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Cloning and analysis of expression profile of 13 *WRKY* genes in rice

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Abstract **The transcriptional factor WRKY proteins contain the highly conserved amino acid sequence WRKYGQK as well as the novel zinc-finger-like motifs Cys_2His_2 or $Cys_2HisCys$. A search of the rice genome identified 97 genes encoding WRKY proteins. Of these 97 WRKY homologs found in rice, 13 cDNAs encoding WRKY proteins were consequently isolated from a rice cDNA library constructed from 4℃-treated shoots by probing for the conserved WRKY domain. Northern blotting analysis revealed that 10 of 13 *OsWRKY* genes were differentially regulated in plants that were treated by the four following abiotic stress factors: NaCl, PEG, cold (4℃) and heat (42℃). The resulting expression profiles exhibited great differences in both the manner and timing of their response to the four different abiotic treatments. The difference of gene expression profiles suggested the different physiological functions among the *WRKY* genes.**

Keywords: rice, transcriptional factor *WRKY* genes, abiotic stress factors, gene expression profiles.

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The transcriptional factor WRKY protein superfamily, which to date is found exclusively in plants, is defined by the presence of the conserved amino acid sequence: WRKYGQK, along with either a Cys_2His_2 or $Cys_2HisCys$ zinc-finger-like motif at its N-terminal end[1,2]. The WRKY proteins regulated the expression of target genes by specifically binding to the (T)TGACC (A/T) (W box) DNA sequences in their promoter region[3]. A number of studies had shown that the WRKY proteins had a regulatory function in plants. First, the WRKY proteins have been shown to regulate a plant's response to pathogen attack[4−10]. For instance, the group Ⅲ *Arabidopsis* WRKY proteins have been shown to be involved in different defense signaling pathways[8]. For example, the AtRRS1-R (WRKY 16) protein has a typical nucleotide-binding site (NBS), DNA-binding domain (WRKYGQK), a putative nuclear localization signal and a leucin-rich repeat (LRR) domain that is typical of disease-resistance genes. Furthermore, AtWRKY16 gene is not only a typical R gene whose role is limited to the resistance of infection by *Ralstonia solanacearum ingress*, but also plays important roles in the pathogen-induced signaling pathways[9] which uncover the plant's nucleus as the new battlefield of plant defense[11]. Another example of a WRKY protein in the regulation of defense-related signal pathways is AtWRKY70. AtWRKY70 is a protein known to repress the jasmonate-mediated signal pathway by activating the salicylate-mediated signal pathway, making it a node of convergence for the two signal pathways in plant defense[10]. Second, WRKY proteins have also been shown to be involved in regulating several important roles ranging from regulating a plant response to abiotic stress to regulating a plant's morphologic establishment[12−14]. Several convincible examples of this trend have been found in *Arabidopsis*, potato, legume and barley. AtWRKY6 protein, for example, controls plant senescence and pathogen defense by specifically binding to the W-box in the promoter of the *senescence-induced receptor-like kinase (SIRK)* gene in *Arabidopsis*[12]. While the AtWRKY53 protein plays a regulatory role in the early events of leaf senescence[13]. Another protein AtWRKY44 is involved in the development of leaf trichome and seed coat[14]. Moreover, potato WRKY protein is associated with quantitative resistance to potato late blight since the pathogen induced its expression[15,16]. Legume WRKY proteins have been shown to regulate seed dormancy and drought tolerance[17], while the Barley SUSIBA2 (WRKY) protein binds specifically to both SURE (sugar responsive) and also the W-box in the promoter of *iso1* (*isoamylase*1) gene to regulate the sugar signaling pathway[18].

Rice is an important crop with surprisingly few, detailed reports about the functions of the rice *WRKY* gene superfamily. Previously, 77 *WRKY* genes (*OsWRKY1* to *OsWRKY77*) were identified in rice from a search of its genome, and it was consequently demonstrated that the *OsWRKY71* gene acts as a transcriptional repressor in aleurone cells, and was required for GA signaling[19]. In the present study, we have re-examined the complete rice genomic sequences and identified another more than 20 additional WRKY genes and consequently, summarized the information of all currently known rice *WRKY* genes (Table 1). As the first step towards understanding the biological functions of the rice *WRKY* gene superfamily, we also cloned 13 full length WRKY cDNAs by using RT-PCR and cDNA library screening. The respective expression profiles were determined by Northern blotting analysis and it was found that 10 of 13 WRKY genes were differentially regulated by 4 different abiotic stress factors.

1 Materials and method

(ⅰ) Materials. Rice seeds (Dianxun 8) were kindly provided by Rice Research Institute, Yunnan Agriculture

Table 1 Identified members of the WRKY superfamily of transcriptional factors in rice

WRKY	Group	Chr	cDNA/EST	Gene	BAC.ORF	GenBank
1	2	1	AK105509	BK005004	P0034C11.1	AP002865
			C26525	NM_188767	OSJNBa0004G10.20	AP003074
					OSJNBa0049B20.9	AC007789
2	2	10	AK108346	BK005005	OSJNBa0056G17.18	AC018727
3	3	3	AK069091	BK005006	OSJNBa0040E01.4	AC079887
			AY341859			
4	1	3	AY341852	BK005007	OSJNBa0040E01.10	AC079887
			AY341848		OSJNBb0048A17.12	AC084282
5	2	5		BK005008	OJ1127_B08_85530~94442 bp	AC093490
6	2	3		BK005009	OSJNBa0052F07.29	AC104321
				NM_185053	OSJNBa0094F01.2	AC093713
7	2	5		BK005010	OJ1741_B01_120642~121661 bp	AC097112
8	2	5	AK109568	BK005011	OSJNBb0035N21.13	AC134929
			AY341857		OJ1651_G11_109415~111400 bp	AC098573
9	2	1		BK005012	AP002480_113596~121836 bp	AP002480
				NM_183590		
10	2	1	AK109578	BK005013	AP002486_106376~107035	AP002486
			AY341854	NM_189615		
11	2	1	AK108745	BK005014	P0688A04.2	AP002839
			AY341856	NM_192891	P0006C01.17	AP002744
12	2	1	AK111416	BK005015	P0006C01.4	AP002744
				NM_192878		
13	2	1	AK067329	BK005016	P0481E12.40	AP003076
			AK099515	NM_191228		
			AK060018			
14	2	1	AK109770	BK005017	P0435H01.23	AP003142
			D24303 C26098	NM_191885		
15	3	1		BK005018	P0694A04.6	AP003294
				NM_192434		AP003212
16	2	1	AU164995	BK005019	P0003E08.17	AP003222
			AY341855	NM_191908		
			AY341844			
17	2	1	AK110625	BK005020	P0698H10.6	AP003298
			AY341842	NM_189473	P0518C01.28	AP003277
18	3	10		BK005021	OSJNBa0073L20.17	AC099774
						AE017076
19	3	5	AK108389	BK005022	AC104284_149826~159453	AC104284
20	3	1		BK005023	P0485B12.3	AP003348
				NM_190349	P0703B11.11	AP003302
21	3	1	AK108657	BK005024	P0703B11.23	AP003302
				NM_190360	P0485B12.15	AP003348
22	3	1		BK005025	P0703B11.5	AP003302
				NM_190343		
23	2	1	AK108909	BK005026	B1060H01.7	AP003560
			AY341845	NM_191553	OSJNBb0036G09.13	AP003309
24	1	1	AK107199	BK005027	P0439E11.17	AP003315

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WRKY	Group	Chr	cDNA/EST	Gene	BAC.ORF	GenBank
			AY341849	NM_190410		
25	2	8		BK005028	OJ1449_H02.26	AP004648
				BK005047		
26	2	1	AK108555	BK005029	B1131B07.13	AP003408
				BK005062		
				NM_191743		
27	2	1		BK005030	P0700A11.6	AP003300
				NM_192540	P0712E02.25	AP003492
28	2	6	AK106282	BK005031	AP003517_105914~110036 bp	AP003517
			AK119644			
29	3	7	AY341858	BK005032	OJ1123_C12.16	AP003746
				NM_186188		
30	1	8	AK065518	BK005033	OJ1118_A06.2	AP 003873
			AK062027			
31	2	6		BK005034	AP003951_62471~61311 bp	AP003951
32	2	2		BK005035	OJ1079_F11.32	AP004080
					OJ1353_F08.1	AP004058
33	3	9		BK005036	AC090056_108227~115569 bp	AC090056
				BK005041	AP006758_46362~53710 bp	AP006758
34	2	2	AK072906	BK005037	C90ERIPDM	AJ307662
					AP002485_101481~104757 bp	AP002485
					AP004175_92970~96132 bp	AP004175
35	1	4	AY341843	BK005038	OSJNBa0089K21.12	AL606441
			AY341851			
36	2	4	AK073695	BK005039	OSJNBb0038F03.13	AL606728
37	2	4	AK110912	BK005040	OSJNBa0093F12.9	AL607004
38	3	1		NM_190356	P0703B11.18	AP003302
					P0485B12.10	AP003348
39	2	2	AK107047	BK005042	AP004683_92372~91070 bp	AP004683
			AK066775			
			AK119593			
40	3	11		BK005043	AC123514_61109~66356 bp	AC123514
41	3	11	AK121102	BK005044	AC135644_29371~43575 bp	AC135644
			AK066053	BK005066	AC135644_36528~43186 bp	AC135644
42	2	2	AK110587	BK005045	OSJNBa0035A24	AP005514
43	2	5		BK005046	AC120986_98432~100880 bp	AC120986
44	3	1		NM_190346	P0703B11.8	AP003302
45	3	5	AK066255	BK005048	AC136227_84441~86584 bp	AC136227
			AK103959			
			AK105939			
			AK063697			
46	3	12	AK073243	BK005049	BX000500_55289~66775 bp	BX000500
47	3	7	AK110900	BK005050	OSJNBa0008J01.9	AP005099
48	3	5		BK005051	AC144737_70702~72279 bp	AC144737
49	2	5		BK005052	AC120986_11783~17456 bp	AC120986
					AC121360_97245~10298 bp	AC121360
50	3	11		BK005054	AC123514_67325~73999 bp	AC123514

(*To be continued on the next page*)

(*Continued*)

WRKY	Group	Chr	cDNA/EST	Gene	BAC.ORF	GenBank
51	2	4	AK100954	BK005053	AL731615_115602~116669 bp	AL731615
			AK121494			
52	3	11		BK005055	AC123514_26311~31095 bp	AC123514
53	1	5	AK121190	BK005056	AC135424_100279~102647 bp	AC135424
54	3	5		BK005057	AC144737_88078~93505 bp	AC144737
55	3	3	AK101653	BK005058	AC118674_13108~14365 bp	AC118674
56	2	1		BK005059	P0406G08.15	AP003240
				NM_190559		
57	2	12	AY341860	BK005060	BX000503_99939~102956 bp	BX000503
58	2	5		BK005061	AC108499_75782~74924 bp	AC108499
59	2	1	AK108755	NM_189575	P0666G04.23	AP003047
			AY341853			
60	2	3	AU162739	BK005063	OSJNBa0075A22.16	AC133859
61	1	11		BK005064	AC135644_81326~82302 bp	AC135644
62	2	9	AK067834	BK005065	AP005784_63011~61549 bp	AP005784
					AP005417_14088~15800 bp	AP005417
63	2	1	AU071032	NM_189614	AP002486_100109~101310	AP002486
			AU091465			
			AU065466			
			AU082723			
64	3	12		BK005067	BX000500_43384~44622 bp	BX000500
65	3	12		BK005068	BX000500_30169~32408 bp	BX000500
66	2	2	AK073100	BK005069	P0459B01.19	AP004778
67	2	5	AK066252	BK005070	AC135431_35305~36416 bp	AC135431
68	2	4	AK061266	BK005071	OSJNBb0015N08.8	AL662996
			AK072938			
69	3	8	AK111606	BK005072	P0434E03.6	AP004689
70	1	5	AK119867	BK005073	AC135924_97551~99972 bp	AC135924
71	2	2	AY541677	BK005074	OJ1297_C09_69154~74807	AP004087
72	2	11	AK108860	BK005075	AC136787_74368~77493 bp	AC136787
73	2	6		BK005076	AP003767_4086~8191 bp	AP003767
74	3	9	AK065265	BK005077	AP005128_86725~88348 bp	AP005128
75	3	5		BK005078	AC136224_148851~156476 bp	AC136224
76	2	9	AK068337	BK005079	AP005784_44950~46052 bp	AP005784
			AK059966			
			AY323479			
			AF467736			
77	2	1	AK108522	BK005080	AP003341_104809~105753 bp	AP003341
			AY341846	NM_192521	AP003492_45486~46430 bp	AP003492
					OSJNBb0024F06.15	
78	2	1		NM_188117	AP001072_110726~118434	AP001072
79	3	3	AK105244		AC125496_26812~28598 bp	AC125496
80	2	3	AK109795		AC128647_133246~137444 bp	AC128647
					OSJNBb0006O08.24	AC120506
81	1	3	AF193802		AC105743_72913~83236 bp	AC105743
			AY302436			
82	3	5			AC135420_76117~86167 bp	AC135420

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WRKY	Group	Chr	cDNA/EST	Gene	BAC.ORF	GenBank
83	2	5			OSJNBb0035N21.7	AC134929
84	3	5			OSJNBa0018K15.11	AC144737
85	3	6		NM_185588	P0702F05.11	AP005828
86	2	6		NM_185324	AP001129_68622~70535 bp	AP001129
87	1	7	AK070537		OJ1127_E01.114	AP003747
			AY341850			
88	1	7	AY341847		P0453E05.117	AP004275
						AP004265
89	1	8	AK073491		OJ1118_F05.13	AP004158
90	2	9	AK065078		AP005392_105750~108428 bp	AP005392
			AK103745			
91	3	11	AK073243		AC123514_37188~40536 bp	AC123514
92	2	11			BX000496_51548~54565 bp	BX000496
93	3	11			AC123514_59764~60804 bp	AC123514
94	2	12	AK070648		AL731743_63844~65872 bp	AL731743
			AK104890			
95	3	12	AK102093		BX000500_50962~51739 bp	BX000500
96	1	12			AL731759_46690~51097 bp	AL731759
97	3	12			BX000500_78237~79317 bp	BX000500

University. [^{32}P]-dATP (>3000 Ci/mmol) was obtained from Beijing Furui Biotechnology Co., Ltd. cDNA synthesis and library construction kits were purchased from BD Clontech. All chemicals were obtained from Shanghai Sangon Biotechnology Co., Ltd. and TaKaRa Biotechnology (Dalian) Co., Ltd.

(ⅱ) Sequence analysis. The amino acid sequences of 97 members of the rice WRKY superfamily and an outgroup from *Avena sativa* AsWKRY3 were aligned by the sequence alignment program CLUSTER W package[20]. A neighbor-joining tree of rice WRKY proteins was constructed with PAUP* 4.0b10[21] using bootstrap to measure support of clades.

(ⅲ) Abiotic stress treatments. Five-week-old seedlings grown on soil were used for treatments under different abiotic stress factors. Seedlings were exposed to cold (4℃), heat (42℃), high-salinity (250 mmol/L^{-1} NaCl), or 25% PEG. Shoot tissues were harvested in 0, 1, 2, 4, 8 and 12 h after abiotic stress treatments, respectively, and frozen in liquid nitrogen and stored at –80℃ for further analysis.

(ⅳ) RNA isolation, cDNA library construction and *WRKY* cDNA clones. Total RNA was isolated as previously described[22]. cDNA library was constructed from cold (4℃)-treated plants for 2 h as suggested by the manufacturer (BD Clontech). A degenerate primer: AGAATTCTGGAGRAARTACGGMCAR (R:A or G; M:G or C) that corresponds to the conserved WRKYGQK amino acid sequence was used for RT-PCR amplification in conjunction with an Poly d(T) primer. The RT-PCR amplified products were inserted into a pMD18-T vector (TaKaRa), and subsequently sequenced. Finally, the rice cDNA library was screened and 13 full length *WRKY* cDNAs were cloned by using the partial DNA sequence of WRKY as a probe.

(ⅴ) Northern blotting analysis. For Northern-blotting analysis, total RNA (20 μg) was separated on agarose-formaldehyde gel and blotted onto nylon membranes following standard procedures[23]. The membranes were hybridized with (α-^{32}P)-dATP-labeled gene-specific probes. Hybridization was performed in PerfectHyb Plus Hybridization Buffer (Sigma) for 16 h at 68℃. The membranes were then washed once for 10 min with 2×SSC and 0.5% SDS, twice for 20 min with 0.5×SSC and 0.1% SDS and then once for 20 min with 0.1×SSC and 0.1% SDS at 68℃, and exposed to X-ray films at –80℃.

2 Results

(ⅰ) Identification and phylogenetic classification of WRKY proteins. Previously, 77 rice *WRKY* genes (*OsWRKY1-OsWRKY77*) were identified by searching for genomic sequences that were available at that time[19]. Recently, we re-examined the almost complete rice genome and identified an additional 24 novel *WRKY* genes containing the conserved amino acid sequence WRKYGQK in WRKY proteins (Table 1). Sequence analysis showed that the previously identified *OsWRKY25* and *OsWRKY44*, *OsWRKY26* and *OsWRKY59*, *OsWRKY33* and *OsWRKY38*, *OsWRKY41* and *OsWRKY63* are the same genes, respectively. Therefore, the 24 new identified *WRKY* genes were designed as *OsWRKY38*,

OsWRKY44, *OsWRKY59*, *OsWRKY63* and *OsWRKY78* to *OsWRKY97*, respectively (Table 1).

Based on the conserved WRKYGQK amino acid sequences and zinc finger-like motifs, WRKY proteins can be classified into three groups as seen in *Arabidopsis*[1,2]. Therefore, rice WRKY proteins were classified into three distinct groups according to the WRKY classification principle used in *Arabidopsis*. Group Ⅰ contains two WRKY domains (WRKYGQK) and one Cys_2-His_2-type zinc finger motif. Group Ⅱ possesses one conserved WRKY domain and one such zinc finger motif. In contrast, group Ⅲ owns one WRKY domain and one Cys_2-His/Cys-type motif. According to this classification principle, we summarized the name, classification, BAC library and cDNA or EST clone of all 97 rice WRKY genes (Table 1). The 97 rice WRKY sequences were multiply aligned using the computer program CLUSTER (Megalign 5.01). The resultant phylogenetic tree is shown in Fig. 1. Notably, 20 groups of WRKY proteins display a very close relationship (>95%) with strong support (Fig. 1). The 97 WRKY proteins were grouped into 13 clusters according to the similarity in amino acid sequences. Group Ⅰ and Group Ⅲ WRKY proteins were placed into 1 (Cluster Ⅰ) and 2 (Cluster Ⅲa and Ⅲb) clusters, respectively, whereas Group Ⅱ WRKY proteins were classified into 10 clusters (Cluster Ⅱa to Ⅱj) (Fig. 1).

The majority of the whole rice WRKY protein superfamily has the typical WRKY domain, WRKYGQK, but there are still 12 proteins containing the alternative WRKY domain sequences: WRKYGKK or WRKYGEK. Moreover, the WRKY proteins possessing either WRKYGKK or WRKYGEK sequences were classified into Group Ⅱ and Group Ⅲ, respectively (Table 1, Fig. 1). Interestingly, WRKY proteins whose WRKY domain sequence is WRKYGEK has not yet to be identified in the WRKY protein superfamily from the genomes of *Arabidopsis* or any other plants so far. This leads us to speculate that the WRKY proteins containing the WRKYGEK domain in rice might have evolved from other WRKY proteins and might have some novel biological functions.

Comparison analysis in amino acid identity among the 97 WRKY proteins revealed 3 pairs of WRKY proteins with high identity (over 80%) at the amino acid level: WRKY93/WRKY95, WRKY40/WRKY64 and WRKY50 /WRKY65. The identity at the amino acid level between the 3 pairs of WRKY proteins which are located on chromosome 11 and chromosome 12, respectively, were 99%, 91% and 81%. *WRKY93*, *WRKY40* and *WRKY50* genes are distributed in tandem on BAC clone AC123514_ 73999bp-59764bp (which comprises about a 14.2 kb region on chromosome 11). Moreover, the *WRKY95*, *WRKY64* and *WRKY65* genes are distributed on BAC clone BX000500_51739bp-30169bp (which comprises about a 21.5 kb region on chromosome 12). The identity at the deoxyribonucleotide sequence level was up to 93% between the two chromosomes partial region, indicating that some DNA recombination or exchange may have occurred between chromosome 11 and chromosome 12.

(ⅱ) Cloning of cDNA which encodes WRKY proteins. 13 *WRKY* cDNA were cloned by screening the cold (4℃)-treated cDNA library. 9 of 13 *WRKY* cDNA clones (Os*W8*, *12*, *13*, *14*, *16*, *17*, *23*, *26* and *45*) had a high copy number (roughly 10—30 positive clones/10^8 cDNA library clones) in the cold-treated library, but another 4 *WRKY* cDNAs (*OsW9*, *21*, *24* and *30*) had a low amount of copies (roughly 5—10 positive clones/10^{10} cDNA library clones). These results showed that the expression of the 9 *WRKY* genes might be induced by stress and play regulatory roles in response to various abiotic stresses in rice. The 13 rice *WRKY* genes have had cDNA or EST sequences deposited in GenBank database as shown by searching the database with Blastn program (Table 1). Based on the information of WRKY cDNAs and ESTs from different tissues in GenBank database, the expression of 13 *WRKY* genes might be in panicles, callus, yellow seedling, mature shoots and young roots, respectively (Table 1).

(ⅲ) Expression profiles of 13 *WRKY* genes induced by high salinity. In order to understand the molecular biological function of 13 rice *WRKY* genes, we analyzed their expression profiles in response to abiotic stress factors. First, the expression profiles of the 13 *WRKY* genes were analyzed by Northern blotting hybridization in response to high salinity (250 mmol/L^{-1} NaCl) (Fig. 2). The expression profiles of 9 *WRKY* genes were influenced by high salinity (250 mmol/L^{-1} NaCl), but that of another 4 *WRKY* genes (*OsW9*, *21*, *24* and *30*) were not induced. Time courses reveal transcript levels of 8 *WRKY* genes (*OsW8*, *12*, *13*, *16*, *17*, *23*, *26* and *45*) initially increased ~2 to 4-fold following treatment compared to control plants, but these levels declined after 8 or 12 h treatment, especially for the *OsW8*, *OsW16 and OsW17* genes. Alternatively, the expression levels of *OsW45* gene displayed two peaks at 2 and 12 h after treatment compared to the *OsW14* gene, whose transcript levels decreased gradually after treatment during the time course.

(ⅳ) Expression profiles of 13 *WRKY* genes induced by drought. The expression profiles of the 13 *WRKY* genes induced by drought (25% PEG) are shown in Fig. 3. 10 *WRKY* genes were regulated by PEG (25%) with no effect on the remaining 3 *WRKY* genes (*OsW9, 21* and *24*). Transcript levels of *OsW8*, *OsW13*, *OsW16*, *OsW23*, *OsW26*, *OsW30* and *OsW45* increased, instantaneously, roughly 2 to 4-fold. However, the expression levels of *OsW13* and *OsW23* genes remained elevated level 12 h after treatment unlike the *OsW16* gene whose transcript levels continued to increase. Unlike *OsW13* and *OsW23*, the expression of *OsW8*, *30* and *OsW45* genes declined

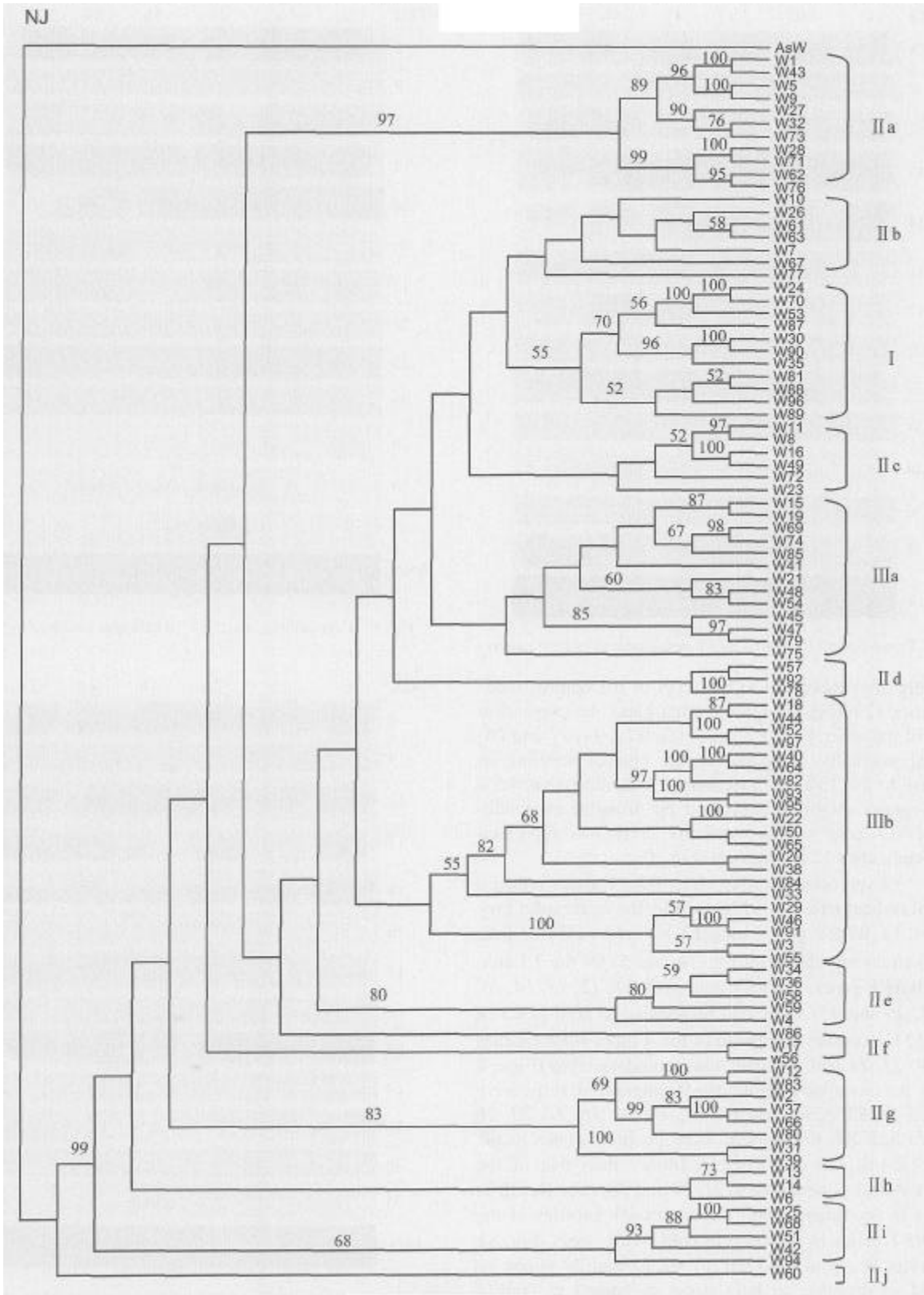

Fig. 1. Phylogenetic tree of the WRKY proteins from rice. Bootstrap values (>50%) are indicated above the branches.

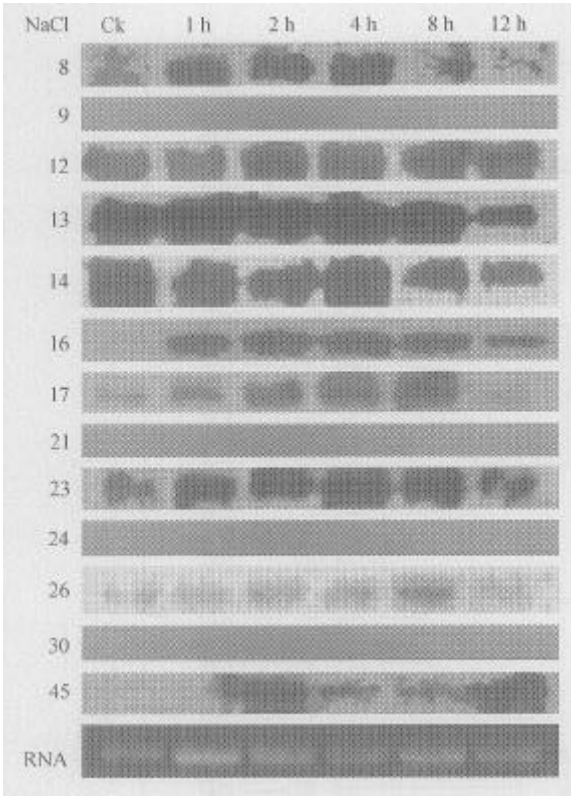

Fig. 2. Expression profiles of 13 *WRKY* genes induced by high salinity.

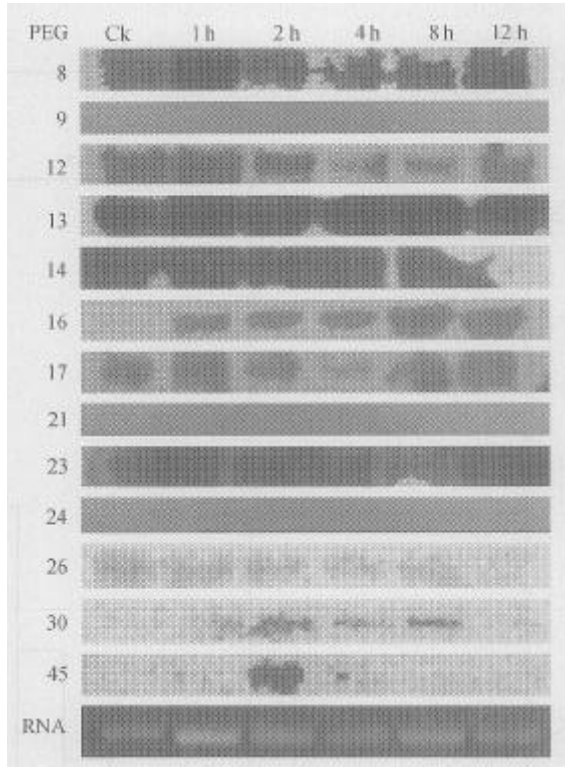

Fig. 3. Expression profiles of 13 *WRKY* genes induced by PEG.

gradually after treatment to the level of the control seedlings after 12 h (Fig. 3). On the other hand, the expression levels of the other 4 *WRKY* genes (*OsW12*, *14*, *17* and *26*) reduced gradually compared to the control seedling in response to the 25% PEG treatment, indicating that the 4 *WRKY* genes might be repressed by drought; especially the *OsW14* gene whose transcript level was repressed completely after 12 h treatment (Fig. 3).

(**v**) Expression profiles of 13 *WRKY* genes induced by cold or heat stresses. Meanwhile, the expression profiles of 13 *WRKY* genes induced by cold (4**℃**) or heat (42**℃**) stress are shown in Figs. 4 and 5. Of the 13 analyzed *WRKY* genes, 9 *WRKY* genes (*OsW8*, *12*, *13*, *14*, *16*, *17*, *23*, *26* and *45*) were also responsive to cold (4**℃**) or heat (42**℃**) stresses compared to the 4 other *WRKY* genes (*OsW9*, *21*, *24* and *30*) which were undetectable (Figs. 4 and 5). As mentioned before, the 9 temperature responsive induced *WRKY* genes (*OsW8*, *12*, *13*, *14*, *16*, *17*, *23*, 26 and *45*) had 200 to 300-fold more positive clones in the cold (**4℃**)-treated (2 h) cDNA library than that of the other 4 *WRKY* genes (*OsW9*, *21*, *24* and *30*) (See Result 2). This is in accordance with the expression profiles of the 13 *WRKY* genes in response to cold (4**℃**) stress (Fig. 5). Transcript levels of the *OsW13*, *16*, *17* and *23* genes increased in response to heat stress compared to *OsW17* gene which declined to the level of the control seedling

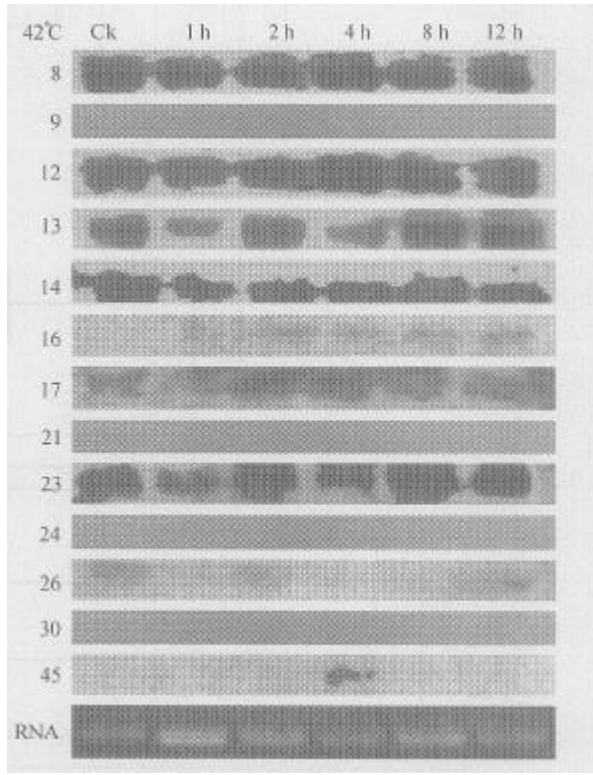

Fig. 4. Expression profiles of 13 *WRKY* genes induced by heat (42**℃**) stress.

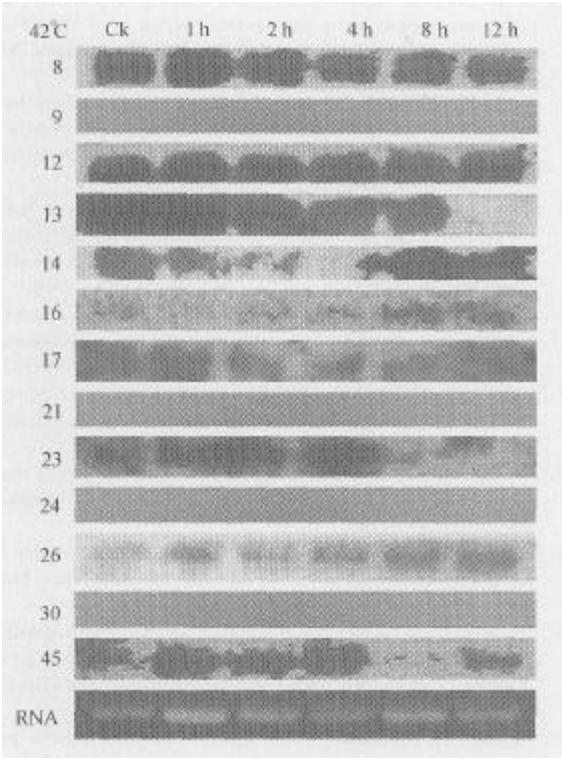

Fig. 5. Expression profiles of 13 *WRKY* genes induced by cold (4℃) stress.

following treatment during the time course (Fig. 4). Expression profiles of *OsW8*, *12* and *14* genes were similar to that of the control seedling in responsive to heat (42℃) stress (Fig. 4). On the other hand, the expression of most *WRKY* genes appear to be repressed with the exception of the *OsW14*, *23* and *45* genes which were induced in response to cold (4℃) stress (Fig. 5). Interestingly, the expression level of *OsW14* gene was repressed at the very onset, but obviously up-regulated following treatment during the time course in the cold (4℃) stress condition (Fig. 5).

3 Discussion

(ⅰ) WRKY gene family has more members in rice genome. Based on the research of Zhang et al., we identified another 24 *WRKY* genes by searching rice genome completely with the highly conserved amino acid sequence WRKYGQK and summarized the name, classification, BAC clones, cDNA or EST clones information for all the 97 currently known rice WRKY genes (Table 1 and Fig. 1). There have been 73 WRKY members identified in the *Arabidopsis*'*s* genome[1,2] but 97 members (24 more genes) have been identified in the rice genome (Table 1 and Fig. 1). Most WRKY members have a typical conserved domain WRKYGQK as seen in the *Arabidopsis* WRKY superfamily. However, there are 3 WRKY members (AtW50, 51 and 59) with the alternative WRKYGKK domain[2]. However, in rice, it has been discovered that a new and uncharacterized conserved amino acid domain WRKYGEK is contained by 7 of the rice WRKY proteins. It is interesting that the zinc-finger-like motif of 7 WRKY proteins whose domain is WRKYGEK is Cys_2-His/Cys-type, not the typical Cys2-His2-type motif. Compared with the WRKY protein superfamily previously identified from the genomes of *Arabidopsis* and other plants, the changes in the number and the sequences of amino acid conserved domain imply that rice WRKY protein superfamily must be more comprehensive and has a more abundant molecular biological function.

(ⅱ) Rice WRKY proteins might regulate abiotic stress responses. The *WRKY* gene transcriptional factor family is a plant-special superfamily. WRKY proteins are thought to specifically bind to the sequence (T)TGACC (A/T) (W box) in the promoter of target genes and regulate the expression of downstream target genes[3]. Present research reports indicate that the biological function of *WRKY* genes mainly involves various protective signal transduction pathways that establish disease resistance and disease response[4,5,7−10]. Rice is a very important crop, but few research reports provide a functional analysis about rice WRKY proteins[19]. In the present research, 13 transcriptional factors *WRKY* genes were cloned from a cDNA library derived from 4℃-treated shoots and the expression profiles were consequently analyzed in response to several abiotic stress factors (Figs. 2—5). 10 of the 13 cloned *OsWRKY* genes were found to be differentially regulated in the plants treated by 4 abiotic stress factors, including NaCl, PEG, cold (4℃) and heat (42℃) (Figs. 2—5). Nevertheless, time courses showed that the expression patterns for the 10 genes differed greatly in their response to different abiotic stress in both a transcriptional and temporal manner (Figs. 2—5). For example, OsW13, 16, 17, 23, 26 and 30 were up-regulated by 2 or 3 abiotic stress factors and down-regulated by 1 or 2 factors (Figs. 2—5). The differences of expression profiles might imply that these *WRKY* genes have different functions, and may play different roles in the responsive to abiotic stress or stress-induced defense signaling pathways. For example, the expression profiles of the *Arabidopsis WRKY* group Ⅲ transcriptional factors (AtW30, 38, 41, 46, 52, 53, 54, 55, 62, 63, 64, 66, 67 and 70) are differentially induced by different pathogens, which revealed that these *Arabidopsis WRKY* genes were involved in different disease resistant response and defense signaling pathways[8]. On the other hand, the differences among the expression profiles of 13 *WRKY* genes revealed that WRKY proteins might be a regulatory node in which a stress factor-mediated signal transduction pathway is up-regulated but consequently down-regulates another stress factor-mediated defense signals. As in the case of the AtW70 protein which was shown to act as a node between two different defense pathways. By activating salicylate-mediated defense sig-

naling pathways, AtW70 would simultaneously act to repress the jasmonate-mediated defense signal transduction in plant defense response[10].

Notably, only *OsW30* gene expression was induced by 25% PEG (Figs. 2—5). The identity and similarity between the OsW30 protein and WRK (WRKY) protein are revealed by Blastp to be of the desert legume *Retama raetam* (GenBank Accession: AF439247) are 38% and 48% respectively. The WRK (WRKY) protein has been shown to have an important biological function in the establishment of drought tolerance in the desert legume *Retama raetam*[17]. This suggests that the *OsW30* gene might have an important regulatory role in the establishment of drought factor-mediated signal transduction pathway in rice. On the other hand, although the expression of *OsW16* gene was induced by all four abiotic stress factors, the inducement activities of 250 mmol/L^{-1} NaCl and 25% PEG were much more remarkable since its expression levels induced by the two stress factors increased ~3 to 4-fold compared to the another two factors of cold (4℃) and heat (42℃) stress. These two stresses resulted in up-regulated expression levels of only 1 to 1.5-fold (Figs 2—5). We suspect that the biological function of *OsW16* gene might mainly be involved in regulating the establishment of high salinity and drought tolerance, and the two stress factors-mediated signal transduction pathways in rice.

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