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Cloning and expression profiles of 15 genes encoding WRKY transcription factor in wheat (*Triticum aestivem* L.)

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Abstract

WRKY proteins are involved in various physiological processes, including biotic and abiotic stress responses, hormone responses and development. However, no systematic identification, expression and function analysis of *WRKY* genes in wheat were reported. In this study, we isolated 15 wheat cDNAs with complete open reading frame (ORF) encoding putative WRKY proteins using in silico cloning. Phylogenetic analysis indicated that the 15 wheat *WRKY* genes belonged to three major WRKY groups. Expression analysis revealed that most genes expressed drastically in leaf, except *TaWRKY10* which expressed in crown intensively. Four genes were strongly up-regulated with the senescence of leaves. Eight genes were responsive to low temperature, high temperature, NaCl or PEG treatment. Moreover, differential expression patterns were also observed between wheat hybrid and its parents, and some genes were more responsive to PEG treatment in the hybrid. These results demonstrated that wheat *WRKY* genes are involved in leaf senescing and abiotic stresses. And the changed expression of these *WRKY* genes in hybrid might contribute to the heterosis by improving the stress tolerance in hybrids.

Keywords: Wheat; WRKY transcription factor; Gene cloning; Gene expression; Heterosis

1. Introduction

The WRKY transcription factor was broadly investigated in plants in the past 20 years. Since Ishiguro and Nakamura [1] identified the first WRKY protein in sweet potato (*Ipomoea batatas*), many members of this family have been cloned and characterized in more than 10 higher plant species, including wild oats (*Avena fatua*) [2], parsley (*Petroselinum crispum*) [3], *Arabidopsis thaliana* [4], tobacco (*Nicotiana tabacum*) [5–8], potato (*Solanum tuberosum*) [9,10], winter bittersweet nightshade (*Solanum dulcamara*) [11], orchardgrass (*Dactylis glomerata*) [12], desert legume (*Retama raetam*) [13], barley (*Hordeum vulgare*) [14], cotton (*Gossypium arboreum*) [15], rice (*Oryza sativa*) [16] and coconut (*Cocos nucifera*) [17]. Recently, some members of WRKY family were also identified in lower plants such as ferns (*Ceratopteris richardii*) and mosses (*Physcomitrella patens*) [18], even in a smile mode (*Dictyostelium discoideum*) and a unicellular protest (*Giardia lamblia*) by searching all of their available sequence data [19].

WRKY proteins are characterized by their WRKY domain of about 60 amino acids, which comprises of absolutely conserved sequence WRKYGQK followed by a Zinc finger motif. The members of WRKY transcription factor (TF) were grouped into three distinct groups depending on the number and type of their WRKY domains. WRKY proteins with two WRKY domains belong to group I. The members of group II and group III have only one WRKY domain. Generally, the WRKY domains of group I and

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group II members have the same type of finger motif C_2H_2, whereas in the group III, the WRKY domain contains a C_2HC motif. WRKY proteins were further classified into different subgroups based on their phylogenetic clades [20–22]. For group I, sequence-specific binding of WRKY proteins to their target DNA sequences is mediated by C-terminal WRKY domain, but the function of the N-terminal WRKY domain remains to be determined, which might participate in enhancing the affinity or specificity of these proteins binding to their target sites. The single WRKY domains of group II and group III are functionally equivalent to the C-terminal WRKY domains of group I. WRKY domain can effectively bind to the W box ([T][T]TGAC[C/T]) in the promoter of the target gene to modulate transcription [20].

In plant, *WRKY* genes are considered to play regulatory functions in defense against biotic and abiotic stresses, such as the infection of bacteria [6,9,23–26], fungi [27], oomycetes [10,28] and viruses [5], treating with salicylic acid (SA) [6,23,29] or H_2O_2 [30], mechanical stimulation [31], drought [13,32], cold [11], wounding [7], high-salinity [33] and UV radiation [34]. The majority of group III members participate in different plant defense signaling pathways [28]. There is also evidence that some members of WRKY family may play key roles in plant development, such as morphogenesis of trichomes and the tannin synthesis in the seed coat [35], fruit maturation [15], embryo development [36], senescence [37–39], dormancy [13], maturation of root cell [40]. Furthermore, a role in hormone signaling was also observed for *OsWRKY71* and *OsWRKY51* which were ABA-inducible and could repress GA signaling transduction in aleurone cells [41–43].

Presently, the reports related to wheat *WRKY* genes are quite limited. Large-scale expressed sequence tags (ESTs) sequencing of cDNA libraries constructed from wheat plants exposed to various abiotic stresses with an emphasis on cold acclimation revealed that 28 *WRKY* genes expressed differentially under abiotic stresses, among which 21 members were up-regulated and 7 numbers were specifically expressed [44]. Golkari et al. [45] investigated the transcription patterns of infected wheat spikes after inoculation with the fungus *Fusarium graminearum* and found that one *WRKY* gene was up-regulated remarkably in anther. In addition, two wheat *WRKY* genes which were responsive to leaf senescence were also identified using microarray by Gregersen and Holm [46]. However, no systematic identification, expression and function analysis of *WRKY* genes are reported in this major cereal crop so far. In this study, we isolated 15 wheat cDNAs with complete ORFs encoding putative *WRKY* genes, and their expression patterns in different organs at different developmental stages and under distinct abiotic stresses were determined. Moreover, differential expression patterns of these genes were also observed between a hybrid and its parental inbreds. It wound provide abundant information for studying the evolution, biologic functions and regulatory mechanisms of *WRKY* genes in wheat in the future.

2. Materials and methods

2.1. Plant materials

Seedling of wheat (*Triticum aestivem* L.) line 3338 was used for RNA preparation. Seedling leaf, leaves at the stage of tillering and heading, and senescing leaf beginning to turn yellow at milk ripe stage, the root and crown at tillering stage, as well as developing seeds of 12 DAP (days after pollination) were collected and used for RT-PCR analysis. For the treatment of stresses, seeds were germinated in the filter paper, and were then transplanted into bottles. They were cultivated with water and grew in a growth chamber at a relative humidity of 75% and 26/20 °C day and night temperature. The seedlings were used for treatments of different abiotic stresses, including low temperature (3 °C), high salinity (10% NaCl), or osmotic stress (20% PEG). Seedlings of wheat genotype TAM107 were used for high temperature (40 °C) stress treatment. Leaves of a highly heterotic hybrid 3338/6554 and its parents at the tillering stage were also used for expression analysis. All the samples were harvested, frozen in liquid nitrogen and stored at −80 °C for further use.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated by using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA preparations were subjected to DNase digestion in the presence of a recombinant ribonuclease inhibitor. RNA was extracted with phenol and precipitated in ethanol. Two micrograms of total RNA were reverse transcribed into cDNA in 20 μl reactions containing 50 mM Tris–HCl (pH 8.3), 75 mM $MgCl_2$, 10 mM DTT, 50 μM dNTPs, 20 U RNase, 200 U MMV reverse transcriptase (Promega) and 50 pmol Oligo-T(15) nucleotides for 60 min at 37 °C and a final denaturation step at 95 °C for 5 min.

2.3. cDNA cloning

Wheat ESTs homologous to rice *WRKY* cDNAs were retrieved from GenBank, and the overlapped ESTs were assembled into contigs with putative open reading frames (ORFs). Specific primers were designed to amplify these cDNAs. PCR amplifications were performed at 95 °C for 3 min, followed by 40 cycles of 95 °C for 1 min, 63 °C for 1 min and 72 °C for 1.5 min, with a final extension at 72 °C for 5 min. PCR products were separated by electrophoresis on a 1% agarose gel and purified using a DNA purification kit (Sunbiotec, Beijing). The purified DNAs were cloned into the pGEM-T Easy vector (Promega) and sequenced by the Songon Company (Shanghai, China).

2.4. Sequence analysis

BLAST searches were completed in NCBI (http://www.ncbi.nlm.nih.org). Sequence alignment and phyloge-

netic analysis were performed using the software DNAMAN (Version 3.0, Lynnon BioSoft).

2.5. Semi-quantitative RT-PCR analysis

One-tenth of the first-strand cDNA was used as the template in a 20 μl RT-PCR with the following thermal cycling parameters: 95 °C for 1 min, 63 °C for 1 min and 72 °C for 1.5 min. Various PCR cycles were tested to ensure that the reactions had not reached the plateau. Gene specific primers (Table 1) were used for semi-quantitative RT-PCR analysis. A 350 bp *β-actin* gene fragment was amplified as a positive control using the primer pair 5′-CAGCAACTGGGATG ATATGG-3′ and 5′-ATTTCGCTTTCAGCAGTGGT-3′. For quantification, the intensity of the PCR products in the gel was estimated with FluorChem™ 5500 software. Three runs of RT-PCRs were conducted using independently isolated total RNAs.

3. Results and discussion

3.1. Identification and phylogenetic analysis of 15 TaWRKY cDNAs

WRKY genes belong to a gene superfamily encoding transcriptional factors involved in the regulation of various biological processes [43]. To date, 74 and 109 members of the WRKY family have been annotated in *Arabidopsis* and rice, respectively [19,21]. Common wheat (*T. aestivem* L.) is a well-known hexaploid species with genome constitution AABBDD. Consequently, the hexaploid wheat genomic contains triplicate homologous genes derived from the ancestral diploid species, and it should contain more *WRKY* genes than *Arabidopsis* and rice.

We examined all the wheat ESTs homologous to 82 rice *OsWRKY* genes in NCBI database using BLASTN program. Wheat ESTs with over 40% similarities to each *OsWRKY* gene were retrieved, and the overlapping wheat ESTs were assembled into contigs, among which 43 had complete putative ORFs. Specific primers (Table 1) were designed to amplify cDNAs from the crown at tillering stage, and 15 specific cDNAs were obtained and were designated as *TaWRKY10*, *TaWRKY13*, *TaWRKY19-a*, *TaWRKY19-b*, *TaWRKY45*, *TaWRKY46*, *TaWRKY53-a*, *TaWRKY53-b*, *TaWRKY68-a*, *TaWRKY68-b*, *TaWRKY71*, *TaWRKY72-a*, *TaWRKY72-b*, *TaWRKY74-a* and *TaWRKY74-b*, respectively. Sequence analysis indicated that these 15 cDNAs ranged from 621 bp (*TaWRKY72-b*) to 1416 bp (*TaWRKY53-a*) in length. Each *TaWRKY* gene shares over 40% amino acid sequence identity with its homologous WRKY proteins in other crops, with the lowest for *TaWRKY19-b* (42%) and *TaWRKY45* (42%), and the highest for *TaWRKY46* (77%) (Table 2).

To study the homology among the 15 wheat *WRKY* genes obtained in this study, a phylogenetic tree was constructed using the amino acid sequences of these genes together with 10 rice and 10 *Arabidopsis* WRKY proteins (Fig. 1). Phylogenetic analysis indicated that these 15 TaWRKY proteins fall into three groups. The members in group II were the most, including seven wheat WRKY proteins (TaWRKY10, TaWRKY13, TaWRKY68-a, TaWRKY68-b, TaWRKY71, TaWRKY72-a and TaWRKY72-b), which were further assembled into four subgroups (IIa, IIc, IId and IIe) designated by Eulgem et al. [20]. Two TaWRKY53-a and TaWRKY53-b belong to group I, and the other six proteins fall into group III. It was also revealed that four pairs of WRKY proteins had high identity (>90%) for each other at the amino acid level: 94% for TaWRKY53-a and TaWRKY53-b, 97% for TaWRKY68-a and TaWRKY68-b, 96% for TaWRKY72-a and TaWRKY72-b and 99% for TaWRKY74-a and TaWRKY74-b, which implied that they are most probably homologous genes.

Multiple sequence alignment to the WRKY domains of the 15 proteins revealed that the members of group I contain two WRKY domains and the C_2H_2-type Zinc finger motif, and group II members possess one WRKY domain and the same type of finger motif like group I, in contrast, group III owns one WRKY domain and C_2HC-type Zinc

Table 1
Gene-specific primer sequences used for isolating wheat *WRKY* genes and RT-PCR analysis

Gene	Forward primer	Reverse primer
TaWRKY10	5′-AGCTCGTCTGTGCAGTGCACTTAT-3′	5′-TCGTGTACATGCATCCGTGAGATT-3′
TaWRKY13	5′-AGATAGCTCCGTCCGTGTCCGAT-3′	5′-CACTCTGAACAGCGAAATCCCTTTG-3′
TaWRKY19-a	5′-AGGCTAGCTAGGTGCTGAGCTGAAC-3′	5′-ACAGGCTATCATCCTTCACGAACTC-3′
TaWRKY19-b	5′-CTGATCGGCAGGCTAGCTAGGTG-3′	5′-ACAGGCTATCATCCTTCACGAACTCCTT-3′
TaWRKY45	5′-CAGATCACTGGCAGTAGACGGTAGTG-3′	5′-TACACATGGGACGGATGGATG-3′
TaWRKY46	5′-CAGCAGAAACGTCGCCTCTTCTC-3′	5′-GCAGTAATCTCTCGATGTGGATCCTTC-3′
TaWRKY53-a	5′-GACTCTCGAAAAATCTCGCTGCTC-3′	5′-ACATGTAAACGCCACAGGGGAAC-3′
TaWRKY53-b	5′-CTCATTTCATATTGAACTCCCTGCTCCT-3′	5′-TATTGTACACGTGGACCCACATGTAAAC-3′
TaWRKY68-a	5′-AGCGAGCCAAGATCTGCAGAGT-3′	5′-AACTAAGTCAGACGTGCCCGTTG-3′
TaWRKY68-b	5′-AAGATCTGCAGAGCCACAGGTGAC-3′	5′-TTACACGGACATCAGACAGATTGAGAAC-3′
TaWRKY71	5′-ATGGATCCATGGGTCAGCAGC-3′	5′-ATTGATGTCCCTGGTCGGCGATA-3′
TaWRKY72-a	5′-GCACGAGGGCTCAACATATCAC-3′	5′-TGTGCATAGGCATTAATTTAGCCATG-3′
TaWRKY72-b	5′-CAACATATCACGGCCATGGAGAAT-3′	5′-GTGATGTTCATTGAAACATGTGTTGGT-3′
TaWRKY74-a	5′-CGATTTCTCCTCTTCCACACACAC-3′	5′-TGGCGTGTTCTTCCCTCCTCT-3′
TaWRKY74-b	5′-AGAAGCTCCGAGCAGAGAGAGATGG-3′	5′-TCAGACCTGCACTGAGGAAGAACTG-3′

Table 2
15 identified members of the WRKY superfamily of transcriptional factors in wheat

Name	Group	Length of ORF (bp)	Accession number	Sequence similarity	Homology	BLASTX *E*-value
TaWRKY10	II	678	EF368361	WRKY transcription factor 10 [*Oryza sativa*]	94/194 (48%)	4e−35
TaWRKY13	II	846	EF397614	WRKY transcription factor 13 [*Oryza sativa*]	161/304 (52%)	2e−79
TaWRKY19-a	III	798	EF368362	WRKY transcription factor 19 [*Oryza sativa*]	127/280 (45%)	6e−46
TaWRKY19-b	III	768	EF397616	WRKY transcription factor 19 [*Oryza sativa*]	91/213 (42%)	4e−50
TaWRKY45	III	882	EF397613	WRKY transcription factor 45 [*Oryza sativa*]	136/319 (42%)	3e−50
TaWRKY46	III	669	EF368365	WRKY transcription factor 46 [*Oryza sativa*]	85/109 (77%)	2e−44
TaWRKY53-a	I	1416	EF368357	Transcription factor WRKY53 [*Oryza sativa*]	309/497 (62%)	e−152
TaWRKY53-b	I	1323	EF368364	WRKY transcription factor 53 [*Oryza sativa*]	294/491(59%)	e−141
TaWRKY68-a	II	987	EF368360	WRKY transcription factor 68 [*Oryza sativa*]	185/332 (55%)	7e−91
TaWRKY68-b	II	942	EF397617	WRKY transcription factor 68 [*Oryza sativa*]	181/284 (63%)	3e−93
TaWRKY71	II	1068	EF368356	WRKY transcription factor [*Hordeum vulgare*]	241/358 (67%)	e−124
TaWRKY72-a	II	648	EF368358	WRKY transcription factor 72 [*Oryza sativa*]	116/218 (53%)	6e−52
TaWRKY72-b	II	621	EF368363	WRKY transcription factor 72 [*Oryza sativa*]	119/218 (54%)	6e−54
TaWRKY74-a	III	1056	EF368359	WRKY transcription factor 74 [*Oryza sativa*]	192/364 (52%)	1e−79
TaWRKY74-b	III	1056	EF397615	WRKY transcription factor 74 [*Oryza sativa*]	197/364 (54%)	1e−83

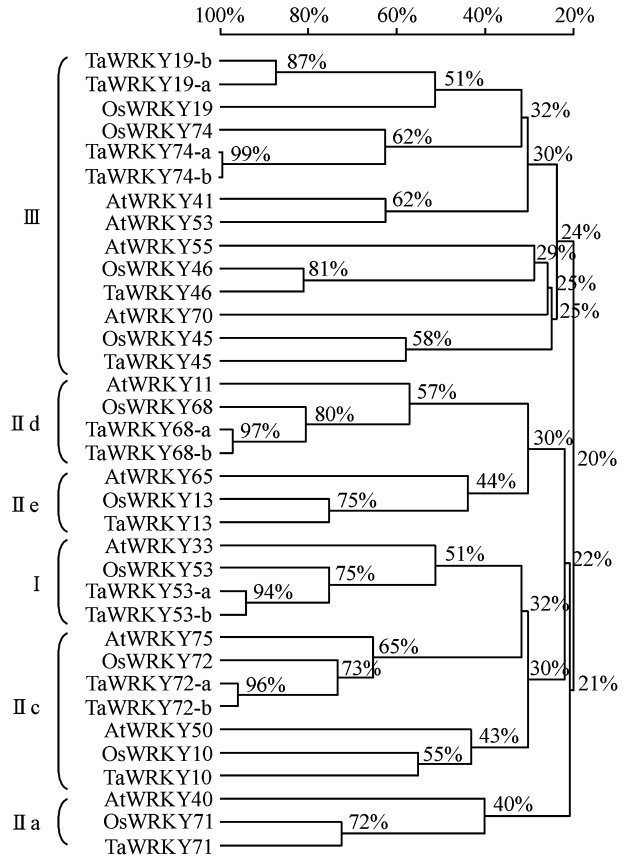

Fig. 1. Phylogenetic tree of WRKY proteins from wheat, *Arabidopsis* and rice. Wheat sequences obtained in this study were used. Rice and *Arabidopsis* sequences are from NCBI database. Accession Nos.: DAA05075 (OsWRKY10), DAA05078 (OsWRKY13), DAA05084 (OsWRKY19), DAA05110 (OsWRKY45), DAA05111 (OsWRKY46), DAA05118 (OsWRKY53), DAA05133 (OsWRKY68), DAA05136 (OsWRKY71), DAA05137 (OsWRKY72), DAA05139 (OsWRKY74), Q9SV15 (AtWRKY11), AAM34736 (AtWRKY33), Q9SAH7 (AtWRKY40), Q8H0Y8 (AtWRKY41), Q9SUP6 (AtWRKY53), Q8VWQ5 (AtWRKY50), Q9SHB5 (AtWRKY55), Q9LP56 (AtWRKY65), AAL13046 (AtWRKY70), AAL50784 (AtWRKY75).

motif (Fig. 2). The majority of the 15 TaWRKY proteins have the typical WRKY domain, "WRKYGQK", but there are three proteins (TaWRKY10, TaWRKY46, TaWRKY72-a) with the alternative WRKY domain sequences: "WRKYGKK", "WRKYGEK" or "WRKYGQE". Previously, Zhang and Wang [19] found nine variations of "WRKYGQK" distributed in 19 distinct WRKY proteins by searching the whole genome of rice. Interestingly, all five proteins with "WRKYGKK" sequence belonged to group IIc and all seven with "WRKYGEK" sequence were classed into group III. In this study, TaWRKY10 with "WRKYGKK" sequence and TaWRKY46 with "WRKYGEK" sequence were also found belonging to group IIc and group III, respectively. It is worthy to note that "WRKYGQE" sequence of TaWRKY72-a, which might derive from other WRKY domains, has not been identified in any other species in previous reports. This sequence variation within the conserved domain suggested that multiple *WRKY* genes should widely represent wheat genome and might play a species-specific role in wheat growth and development.

3.2. Expression of the 15 TaWRKY genes in different tissues

To determine the expression profiles of the 15 *TaWRKY* genes, semi-quantitative RT-PCR was performed. To validate RT-PCR results, amplifications were repeated three times with different cycles (30, 35 and 40) using independently isolated RNA.

We analyzed the expression patterns of the 15 *TaWRKY* genes in different wheat tissues, including leaf, crown, root at tillering stage, and developing seeds of 12 DAP. The results revealed that the expression patterns of these genes were markedly different in different tissues and could be divided into three groups: (1) four genes (*TaWRKY13*, *TaWRKY46*, *TaWRK68-a* and *TaWRKY68-b*) were expressed in all the four tissues tested (Fig. 3a); (2) eight genes (*TaWRKY19-a*, *TaWRKY19-b*, *TaWRKY45*, *TaW-*

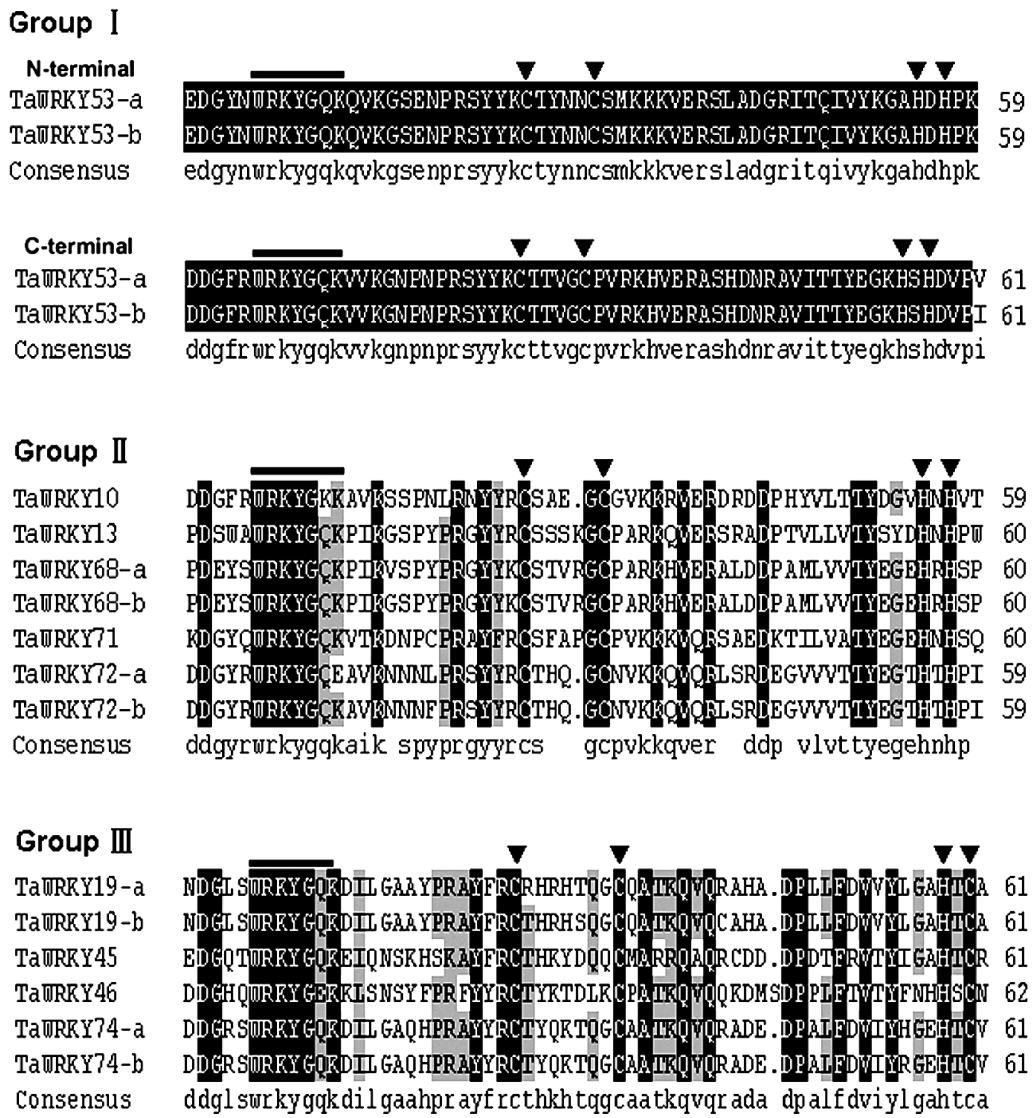

Fig. 2. Multiple sequence alignment of WRKY domains. Cys/His residues of the zinc finger motif are indicated with arrows, WRKY motifs are below the black lines.

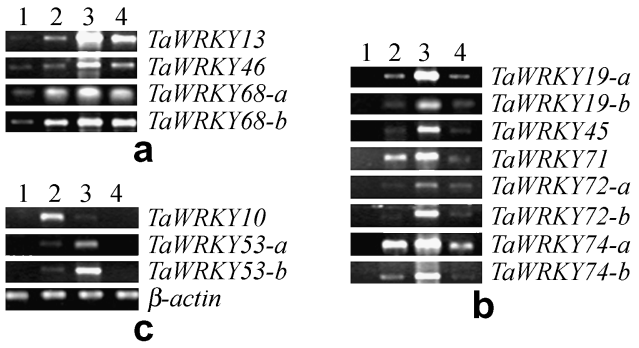

Fig. 3. Expression profiles of 15 *TaWRKY* genes in different tissues (40 PCR cycles). (1) The developing seeds of 12 DAP; (2) crown at tillering stage; (3) leaf at tillering stage; (4) root at tillering stage.

RKY71, *TaWRKY72-a*, *TaWRKY72-b*, *TaWRKY74-a* and *TaWRKY74-b*) were expressed in leaf, root, and crown but not in the developing seeds of 12 DAP (Fig. 3b); (3) the remaining three genes (*TaWRKY10*, *TaWRKY53-a* and *TaWRKY53-b*) were expressed only in leaf and crown (Fig. 3c). Furthermore, *TaWRKY10* was abundantly expressed in crown, whereas the other 14 genes were strongly expressed in leaf.

3.3. Expression of the WRKY genes in leaf at different developmental stage

It was demonstrated that WRKY transcriptional factors played a key role in leaf senescence [37–39]. *WRKY* genes constituted the second largest group of *Arabidopsis* transcription factors in the transcriptome of senescing leaves [47], and the transcript levels of several *Arabidopsis* genes (*AtWRKY4*, *AtWRKY6*, *AtWRKY7*, *AtWRKY11* and *AtWRKY53*) were enhanced in senescent leaves [37–39]. AtWRKY6 protein positively influenced plant senescence and pathogen defense by specifically binding to the W-

box in the promoter of the *SIRK* gene (senescence-induced receptor-like kinase). Senescing leaves of *AtWRKY6* knockout mutants showed a dramatic reduction of *SIRK* expression, and green leaves of *AtWRKY6* overexpression lines showed clearly elevated *SIRK* transcript levels [39]. It was also shown that the AtWRKY53 protein played a regulatory role in the early events of leaf senescence [37]. Further studies indicated that AtWRKY53 could specifically bind senescence-associated genes (SAGs). Overexpression, RNAi and knockout lines of *AtWRKY53* showed accelerated and delayed senescence phenotypes, respectively [48].

We want to determine if the expression of the *TaWRKY* genes identified in this study is regulated in the senescing wheat leaves. Semi-quantitative RT-PCR was carried out using the cDNA from normally grown leaves at seedling stage, heading stage, and senescing leaves when the leaves began turning yellow at milk ripe stage. The results demonstrated that four genes (*TaWRKY10*, *TaWRKY53-b*, *TaWRKY72-a* and *TaWRKY72-b*) were up-regulated following the maturation and senescence of leaf, especially, *TaWRKY10*, *TaWRKY53-b* and *TaWRKY72-b* had very high expression levels in the senescing leaves (Fig. 4a), suggesting that these *TaWRKY* genes might play important roles in regulating leaf senescence in wheat. Whereas the expression levels of the other *TaWRKY* genes were unchanged (Fig. 4b).

A recent study revealed that two wheat ESTs (TIGR contigs: TC238177 and TC237283) of *WRKY* genes were also up-regulated during flag leaf senescence [46]. It should be noted that TC238177 showed higher homology (93%) to *TaWRKY45* gene cloned in this study at the nucleotide level, but expression profile of *TaWRKY45* was constant during leaf senescence, indicating that TC238177 and *TaWRKY45* might be two different genes with distinct functions.

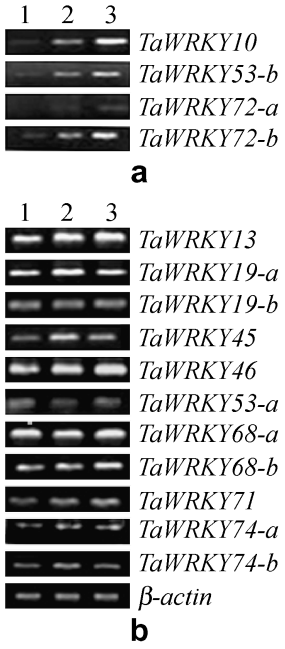

Fig. 4. Expression patterns of 15 *TaWRKY* genes in leaf at different developmental stages (35 PCR cycles). (a) Up-regulated notably with the maturation and senescence of leaves; (b) invariable expression levels. (1) Young leaf at seedling stage; (2) mature leaf at heading stage; (3) senescing leaf beginning to turn yellow at milk ripe stage.

3.4. Response of *TaWRKY* genes to abiotic stresses

Complex and perfect regulation network defending environmental stresses has been arranged in plants. The WRKY transcriptional factor family is functionally significant in this regulatory net, since current data have indicated that a great deal of *WRKY* genes can be induced by many biotic and abiotic stress factors [22,49]. The genes involved in defending processes could be deduced approximately based on their differential expression patterns under different stress factors. Therefore, investigating the differential expression of *WRKY* genes in wheat is not only to contribute to understand their functions, but what is more, to find defense-related genes of wheat in the future.

In this study, wheat seedlings were treated with low temperature (3 °C), high temperature (40 °C), high salinity (10% NaCl), or osmotic stress (20% PEG), respectively. Semi-quantitative RT-PCR analysis showed that seven wheat *WRKY* genes (*TaWRKY13*, *TaWRKY19-b*, *TaWRKY45*, *TaWRKY53-a*, *TaWRKY68-b*, *TaWRKY72-a* and *TaWRKY74-a*) were not responsive to any one abiotic stress, and the other eight what *WRKY* genes were found to be differentially and rapidly regulated under low and high temperatures and high-salinity stresses, suggesting that the eight *TaWRKY* genes might play important roles in defending these abiotic stresses. For different stresses, the expression patterns of the eight genes were also different: in the case of treatment with low temperature (3 °C), six genes (*TaWRKY10*, *TaWRKY19-a*, *TaWRKY53-b*, *TaWRKY71*, *TaWRKY72-b* and *TaWRKY74-b*) were up-regulated at 30 min, and then reduced to the normal levels at 60 min after treatment (Fig. 5a). For high temperature stress, the expression levels of two genes (*TaWRKY19-a* and *TaWRKY71*) were increased at 20 min and maintained till 40 min after treatment (Fig. 5b). Two genes (*TaWRKY10* and *TaWRKY19-a*) were responsive to high salinity, and their expression levels were increased till 40 min after treatment (Fig. 5c). Furthermore, four genes (*TaWRKY10*, *TaWRKY46*, *TaWRKY68-a* and *TaWRKY72-b*) were up-regulated after treatment with PEG at 4 h (Fig. 5d). These differentially induced expression patterns might be caused by the divergent functions of these *TaWRKY* genes in distinct signaling transduction pathways. Furthermore, more than one gene was responsive to each of the abiotic stress, indicating that multiple WRKY members might participate together in signal transduction of each stresses, and they might function cooperatively in the same physiological process. It was also noticeable that four *TaWRKY* genes (*TaWRKY10*, *TaWRKY19-a*, *TaWRKY71* and *TaWRKY72-b*) could be induced by two abi-

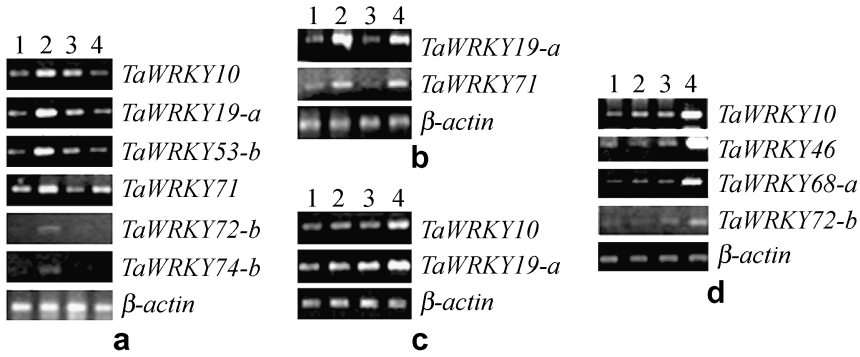

Fig. 5. Expression patterns of *TaWRKY* genes in response to four abiotic stress factors (30 PCR cycles). CK, control plants cultivated only with water under normal condition. (a) Low temperature (3 °C), lanes 1–4 represent CK (30 min), 3 °C (30 min), CK (60 min) and 3 °C (60 min), respectively. (b) High temperature (40 °C), lanes 1–4 represent CK (20 min), 40 °C (20 min), CK (40 min) and 40 °C (40 min), respectively. (c) High-salinity (10% NaCl), lanes 1–4 represent CK (20 min), 10% NaCl (20 min), CK (40 min) and 10% NaCl (40 min), respectively. (d) Osmotic stress (20% PEG), lanes 1–4 represent CK (2 h), 20% PEG (2 h), CK (4 h) and 20% PEG (4 h), respectively.

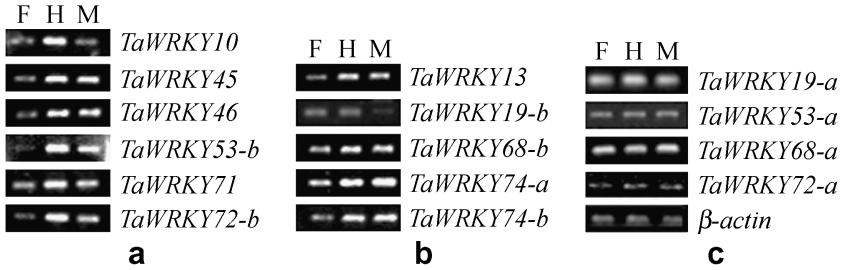

Fig. 6. Differential expression profiles of 15 *TaWRKY* genes in leaves between the hybrid 3338/6554 and its parents at tillering stage (30 PCR cycles). (a) Expressions were up-regulated in hybrid; (b) expressions of hybrid were similar to the highly expressed parent; (c) no significant differences in expression levels between hybrid and its parents. F, H and M represent female parent, hybrid and male parent, respectively.

otic stress factors at least, suggesting that they may be key cross-talk factors throughout the whole regulation network constituting different stress-induced defense signaling pathways.

3.5. Differential expression of TaWRKY genes between hybrid and its parents

Heterosis, recently a hotspot, is defined by its advantage of hybrid performance over its parents in terms of viability, growth and productivity. Previous studies suggested that differential gene expression between hybrid and parents is responsible for the heterosis [50]. In this study, we determined differential expression profiles of these 15 *TaWRKY* genes between hybrid and its parents. Leaves of a highly heterotic hybrid 3338/6554 and its parents at tillering stage were used for investigation. The results indicated that expressions of six genes (*TaWRKY10*, *TaWRKY45*, *TaWRKY46*, *TaWRKY53-b*, *TaWRKY71* and *TaWRKY72-b*) are up-regulated in hybrids (Fig. 6a). Five genes (*TaWRKY13*, *TaWRKY19-b*, *TaWRKY68-b*, *TaWRKY74-a* and *TaWRKY74-b*) were expressed at similar levels to highly expressed parent (Fig. 6b). The remaining four genes (*TaWRKY19-a*, *TaWRKY53-a*, *TaWRKY68-a* and *TaWRKY72-a*) showed no expression difference between hybrids and parents (Fig. 6c). It was also found that for some *TaWRKY* genes, hybrids and parents responded differently to PEG treatment. Under normal growing condition, *TaWRKY46* was down-regulated in hybrid and *TaWRKY68-a* showed a mid-parental expression level, however, when the hybrid and its parents were exposed to 20% PEG for 4 h, no significant differences in expression between hybrid and its parent were observed for *TaWRKY46*, and *TaWRKY68-a* was expressed at a similar level of highly expressed parents, which suggested that hybrid was more responsive to PEG treatment than its parents (Fig. 7).

Since *WRKY* genes play important roles in defense systems of plants, we speculate that their differential expression between hybrids and parents might be related to heterosis through regulating the expression of defense-

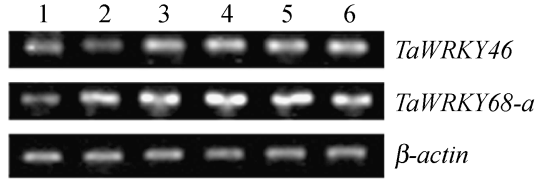

Fig. 7. Differential expression profiles of *TaWRKY46* and *TaWRKY68-a* in the seedling leaves between the hybrid 3338/6554 and its parents under normal growing condition and under 20% PEG treatment (30 PCR cycles). Lanes 1–3 represent female parent, hybrid and male parent grown under normal condition, respectively; lanes 4–6 represent female parent, hybrid and male parent exposed to 20% PEG for 4 h, respectively.

related genes downstream, mediating defense responses against stress and improving the ability of stress tolerance in hybrid. However, the causal relationships between the changed *WRKY* gene expression in hybrid and heterosis still need further investigations.

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