**+/+(2)**		***11g02530***	**+/+(2)**
	03g55164	**–/–**		***11g02540***	**+/+(6)**
5	***05g25700***	**–/–**	**11**	***11g45850***	**+/+(5)**
	05g25770	**+/+(4)**		***11g45920***	**–/–**
6	***05g40060***	**–/–**	**11**	***12g02400***	**–/+(3)**
	05g40070	**–/–**		***12g02420***	**+/+(2)**
	05g40080	**–/+(1)**		***12g02440***	**+/+(5)**
7	***05g50610***	**+/+(1)**		***12g02450***	**+/+(3)**
	05g50700	**–/–**		***12g02470***	**–/–**

[a]See footnotes of Table 1.

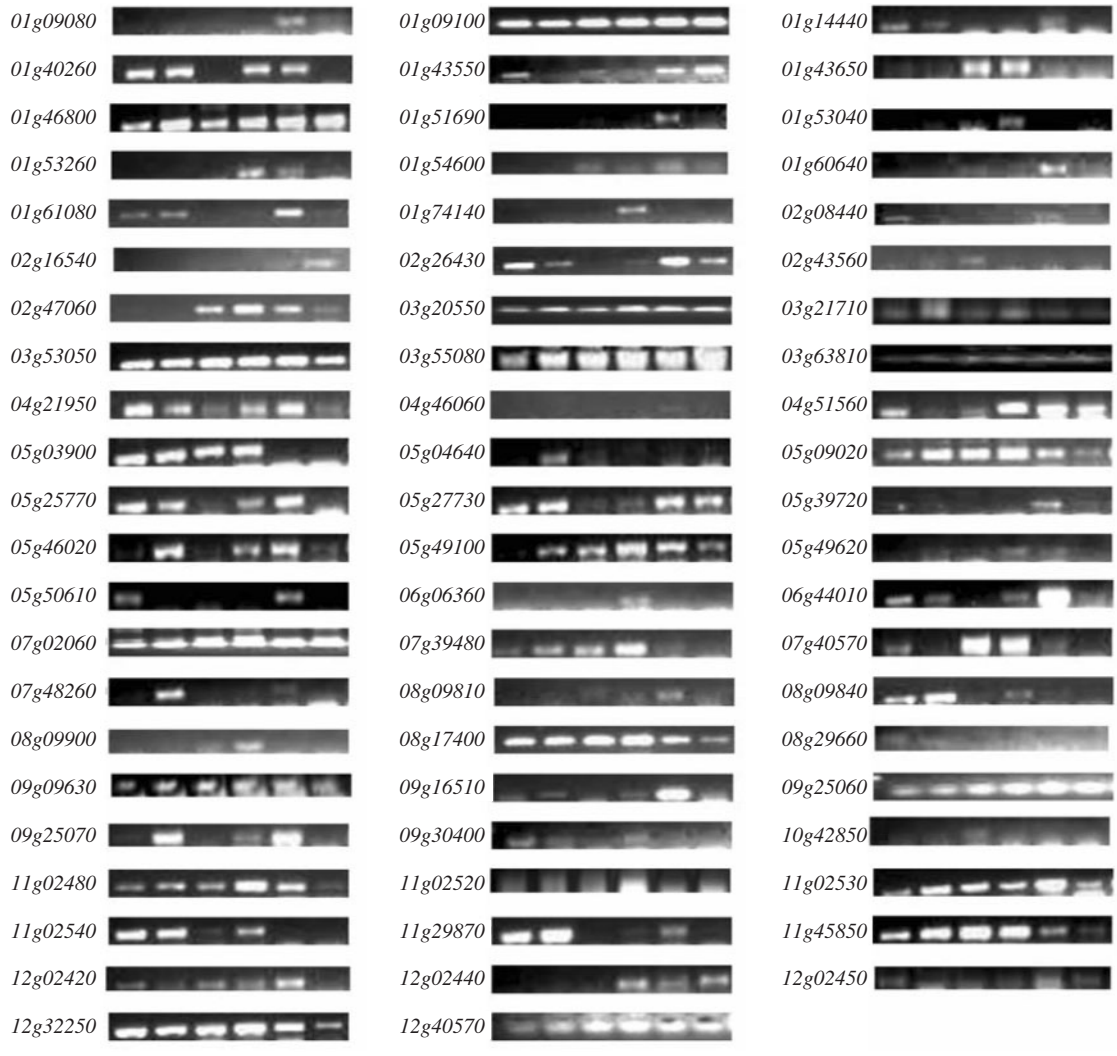


Fig. 2 Expression profiles of *WRKY* genes in various tissues shown by RT–PCR analyses. Six amplified bands from left to right for each *WRKY* gene represent amplified products from young leaves, mature leaves, young panicles, mature panicles, young roots and mature roots.

and four genes showed panicle-specific expression: *01g53040*, *01g74140*, *06g06360* and *08g09900*. Out of 103 genes, the remaining genes exhibited no expression at all in any of these tissues.

Expression patterns under various stress conditions revealed by RT–PCR

To explore the roles of *WRKY* genes under various abiotic stress conditions (cold, drought or salinity) and hormone [ABA, gibberellic acid (GA_3), IAA, methyl jasmonate (MeJA) and SA] treatments, RT–PCRs were carried out to detect the differences in their expression abundance. The results indicated that 68 WRKY members showed expression in one or more samples. Among them, 65 genes were also expressed under normal growth conditions (Fig. 1). Thus, three genes were expressed only under stress conditions, i.e. *01g08710*, *05g40080* and *12g02400*, suggesting specific roles for these genes in stress conditions. The *01g08710* gene was expressed only under cold conditions (Fig. 3A). The *05g40080* gene was induced by drought treatment (Fig. 3B). However, *12g02400* showed expression in drought- (Fig. 3C), MeJA- (Fig. 3D) and SA- (Fig. 3E) treated samples. These expression patterns were also confirmed by real-time PCR analyses (bottom panel in Fig. 3A–E).

Expression analyses under various stresses by real-time PCR

To confirm the results obtained by RT–PCR analysis and to attempt to quantify the expression levels, real-time PCR was performed and the results were compared. In the meantime, in order to demonstrate that various abiotic and phytohormone stimuli in this study (Materials and

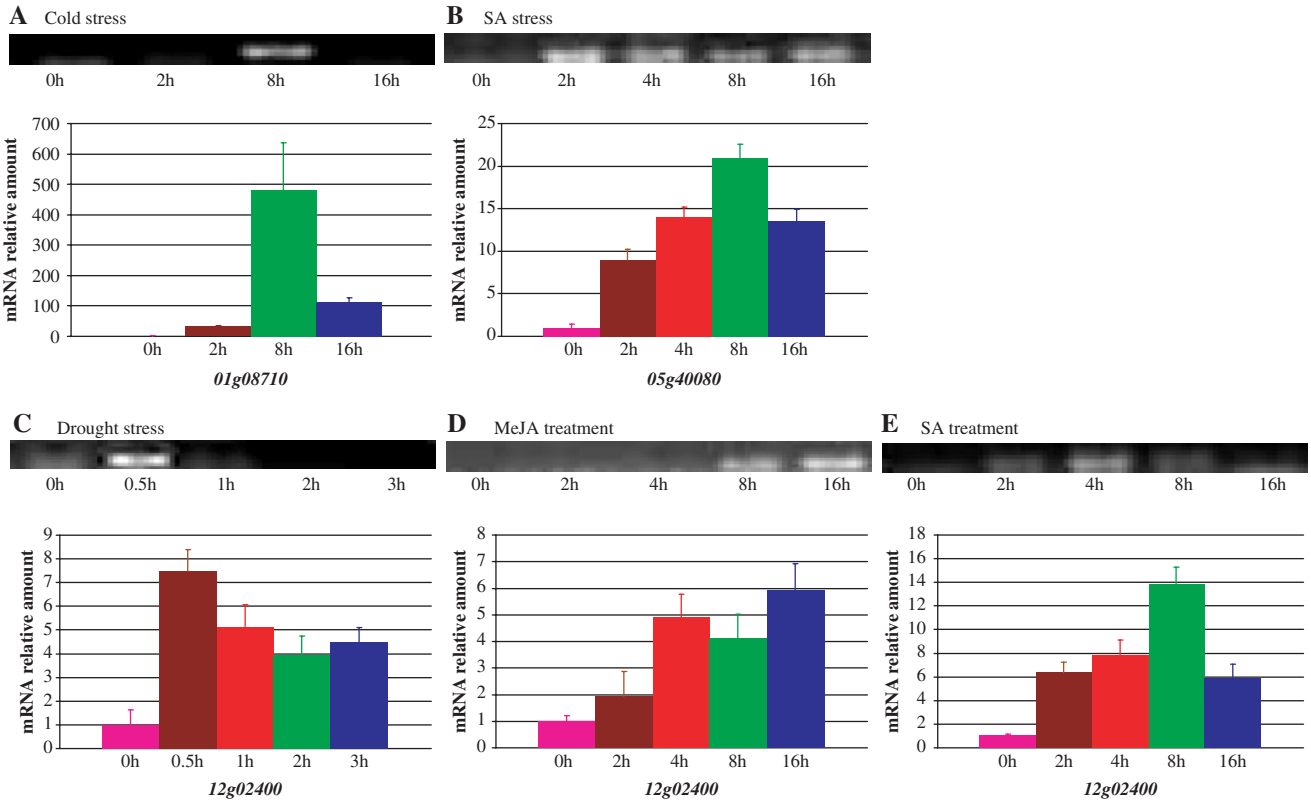


Fig. 3 Expression patterns of three stress-induced *WRKY* genes. In A–E, the top panel shows the RT–PCR result and the bottom panel shows the corresponding real-time PCR result. The relative amount of mRNA (*y*-axis) was calculated as described in Materials and Methods. Thr rice *ACTIN* 1 (*OsAct1*) gene was used as an internal control to normalize the data. (A) Expression patterns of the *WRKY* gene *01g08710* under cold growth conditions. The pink, vermeil, green and blue bars indicate the relative amount of mRNA at 0, 2, 8 and 16 h, respectively, after the cold treatment. (B) Expression profiles of the *WRKY* gene *05g40080* under SA treatment. (C–E) Transcript profiles of the *WRKY* gene *12g02400* under drought, MeJA and SA treatment, respectively. The pink, vermeil, red, green and blue bars in B–E indicates the relative amount of mRNA detected 0, 0.5, 1, 2 and 3 h, respectively, after drought treatment, and 0, 2, 4, 8 and 16 h after MeJA or SA treatments.

Methods) worked properly, eight previously reported genes were employed as positive controls to monitor corresponding treatments by real-time PCR analyses. These results showed that all three abiotic stress-induced and five hormone-induced genes were up-regulated by corresponding stresses/hormone (Supplementary Fig. S1). Thus, samples from various abiotic stress or hormone treatments can be used to analyze the expression profiles of rice *WRKY* genes under appropriate conditions by RT–PCR and real-time PCR. As shown in Fig. 4, expression profiles between RT–PCR and real-time PCR under eight different stresses, cold (Fig. 4A), drought (Fig. 4B), salinity (Fig. 4C), ABA (Fig. 4D), GA_3 (Fig. 4E), IAA (Fig. 4F), MeJA (Fig. 4G) and SA (Fig. 4H), showed no significant difference in the message levels. However, in some instances, no obvious difference was observed in RT–PCR analysis (top panel in Fig. 4C and F) while a significant difference was revealed by real-time PCR analysis (bottom panel in Fig. 4C and F). Therefore, real-time PCR was carried out for critical expression evaluation of 68 genes which showed a detectable expression signal in RT–PCRs. These analyses showed that 54 out of 68 detected genes exhibited significant differences in their expression level under one or more stress conditions or treatments. These genes and their responses to stresses and treatments together with their phylogeny are shown in Fig. 5. Generally, under cold stress, most of the responsive genes showed down-regulation of their expression (Fig. 5). In contrast, most of the stress-responsive genes showed up-regulation under the other seven conditions—drought, salinity, ABA, IAA, GA_3, MeJA and SA. On the other hand, the majority of *WRKY* genes (28 out of 54, 52%) were up-regulated by SA and only eight genes were up- or down-regulated by GA_3 treatment.

By comparing the expression data with the phylogenetic relationship, it was obvious that expression divergence was observed even in closely related genes clustered together in the phylogenetic tree (Fig. 5). For example, the first

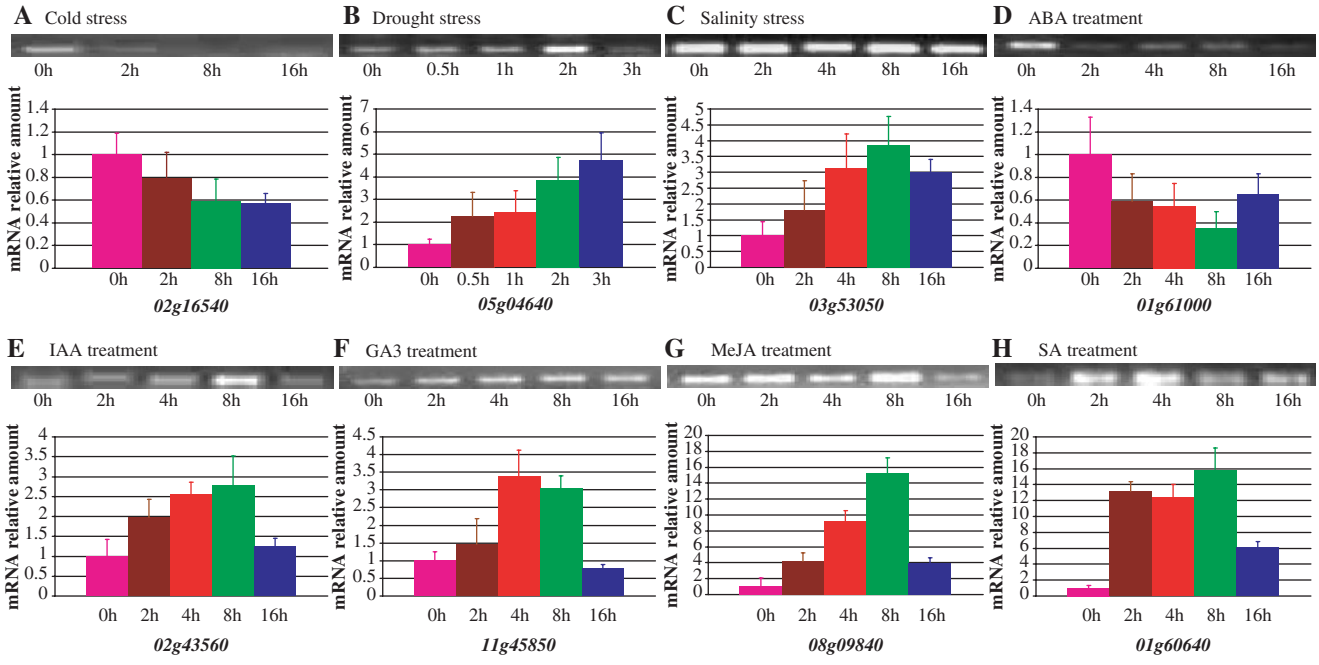


Fig. 4 A comparison of the expression patterns shown by RT–PCR and real-time PCR analyses under eight different stresses. In A–H, the top panel indicates the RT–PCR result and the bottom panel shows the corresponding real-time PCR result. The relative amount of mRNA (*y*-axis) was calculated according to the description in Materials and Methods. A rice *ACTIN* 1 (*OsAct1*) gene was used as an internal control to normalize the data. (A) Expression patterns under cold growth conditions. The pink, vermeil, green and blue bars indicated the relative amount of mRNA detected 0, 2, 8 and 16 h, respectively, after the cold treatment. (B–H) Expression profiles under drought, salinity, ABA, IAA, GA_3, MeJA and SA, respectively. The pink, vermeil, red, green and blue bars indicated the relative amount of mRNA detected 0, 0.5, 1, 2 and 3 h, respectively, after the PEG treatment, and 0, 2, 4, 8 and 16 h after salinity or various hormone treatments.

cluster consisted of the five members *01g43550*, *02g16540*, *10g42850*, *02g47060* and *01g54600*. They exhibited completely different expression patterns in response to different stress conditions (Fig. 5).

WRKY genes regulated by abiotic stresses

Real-time PCR analyses showed that 41 *WRKY* genes were significantly regulated by abiotic stresses including cold, drought and salinity (Fig. 6). Among them, 22 *WRKY* genes were regulated by only one of these stresses (nine by cold or salinity and four by drought, Fig. 6). Another set of 17 genes were regulated by two of these three stresses (Fig. 6). The remaining two genes, *06g06360* and *11g02540*, were regulated by all three stresses (Fig. 6).

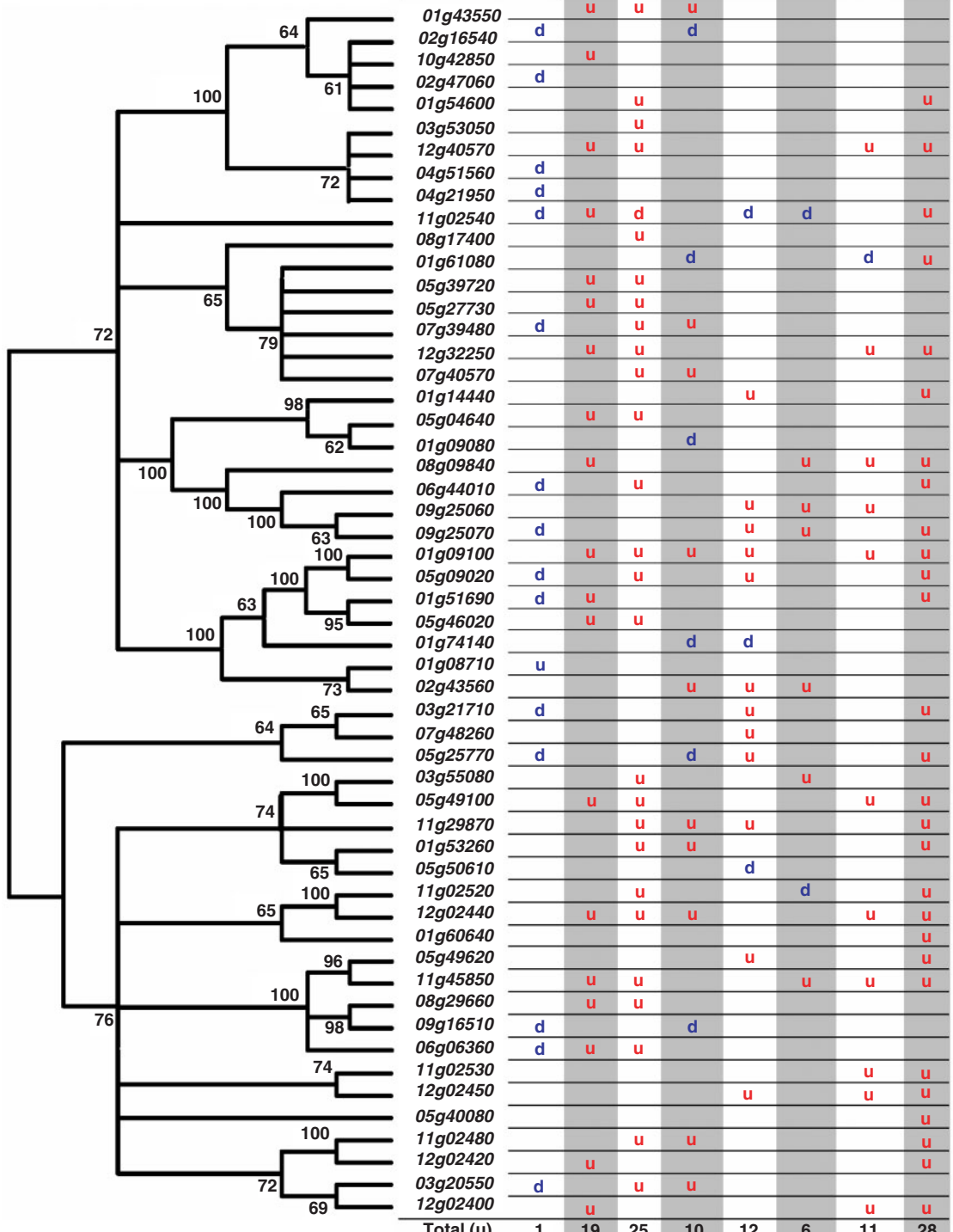
WRKY genes regulated by various hormone treatments

Besides abiotic stresses, various hormones were also shown to play important roles in regulating *WRKY* gene expression. In total, 41 *WRKY* genes were detected with significant differences in their expression levels under various hormone treatments (Fig. 7A). Among them, 16 genes were regulated by only one of the five treatments. Thirteen and 11 *WRKY* genes were expressed in response to two and three treatments, respectively. Only one *WRKY* gene (*01g09100*) was regulated by the four hormones ABA, IAA, MeJA and SA. None of these *WRKY* genes was regulated by all five treatments.

Among the 16 genes regulated by only one hormone treatment, three genes were down-regulated by ABA treatment (*01g09080*, *02g16540* and *09g16510*); the other down-regulated gene was *05g50610* under IAA treatment (Fig. 7B). All the other genes were up-regulated by ABA, IAA, GA_3 or SA, and none of the genes was regulated only by MeJA (Fig. 7B). Among the 13 genes responsive to two hormone treatments, all of them were up-regulated (Fig. 7C). Interestingly, the highest ratio of *WRKY* genes (5 out of 13) was regulated by both MeJA and SA, suggesting the interaction of these two hormones (Fig. 7C). On the other hand, we have detected 11 *WRKY* genes responsive to three treatments with seven different hormone combinations. For each combination, only one or two *WRKY* genes was found to be regulated (Fig. 7D).

WRKY genes regulated by the combination of abiotic stress and hormone treatment

Among 54 stress-responsive *WRKY* genes, 13 genes were regulated by only abiotic or hormone treatment and 28 genes were regulated by both abiotic and hormone



Locus No.	Col	Dro	Sal	ABA	IAA	GA3	MeJA	SA
01g43550		u	u	u				
02g16540	d			d				
10g42850		u						
02g47060	d							
01g54600			u					u
03g53050			u					
12g40570		u	u				u	u
04g51560	d							
04g21950	d							
11g02540	d	u	d		d	d		u
08g17400			u					
01g61080				d			d	u
05g39720		u	u					
05g27730		u	u					
07g39480	d		u	u				
12g32250		u	u				u	u
07g40570			u	u				
01g14440					u			u
05g04640		u	u					
01g09080				d				
08g09840		u				u	u	u
06g44010	d		u					u
09g25060					u	u	u	
09g25070	d				u	u		u
01g09100		u	u	u	u		u	u
05g09020	d		u		u			u
01g51690	d	u						u
05g46020		u	u					
01g74140				d	d			
01g08710	u							
02g43560				u	u	u		
03g21710	d				u			u
07g48260					u			
05g25770	d			d	u			u
03g55080			u			u		
05g49100		u	u				u	u
11g29870			u	u	u			u
01g53260			u	u				u
05g50610					d			
11g02520			u			d		u
12g02440		u	u	u			u	u
01g60640								u
05g49620					u			u
11g45850		u	u			u	u	u
08g29660		u	u					
09g16510	d			d				
06g06360	d	u	u					
11g02530							u	u
12g02450					u		u	u
05g40080								u
11g02480			u	u				u
12g02420		u						u
03g20550	d		u	u				
12g02400		u					u	u
Total (u)	1	19	25	10	12	6	11	28
Total (d)	15	0	1	6	3	2	1	0

Fig. 5 Phylogenetic tree of rice WRKY members and their expression profiles under stressed conditions. (A) A neighbor-joining bootstrap consensus tree of 54 stress-regulated WRKY members. The amino acid sequence of only one WRKY domain (the C-terminal domain was selected if two domains were detected) was used for the phylogenetic tree construction. (B) Expression patterns of 54 *WRKY* genes under abiotic (cold, drought and salinity) and various hormone (ABA, IAA, GA_3, MeJA and SA) treatments. The results were from two biologically replicated real-time PCR analyses. The blue 'd' indicates that the corresponding gene was significantly down-regulated by t-test under a stress treatment. The red 'u' indicates up-regulation by t-test. Col, cold; Dro, drought; Sal, salinity.

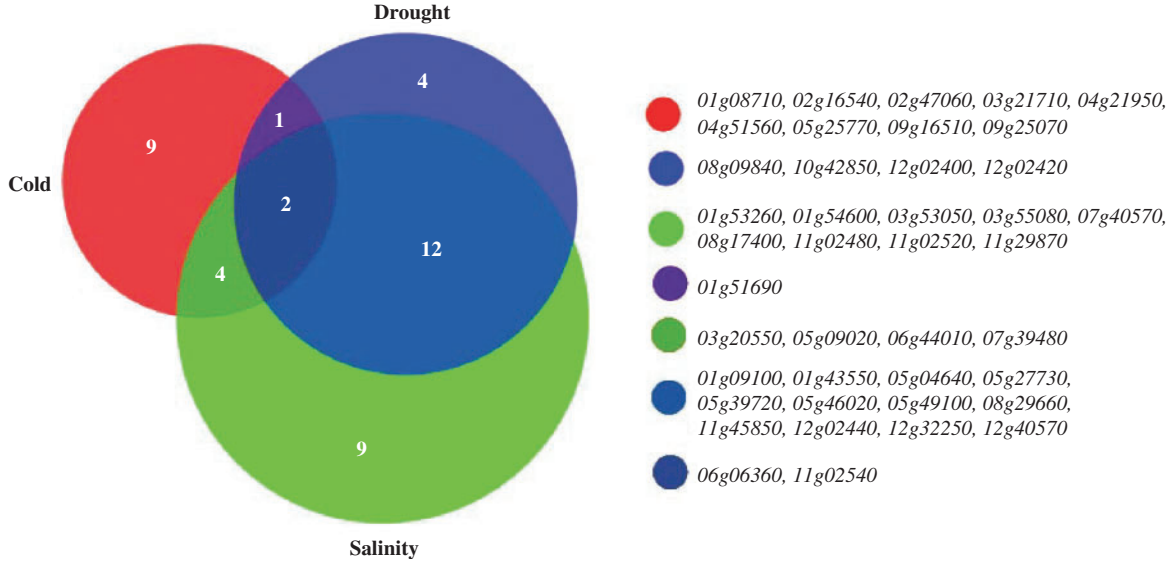


Fig. 6 Summary of the expression analyses of *WRKY* genes under cold, drought and salinity treatment. (A) Venn diagram showing the classification of genes inducible by cold, drought and salinity stresses based on real-time PCR analyses. The inducible genes were classified into various groups showing the numbers with one or more stress-inducible genes. (B) List of the genes in the various groups identified in (A).

treatments (Fig. 8A). These genes are listed in Fig. 8B. This result suggested that some *WRKY* genes might play a role only in the abiotic or hormone signal pathway, and most of the stress-responsive *WRKY* genes might play an important role in abiotic stresses by interacting with hormone signaling.

Expression divergence among fragmentally or tandemly duplicated WRKY genes

To understand the functional divergence of fragmentally duplicated *WRKY* genes, we compared the expression profiles of these genes in different tissues or under various stress conditions. In total, 16 groups of fragmentally duplicated genes exhibited significant divergence in their expression patterns (shown in bold in Table 1). The remaining five groups showed similar expression patterns among different coordinates in each group (Table 1). However, these coordinates exhibited expression divergence in response to various stresses. For example, *01g09100* and its coordinate *05g09020* were expressed in tissues and regulated by stresses; however, the former was regulated by six different stresses while the latter was regulated by only four stresses (Table 1). On the other hand, two fragmentally duplicated genes, *01g18600* and *11g01180* (indicated in Table 1), have evolved into non-WRKY family members. Therefore, most of the fragmentally duplicated genes have shown high expression divergence during evolution.

Similarly, higher expression divergence was also observed among tandemly duplicated *WRKY* genes. We have detected 11 tandem array clusters with a maximum of 10 gene spacers. Cluster 1 contained both *01g09080* and *01g09100*. (Table 2). These two genes exhibited a difference in response to various stresses. The former was down-regulated by ABA while the latter was up-regulated by drought, salinity, ABA, IAA, MeJA and SA (Fig 5A, Table 2). Among the remaining 10 clusters marked in bold in Table 2 (cluster 2–11), at least one gene in each cluster revealed a difference in their expression patterns in different tissues and/or under various stress conditions in comparison with the other members within the same cluster (Table 2). Furthermore, the expression signal was absent for some members of a few clusters. For example, we could not detect any expression signal in six different tissues or under various stress conditions for four *WRKY* genes in cluster 3 (Table 2).

Discussion

Annotation of WRKY genes in rice genome

Several groups have reported the genome-wide identification and annotation of rice *WRKY* genes (Wu et al. 2005, Xie et al. 2005, Zhang and Wang, 2005, Ross et al. 2007). However, different groups reported different numbers (ranging from 83 to 109) of *WRKY* genes in the rice genome. For example, 102 *WRKY* genes were predicted by Wu et al. (2005); 109 *WRKY* genes were identified and four of them were found to encode an incomplete WRKY domain (Zhang and Wang 2005). However, no sequence information was available for these predicted *WRKY* genes.

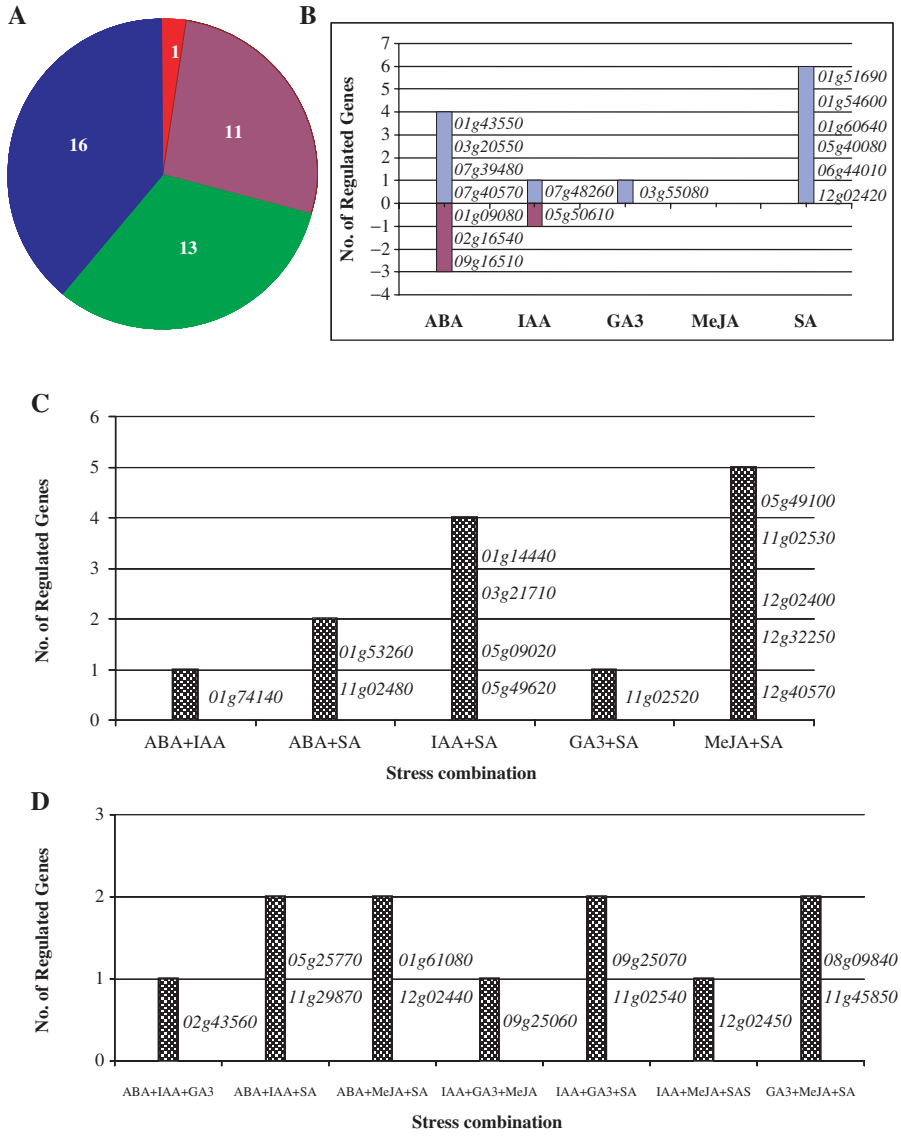


Fig. 7 Summary of expression analyses of *WRKY* genes under various hormone treatments. (A) A pie diagram showing the numbers of genes regulated by a single hormone (shaded in blue), two hormones (green), three hormones (vermeil) and four hormones (red). (B) List of genes regulated by only one of the five hormones. Blue bars indicate the numbers of genes up-regulated by the hormone, and the corresponding genes are listed in the right of the column. Vermeil bars indicate the numbers of genes down-regulated by the hormone. Corresponding genes are also listed in the right of the column. (C) and (D) List of genes regulated by three and four hormones, respectively. Shaded bars in (C) and (D) indicate the numbers of genes up-regulated by three and four hormone combinations. Corresponding genes are listed in the right of this column.

Currently, 83 annotated *WRKY* genes have been deposited in the TIGR database by Xie et al. (2005) (http://rice.tigr.org/tdb/e2k1/osa1/ca/gene_fams/3_5.shtml). In this study, we have identified 103 *WRKY* genes. Compared with the latest annotation of 98 *WRKY* genes (Ross et al. 2007), the member *11g45750* predicted by their group was not included in our study since no WRKY domain was detected in its deduced amino acid sequence. Furthermore, six other members were predicted to encode the WRKY domain (*01g62510*, *04g04300*, *08g09800*, *08g09810*, *08g09840* and *08g09900*). Of these, both *01g62510* and *04g04300* encode only a WRKY domain, and their encoded proteins lacked the zinc finger motifs. The remaining four genes encoded both a WRKY domain and a zinc finger motif. Therefore, we included these six members subsequently in our analysis. RT–PCR and real-time PCR analyses showed that no expression was detected for the three genes *01g62510*, *04g04300* and *08g09800*, both in

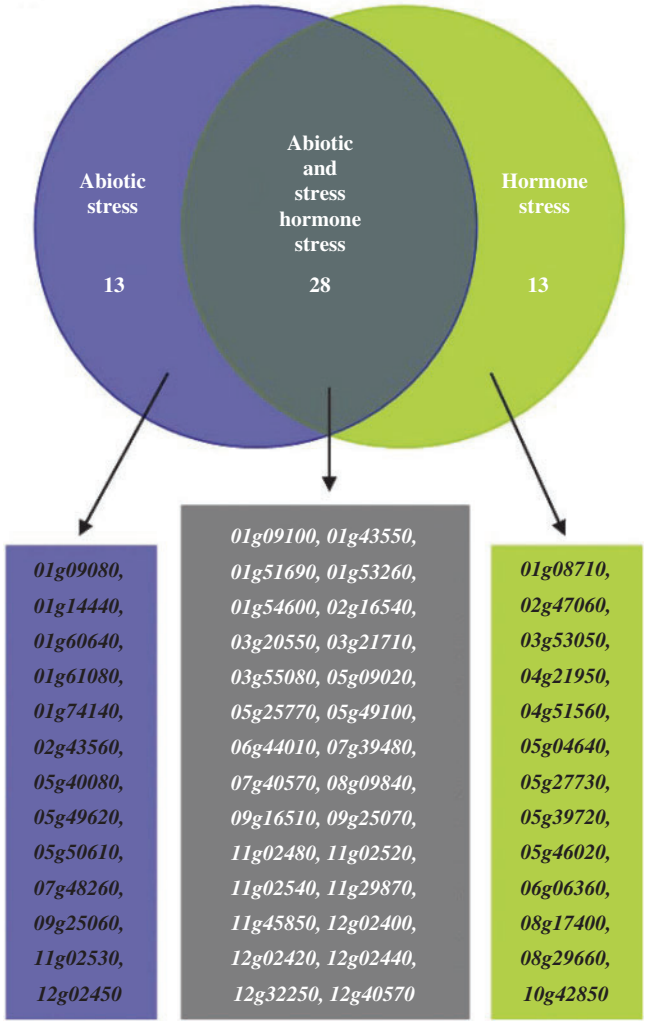


Fig. 8 Summary of expression analyses of 54 stress-regulated *WRKY* genes under both abiotic and various hormone treatments. (A) Venn diagram showing the classification of genes inducible by abiotic or hormone treatments based on real-time PCR analyses. Inducible genes were classified into three groups, i.e. regulated by only abiotic stress (shaded in blue), by only various hormones (shaded in gray) and by both abiotic and hormone combinations (shaded in green). (B) A list of the genes of the three groups identified in (A).

different tissues and under various stress conditions. The remaining three genes were expressed in a few tissues (Fig. 2) and the expression of *08g09840* was up-regulated by drought, GA_3, MeJA and SA (Fig. 5).

Expansion and retention of WRKY genes in rice genome

Based on the numbers of WRKY and zinc finger motifs present in the proteins, rice WRKY members were classified into four groups: (i) group I consisting of proteins with two WRKY domains and two zinc finger motifs (C2H2/C2HC); (ii) group II comprising proteins with one WRKY domain and a C2H2 motif; (iii) group III with members that possessed one WRKY domain and a C2HC motif; (iv) and group IV consisting of proteins with only one WRKY domain or accompanied by an incomplete zinc finger motif (Ross et al. 2007). Recently, a hypothesis has been proposed to explain how different groups of *WRKY* genes have evolved or expanded in plants (Xie et al. 2005, Zhang and Wang 2005, Ross et al. 2007). However, little is known about the expansion of *WRKY* genes in the rice genome. Here, we compared the physical mapping of rice *WRKY* genes with large-scale duplicated regions as well as fragmental or tandem duplications in the rice genome. We found that the majority of rice *WRKY* genes (77.7%) were located in duplicated regions (Fig. 1); 45.6% of *WRKY* genes were fragmentally duplicated and 35% of them were tandemly duplicated. These results suggested that these genome duplications could be regarded as the major mechanism for expansion of this family. After gene duplication, one copy might have been kept or lost (Seoighe et al. 2003); it is of interest to consider how these duplicated genes were retained. We had surveyed expression divergence among these duplicated genes under normal or stressed growth conditions and our results showed that high expression divergence had occurred not only among fragmentally duplicated genes but also among tandemly duplicated genes (Tables 1, 2). These results suggested that high expression divergence was one of the mechanisms for the retention of these duplicated genes.

Possible roles of WRKY genes under normal growth conditions

We have investigated the expression patterns of *WRKY* genes in six different tissues of rice plants grown under normal conditions by RT–PCR analyses. The expression data showed that several of them were expressed in all tested tissues; a number of them showed tissue-specific expression patterns (Fig. 2). These expression profiles suggested divergence in biological functions of *WRKY* genes in plant development. The RT–PCR analyses also showed that only around 63% of *WRKY* genes showed expression in at least one of the six tested tissues. The remaining 37% of these genes with no detectable expression signal might be expressed in other tissues such as stem and seeds, or their expression level could be too low to be detected by RT–PCR. Alternatively, these genes could be stress inducible or pseudogenes.

To validate the expression data, we have developed transgenic plants ectopically expressing a *WRKY* gene *01g51690* based on its induction of gene expression in drought-stressed seedlings. This gene also showed young panicle-specific expression (Fig. 2) under normal growth conditions. Four independent overexpression lines were selected for phenotypic investigation. Our preliminary

results showed that three of these lines exhibited complete sterility and, as a result, no viable seeds were obtained, and the fourth line was found to produce only a few viable seeds (data not shown). Upon further investigation, their pollen showed normal viability, which suggested that the female part of these transgenic lines may be defective. These results suggested that the gene might play important roles during development of female reproductive organs. In addition to this, since the transcript of this gene is induced in drought-stressed seedlings, it can be speculated that this gene may be involved in stress signaling during drought conditions.

WRKY gene expression in response to abiotic stresses

WRKY TFs have been reported to be involved in various abiotic stress signaling pathways (Rizhsky et al. 2002, Seki et al. 2002, Mare et al. 2004, Zou et al. 2004, Hwang et al. 2005). In Arabidopsis, at least four *WRKY* genes were shown to be involved in the drought, cold or salinity response (Seki et al. 2002). Upon salinity stress, the levels of 18 *WRKY* transcripts in Arabidopsis were induced >2-fold and those of eight *WRKY* genes were repressed, as shown by microarray analysis (Jiang and Deyholos 2006). In rice, 10 *WRKY* genes were differentially regulated by NaCl, polyethylene glycol (PEG), cold or heat (Qiu et al. 2004). In this study, we have detected 41 rice *WRKY* genes with significantly higher or lower expression by real-time PCR analyses, when subjected to cold, drought or salinity stresses. Among them, 16, 19 and 26 *WRKY* genes were regulated by cold, drought and salinity, respectively (Fig. 5). To our knowledge, this is the first report on the genome-wide expression analyses of rice *WRKY* genes under various abiotic stresses.

Since abiotic stress-regulated genes act either in an ABA-dependent or an ABA-independent manner (Oh et al. 2005), we investigated the relationship between ABA- and abiotic stress-regulated *WRKY* genes. The result showed that among 16 ABA-regulated *WRKY* genes, 12 genes were also regulated by cold, drought or salinity (Fig. 5). This result suggested that the majority of rice *WRKY* genes might be involved in the abiotic stress signal pathway in an ABA-dependent manner.

WRKY gene signaling pathways mediated by various hormones

Previous reports showed that ABA, GA_3 and SA have been involved in the WRKY-mediated hormone signal pathway during seed germination (Zhang et al. 2004, Xie et al. 2005, Xie et al. 2006, Xie et al. 2007). We also surveyed expression patterns of *WRKY* genes under treatment with these three hormones at the seedling stage. Our data showed that only one *WRKY* gene (*02g43560*) was regulated by both ABA and GA_3 (Fig. 5). Five genes were co-regulated by GA_3 and SA; seven genes were co-regulated by ABA and SA. These results suggested that *WRKY* genes may play a role in hormone signaling pathways during the seedling stage.

On the other hand, accumulating evidence has shown that many *WRKY* genes positively or negatively regulate disease resistance in plants (Eulgem et al. 2000, Ulker and Somssich 2004, Eulgem 2006, Eulgem and Somssich 2007, Ross et al. 2007). In rice, at least four *WRKY* genes were reported to be involved in disease: *OsWRKY03* (Liu et al. 2005), *OsWRKY13* (Qiu et al. 2007), *OsWRKY45* (Shimono et al. 2007) and *OsWRKY71* (Liu et al. 2007). Since local and systemic defense responses in plants are controlled by the mutually antagonistic hormones SA and jasmonic acid (Bostock 2005), expression analyses of *WRKY* genes were carried out to investigate the response to SA or MeJA. In rice, at least seven *WRKY* genes were differentially regulated by SA or jasmonic acid (Ryu et al. 2006). We have detected 28 *WRKY* genes with significant difference in their transcript levels following SA treatment (Fig. 5). Among the 12 MeJA-regulated *WRKY* genes, 11 were also regulated by SA, suggesting an interaction between these two hormone signaling pathways and signifying the importance of rice *WRKY* genes in plant defense responses.

Materials and Methods

Database search for genes encoding WRKY domain

Since 98 *WRKY* genes were predicted and were available (Xie et al. 2005, Ross et al. 2007), we used these *WRKY* genes as the representative sequences for BLAST searches. The domain amino acid sequences of the representative *WRKY* genes were used to identify other *WRKY* genes with a high similarity in the rice genome using the TBLASTN and BLASTP programs in the TIGR (http://tigrblast.tigr.org/euk-blast/index.cgi?project=osa1, Version 5 of the TIGR rice pseudomolecules and genome annotation) database. All predicted *WRKY* genes were used for similarity searches again in order to confirm these predicted genes and to detect new candidates.

Identification of domains in the predicted WRKY genes

In order to confirm the presence of conserved domains in these predicted genes and use their sequences for phylogenetic analysis, domains in predicted *WRKY* genes were identified using the Pfam program (http://www.sanger.ac.uk/). The 'Conserved domains' search program in the NCBI database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was also employed to detect the WRKY domain. Domain searches with two programs were performed under a default E-value level (0.01).

Sequence alignment and phylogenetic analysis

The DNASTAR program was used for preliminary sequence manipulations. The sequence alignment was generated using ClustalX (Version 1.8, Thompson et al. 1997). The aligned amino acid sequences formed the basis for the phylogenetic analysis according to the description by Jiang and Ramachandran (2006).

Mapping and detecting of duplicated genes

Physical mapping and detection of duplicated *WRKY* genes were carried out according to our previous description (Jiang et al. 2008).

Plant materials and stress treatments

Japonica rice (*Oryza sativa* cv. Nipponbare) was used for all experiments. The rice plants were grown under greenhouse conditions. Two-week-old seedlings were used for all abiotic and hormone treatments.

For the drought treatment, seedlings were treated with 30% PEG, and whole plants were collected at various time intervals (0, 0.5, 1, 2 and 3 h), and then frozen in liquid nitrogen for RNA preparation. For salinity and cold treatments, seedlings were subjected to a 250 mM NaCl solution and 4°C conditions, respectively. Samples for RNA extractions were collected at 0, 2, 4, 8 and 16 h time intervals. For hormone treatments, seedlings were subjected to various hormone solutions (ABA, GA_3, IAA, MeJA and SA at the same concentration of 100 μM). Samples were then collected at 0, 2, 4, 8 and 16 h intervals respectively.

Primer selection, RT–PCR and real-time PCR

All primers used in this study were designed by Applied Biosystems Primer Express® software and then submitted to the NCBI database for a BLAST search. All non-specific primers shown by the search results were eliminated instead of re-designing primers with specific binding to the corresponding genes. Thus, the results from RT–PCR or real-time PCR analysis might represent the expression pattern of specific *WRKY* genes. Supplementary Table S1 lists all the primer sequences used in the present study.

Total RNA samples were prepared using a QIAGEN RNeasy Midi Kit. The first-strand cDNA was synthesized using an Invitrogen kit. RT–PCRs were performed in 20 μl reaction mixtures with 20 ng of first-strand cDNA, 200 μM of each dNTP, 2.5 mM of $MgCl_2$, 0.5 μM of each primer, 1 U of *Taq* DNA polymerase and 1 × PCR buffer provided by Qiagen. These reactions were carried out using PTC-100 thermocyclers. The following program was used for RT–PCR: 94°C for 2 min followed by 35 cycles at 94°C for 10 s, 59°C for 10 s and 72°C for 25 s, followed by a 2 min extension step at 72°C. PCR products (10 μl) were visualized in a 1.6% agarose gel and all pictures were taken in the BIORAD UV-Gel documentation system using Quantity one 1-D Analysis software.

The real-time PCR analyses were carried out according to the description by Jiang et al. 2007.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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