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A wheat salinity-induced WRKY transcription factor TaWRKY93 confers multiple abiotic stress tolerance in *Arabidopsis thaliana*

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ABSTRACT

Wheat is an important crop in the world. But most of the cultivars are salt sensitive, and often adversely affected by salt stress. WRKY transcription factors play a major role in plant responses to salt stress, but the effective salinity regulatory WRKYs identified in bread wheat are limited and the mechanism of salt stress tolerance is also not well explored. Here, we identified a salt (NaCl) induced class II WRKY transcription factor *TaWRKY93*. Its transcript level was strongly induced by salt (NaCl) and exogenous abscisic acid (ABA). Over-expression of *TaWRKY93* in *Arabidopsis thaliana* enhanced salt (NaCl), drought, low temperature and osmotic (mannitol) stress tolerance, mainly demonstrated by transgenic plants forming longer primary roots or more lateral roots on MS plates supplemented with NaCl and mannitol individually, higher survival rate under drought and low temperature stress. Further, transgenic plants maintained a more proline content, higher relative water content and less electrolyte leakage than the wild type plants. The transcript abundance of a series of abiotic stress-related genes was up-regulated in the *TaWRKY93* transgenic plants. In summary, *TaWRKY93* is a new positive regulator of abiotic stress, it may increase salinity, drought and low temperature stress tolerance through enhancing osmotic adjustment, maintaining membrane stability and increasing transcription of stress related genes, and contribute to the superior agricultural traits of SR3 through promoting root development. It can be used as a candidate gene for wheat transgenic engineering breeding against abiotic stress.

1. Introduction

Abiotic stress such as salt, drought, and low temperature often adversely affect plant growth and development, thus imposing constraints on crop productivity [1–3]. To adapt to these abiotic stresses, plant has evolved corresponding mechanisms on different levels. On the molecular level, the induction of stress-associated transcription factors (TFs) is of great importance, for transcription factors being often evolved in different complex signalling pathways [4].

WRKYs comprise a large family of plant TFs (transcription factors) [5]. They are characterized by the presence of a 60-residue-domain defined by the oligopeptide motif WRKYGQK at the N terminus and the Cys2His2 or Cys2HisCys zinc finger motif at their C terminus [6,7]. Based on these conserved domains and their different assembling, WRKYs are categorized into distinct groups [6–8]. To date, the key roles of WRKY TFs are known in regulating the plant response to pathogen infection and in certain developmental processes [7]. But now, more and more WRKYs evolved in abiotic stress tolerance have been identified [5,9–11]. They responded to abiotic stress independently of ABA or in a dependent manner [5]. Over-expression of these WRKYs resulted in enhanced abiotic stress tolerance [12–15]. For example, *AtWRKY25* and *AtWRKY33* increased salinity tolerance in *Arabidopsis thaliana* [16]. *VvWRKY11* enhanced osmotic stress tolerance in grape [14]. *OsRWKY45* conferred both disease resistance and drought tolerance in rice [17].

Wheat is an important crop for people's food in the world. But because of the large and complicated genome, as well as the uncompleted genomic sequencing information, functional genomics research of wheat has been lagged much behind. Although some of WRKYs have been isolated in wheat, such as Wu et al. isolated15 WRKY genes [18]. Niu et al. identified two WRKYs *TaWRKY2* and *TaWRKY19* and proved that both of these two genes respond to salinity and drought stress [19]. Considering the large genome of

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wheat and the complexity of salt stress tolerance mechanism, the identification of salinity related WRKYs in bread wheat and the elucidating of their salt stress tolerance mechanism are still challenging.

The bread wheat cultivar Shanrong No. 3 (cv. SR3) bred from an asymmetric somatic hybrid made between cv. JN177 and tall wheatgrass (*Thinopyrum ponticum*) expresses a good level of both salinity and drought tolerance [20]. Transcriptomic analysis of the root of cv. SR3 seedlings has identified a number of salinity-induced TFs, including WRKYs [8]. Here we give a detailed description of the properties of one of these salt inducible WRKY genes, namely *TaWRKY93*.

2. Materials and methods

2.1. Plant materials and stress treatments

Grain of cv. SR3 were germinated at 25 °C under a 16 h photoperiod, and two week old seedlings were transferred into half strength MS [21] medium supplemented with either 200 mM NaCl or 100 μM ABA. Control plants were raised in the nutrient medium without any supplementation. Seedling roots and leaves were harvested at specific times after the imposition of the stress, and used for RNA extraction.

Abiotic stress treatments of both the wild type *A. thaliana* ecotype Col-0 and selected TaWRKY93 transgenic plants were imposed by transferring five day old seedlings onto MS agar plates supplemented with either 100 mM NaCl, 150 mM mannitol, 50 mM KCl, 10 mM LiCl or 2 μM ABA. The plates were stored vertically allowing seedlings growing for seven days under a 16/8 h light/dark, 22/20 °C, 60% humidity regime. To impose drought stress, water was withheld for 14 days from two week old soil-grown plants, after which the plants were re-watered. To impose low temperature stress, three week old seedlings were acclimated at 4 °C for 24 h, then exposed to −6 °C for three days, and finally allowed to recover at 25 °C for four days.

2.2. Nucleic acid extraction, cDNA synthesis and quantitative RT-PCR (qRT-PCR) analysis

Genomic DNA was extracted from cv. SR3 seedlings using the CTAB method. Total RNA was extracted from both *A. thaliana* and wheat seedlings using the TRIZol reagent (Invitrogen), as recommended by the manufacturer. The first cDNA strand was synthesized from 2 μg mRNA template in 20 μl volume using a primerscript RT reagent kit with gDNA eraser (Takara), and the subsequent qRT-PCR was performed in a 10 μl volume comprising 1 μl diluted (1:10 v/v) cDNA, 5 μl 2 × SYBR Ex Taq mix (Takara) and 0.2 μM forward and reverse primers (primer sequences given in Table S1). The reactions comprised a denaturation step of 95°C/60 s, followed by 45 cycles of 94°C/30 s, 56°C/30 s, 72°C/30 s. *TaActin* and *AtTubulin* were used as internal references for wheat and *A. thaliana*, respectively. The relative abundance of transcript was estimated using the $2^{-\Delta\Delta Ct}$ method. Three biological replicates and three technical replicates for each sample were performed.

2.3. Gene isolation and sequence analysis

The full length cDNA of *TaWRKY93* was cloned using PCR from a cv. SR3 root full length cDNA library, constructed using the pBluescript (+) vector system. The forward primer (see Table S1) targeted a sequence close to the 3′ end of the gene and the reverse primer targeted vector sequence at the 5′ end of the insert. Based on the isolated cDNA sequence information, a pair of primers were designed to amplify the full open reading frame of *TaWRKY93* and the genomic copy (see Table S1). Predicted protein and DNA sequences were analysed by BLAST (http://www.ncbi.nlm.gov/blast), and a phylogenetic analysis against heterologous WRKY proteins was carried out using DNAman v5.2.2 software.

2.4. Production of transgenic A. thaliana plants

The full length open reading frame of *TaWRKY93* was cloned into the *Xba*I/*Sac*I site of the binary vector pCAMBIA super 1300 under

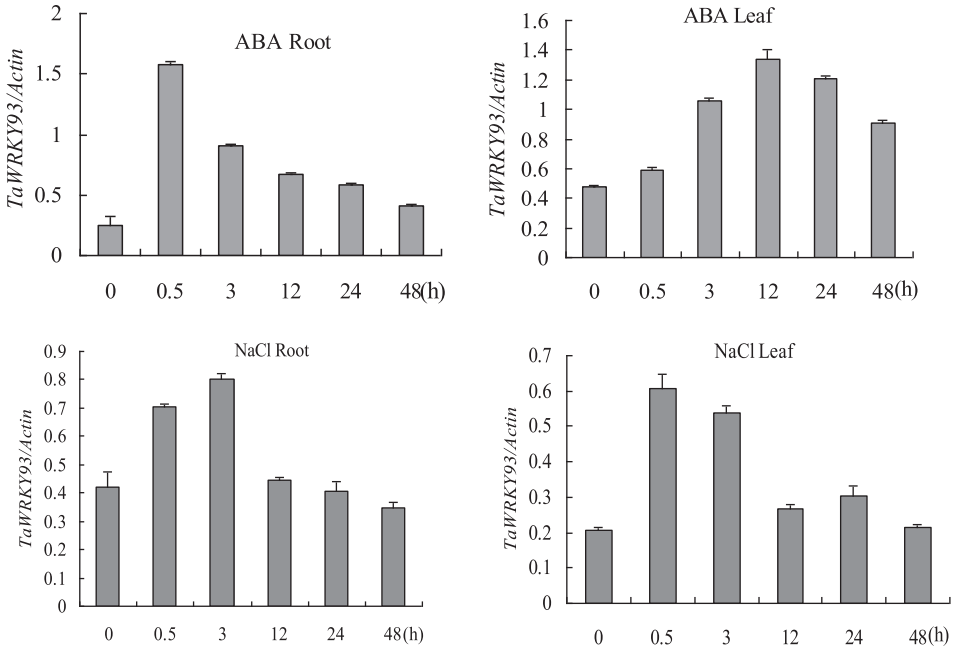

Fig. 1. Transcription of *TaWRKY93* in two week old cv. SR3 wheat seedlings exposed to either salinity stress or ABA treatment. *TaActin* was used as the reference gene.

the control of CaMV35S promoter. Then the recombinant plasmid was transformed into the *Agrobacterium tumefaciens* strain GV3101. *TaWRKY93* transgenic Arabidopsis ecotype Columbia plants (Col-0) were generated through *Agrobacterium* mediated transformation using floral dipping method [22]. Three randomly independent homozygous transgenic lines were selected for subsequent abiotic stress tolerance tests.

2.5. Measurement of leaf proline content, relative electrolyte leakage and water loss rate

Free proline content in the leaf of three week old plants grown under normal conditions (a 16/8 h light/dark, 22/20 °C, 60% humidity regime) was determined using the method given by Bates LS [23]. To determine relative electrolyte leakage, 0.5 g leaves sampled from three week old seedlings were divided into two groups, with one half held at 42 °C for 1 h and the other at 25 °C in distilled water. The leaves were rinsed twice, vacuum-infiltrated in 15 ml distilled water for 30 min and then held in water for a further 3 h. The electrical conductivity of the solution (C_1) was then determined, the solutions (including the leaves) boiled for 15 min, cooled to room temperature, and the conductivities re-measured (C_2). The relative electrolyte leakage was calculated from the expression $(C_1-C_0)/(C_2-C_0)$, where C_0 represented the conductivity of distilled water. To determine the relative water loss rate, water was withheld for two weeks from three week old soil-grown seedlings; rosette leaves were collected every other day and their relative water content (RWC) measured as described by Brini et al. [24]. Mean trait values were derived from three replications.

2.6. Transcription analysis of abiotic stress-related genes

Wild type and *TaWRKY93* transgenic *A. thaliana* plants were grown under the normal conditions for two weeks. Total RNA was isolated from their leaves, converted into cDNA and subjected to qPCR (as described above) using primers directed at the abiotic stress-related genes *ABF3*, *ABI1*, *ABI2*, *ABI3*, *DREB2A*, *RD19A*, *ICE1*, *RD21*, *ABA1* and *P5CS*. The primer sequences used are given in Table S1.

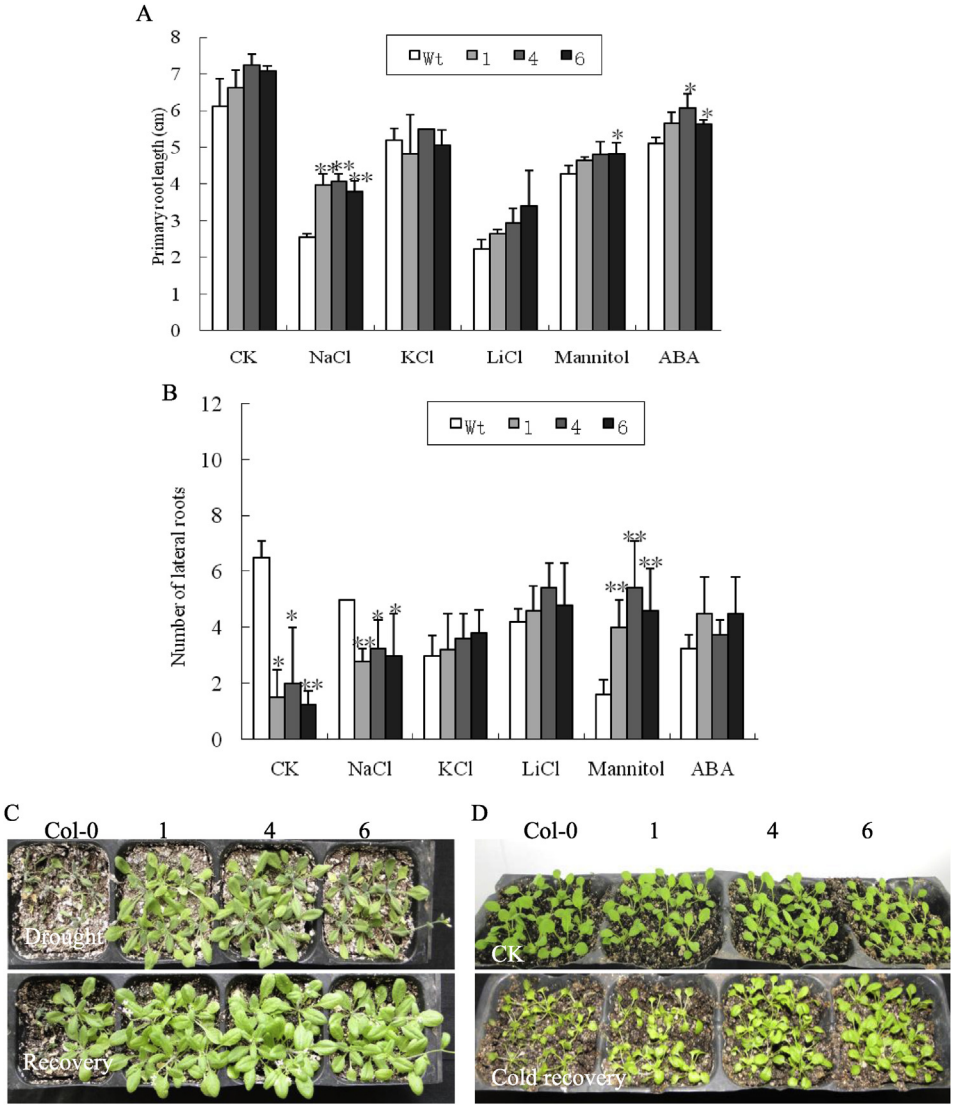

Fig. 2. Phenotype of *TaWRKY93* transgenic and wild type *A. thaliana* plants following exposure to various stresses. Wt: Col-0 ecotype. 1, 4, 6: three independent *TaWRKY93* transgenic lines. (A) the number of lateral roots, (B) the primary root length. *, **: significant at $P < 0.05$, $P < 0.01$. T-line bar: standard deviation. (C) The effect of drought on plant phenotype. (D) The effect of freezing tolerance on phenotype. CK: wild type and transgenic seedlings grown at 25 °C, Cold recovery: seedlings exposed to −6 °C for 3 d, followed by a 4 d recovery at 25 °C.

3. Results

3.1. The structure and phylogeny of TaWRKY93

The new identified WRKY transcription factor with the Genbank accession number JX679079 was named *TaWRKY93* following *TaWRKY1-92* that had been used for other WRKY-related genes or ESTs in public database. The full length cDNA sequence was 1618 bp in length, including a 1053 bp open reading frame (Fig. S1A). Comparison with the genomic sequence revealed a 109 bp intron located 158 bp downstream of the transcription start codon (Fig. S1C). The putative TaWRKY93 protein contained a single WRKY domain (WRKYGQK) and a putative zinc finger motif (C-X5-C-X23-H-X1-H) (Fig. S1A), placing it as a group II member of the WRKY family. The WRKY domain of TaWRKY93 was highly similar to those presenting in the abiotic stress-responsive WRKYs, such as, HvWRKY38 in barley and OsWRKY08 in rice (Fig. S1B, D), indicating a contribution of TaWRKY93 to wheat's abiotic stress response.

3.2. Transcription of TaWRKY93 in response to salinity and exogenous ABA

qRT-PCR analysis showed that *TaWRKY93* was strongly and rapidly induced by the presence of either 200 mM NaCl or 100 μM ABA (Fig. 1). The transcript level increased within 0.5 h exposure to the NaCl or ABA treatment and peaked at 0.5 h in the root, 12 h in the leaf after ABA treatment. The equivalent maximum transcript level was got after 3 h and 0.5 h salinity treatment in the root and leaf, respectively. After peaking, the levels of transcript gradually fell back to the background level (Fig. 1).

3.3. The heterologous expression of TaWRKY93 enhances the abiotic stress tolerance of A. thaliana

TaWRKY93 transgenic *A. thaliana* lines were firstly confirmed using RT-PCR analysis based on the *TaWRKY93* gene-specific primers. Then three randomly independent transgenic lines were selected for an evaluation of abiotic stress or ABA tolerance. Compared with wild type plants, the *TaWRKY93* over-expression transgenic plants formed longer primary roots in the presence of 100 mM NaCl and a higher number of lateral roots in the presence of 150 mM mannitol (Fig. 2A, B, Fig. S2). No clear difference in the phenotype was detected for plants challenged with either 2 μM ABA, 50 mM KCl or 10 mM LiCl (Fig. 2A, B, Fig. S2). This implied that the heterologous, constitutive expression of *TaWRKY93* enhanced salt (NaCl) and osmotic stress tolerance. After exposed to drought stress for a period of two weeks, the wild type plants showed severely wilting, while the transgenic lines remained turgid; during the recovery period, half of the wild type plants died, whereas all the transgenic plants recovered fully and reached flowering before any of the surviving wild type plants (Fig. 2C). Cold temperature stress tolerance was also examined in *TaWRKY93* transgenic plants, at the start of the stress period, the wild type and the transgenic plants were indistinguishable in the phenotype, but after the 3 d exposure to −6 °C and the subsequent 4 d recovery period, over half of the wild type seedlings appeared wilted, while all the transgenic line seedlings appeared undamaged (Fig. 2D). These results demonstrated that *TaWRKY93* also contributed to drought and low temperature stress tolerance.

3.4. TaWRKY93 transgenic plants showing favourable physiological parameters

To elucidate the better abiotic stress tolerance of *TaWRKY93* transgenic plants, several physiological parameters were tested. The results showed that under the control conditions, *TaWRKY93* transgenic plants could accumulate more proline than the wild type plants (Fig. 3A). At the same time, the transgenic lines had a lower RWC (rate of water loss) than the wild types during dehydration process (Fig. 3B). As for electrolyte leakage, under control conditions, they were similar in the wild type and the transgenic plants, but within 1 h of the imposition of drought stress, its level in the transgenic plants was lower than that in the wild types, hence, leading to a lower relative electrical conductivity in the transgenic plants (Fig. 3C). These results indicated that *TaWRKY93* transgenic plants increased abiotic stress tolerance through maintaining favourable physiological parameters.

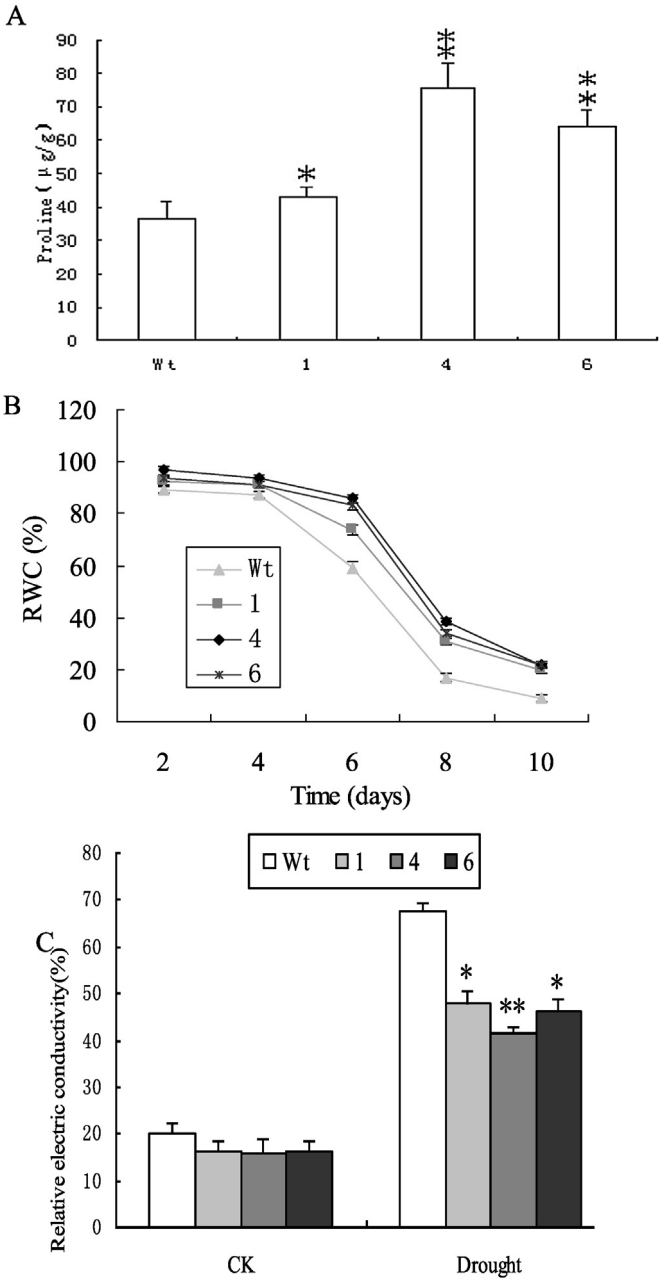

Fig. 3. Physiological parameters in *TaWRKY93* transgenic and wild type *A. thaliana* plants. (A) Leaf proline content under normal conditions, (B) relative water content in drought-stressed seedlings, (C) electrolyte leakage from the leaf of drought-stressed seedlings. Wt: wild type, 1, 4, 6: *TaWRKY93* transgenic lines.

3.5. The heterologous expression of TaWRKY93 altered the transcription of key abiotic stress responsive genes

The expression of some stress-related genes was examined under control conditions in *TaWRKY93* transgenic plants. The results showed that, compared with wild type plants, except the level of *ABA1* was comparable between transgenic and the wild type plants, the transcript abundance of examined abiotic stress-related genes, such as *ABF3*, *ABI1*, *2*, *3* (ABA-dependent), *DREB2A*, *RD19A*, *ICE1*, *RD21* (ABA-independent) and *P5CS* (involved in proline synthesis) was higher in the *TaWRKY93* transgenic plants (Fig. 4). This indicated that *TaWRKY93* enhanced abiotic stress tolerance through up-regulating of abiotic stress related genes.

4. Discussion

Transcription factors play a critical role in regulation of plant responses to abiotic stress [25]. More and more results demonstrated that over-expression of stress responsive transcription factors is a promising strategy for improving abiotic stress tolerance in crop [26,27]. Some members of WRKY family have been found to be responsive to various abiotic stresses [28]. In order to offer effective salt stress tolerance candidate genes for wheat genetic engineering, here a wheat salt inducible class II WRKY TF *TaWRKY93* was characterized. Transcription analysis demonstrated that *TaWRKY93* was rapidly induced by NaCl and exogenous ABA within 0.5 h after treatments, which coincided with our previous microarray results reporting *TaWRKY93* was an early NaCl responsive gene. Transgenic lines over-expressing *TaWRKY93* developed longer primary roots exposed to 100 mM NaCl, survived better under drought stress, recovered faster after low temperature treatment in contrast to non-transgenic plants, revealing that *TaWRKY93* confers multiple abiotic stress tolerance. Mahajan et al. reported that a number of TFs associated with enhancing salinity stress tolerance at the same time also have a positive effect on drought and low temperature tolerance, for all these stresses induce an element of osmotic stress [29]. In this study, *TaWRKY93* transgenic plants produced more lateral roots under 150 mM mannitol (Fig. 2B), elevated transcript level of *P5CS1*(Fig. 4), produced a more proline content (Fig. 3A), indicating that the superior salt stress tolerance of *TaWRKY93* transgenic plants also may be achieved by the enhanced osmotic stress tolerance through heterologous expression of *TaWRKY93*.

Osmoltye accumulation under water deficit condition can maintain root growth and consequently lead to reaching water that may be available deeper in the soil profile, at last benefits to crop yield [30]. The bread wheat cultivar cv. SR3 has a higher productivity than its parent bread wheat cv. Jinan 177, showed a superior root system and a better level of salinity and drought tolerance ability, [20]. We suggest that *TaWRKY93* may contribute to the superior agricultural traits of SR3 through promoting root development via enhancing osmotic adjustment ability. Some other abiotic stress related WRKY TFs regulating root development were also isolated in other plant species. Yu et al. reported that over-expression of the salinity-induced TF *OsWRKY08* induced the development of lateral roots and promoted the length of the primary ones [13]. Devaiah et al. also showed the suppression of *AtWRKY75* reduced the uptake of inorganic phosphorus, at the same time increasing the length of the lateral roots and the number of lateral root hairs [31]. The mechanism of *TaWRKY93* regulating root growth is undertaking.

A higher RWC indicates that the plant was better able to maintain its moisture status and so avoid dehydration. *TaWRKY93* transgenic plants survived better under drought stress (Fig. 2C) and recovered faster after low temperature treatment (Fig. 2D), which is consistent with the higher RWC under drought stress. Cell membrane is the primary site of abiotic stress damage in plants, and electrolyte leakage is a key physiological marker providing a measure of membrane stability [32]. The *TaWRKY93* transgenic plants maintained a lower relative electrical conductivity in contrast to the wild type ones, implying that *TaWRKY93* may be benefit to abiotic stress tolerance through the actions of maintaining the membrane stability (Fig. 3C).

ABA is a key hormone related to abiotic stress tolerance [33]. Plant responds to abiotic stress in an ABA-dependent or independent pathway. WRKYs are known to be a key component in ABA signalling [5]. *TaWRKY93* was induced by exogenously applied ABA (Fig. 1), its heterologous expression in *A. thaliana* up-regulated *ABF3*, *ABI1*, *2* and *3* (ABA-dependent) and *DREB2A*, *RD19A* and *RD21* (ABA-independent) (Fig. 4), thus *TaWRKY93* appears to be a regulator involved both in ABA-dependent and -independent pathways. The similar findings were also found in the functions of *TaMYb73* and *TaCHP* transcription factors. The former regulated *ABF3, CBF3*, *RD29A* and *RD29B*, the latter acted on *CBF3*, *DREB2A*, *ABI1* and *ABI2* [9,10,34]. Rushton et al. also pointed out that a single WRKY TF can be involved in regulating several seemingly disparate

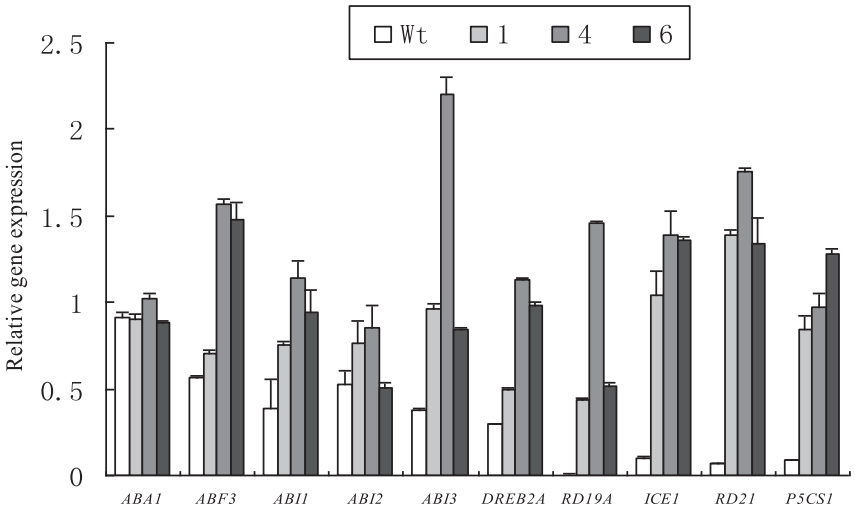

Fig. 4. The induction of a selection of genes involved in the abiotic stress response in ten day old *TaWRKY93* transgenic *A. thaliana* seedlings.

processes [7]. This may in part explain the function of *TaWRKY93* in up-regulating of marker genes involved in both ABA-dependent and -independent pathways in transgenic plants. I*CE1* and DREBs have been shown to be involved in cold stress through regulating the transcription of the low temperature responsive genes [3,35,36]. In *TaWRKY93* transgenic plants, *ICE1* and *DREB2A* were also up-regulated (Fig. 4), indicating the reason that *TaWRKY93* transgenic plants enhanced low temperature tolerance (Fig. 2D).

In summary, *TaWRKY93* is a new positive regulator of abiotic stress. It may increase salt, drought and low temperature stress tolerance through enhancing osmotic adjustment, maintaining membrane stability and increasing transcription of stress related genes, and contribute to the superior agricultural traits of SR3 through promoting root development.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.128.

Transparency document

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