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Wheat TaWRKY10-1 is involved in biological responses to the salinity and osmostresses in transgenic Arabidopsis plants

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Abstract

Wheat *TaWRKY10* is an uncharacterized group II member of the WRKY superfamily transcription factor and is regulated by high salinity and PEG. In order to study the diverse biological functions of *TaWRKY10*, we cloned a 783 bp cDNA fragment of this gene from the wheat accession Yangmai 158 (*Triticum aestivem* L.). Subsequent sequence analysis revealed that the cloned fragment contains UTRs and coding region and shows 96% sequence identity to the *TaWRKY10* gene in Genbank. The amino acid sequences analysis identified 15 amino acid differences, mostly at the C-terminus of this protein of 222 amino acids, compared with the TaWRKY10, suggesting that it is likely to be a novel allele of *TaWRKY10*. Hence we designated it as *TaWRKY10-1*. RT-PCR results showed that *TaWRKY10-1* gene was up-regulated by NaCl and PEG in wheat. To further elucidate its function, we constructed a chemical-inducible *TaWRKY10-1* over-expression plasmid, pKIGW-TaWRKY10-1, via Gateway cloning technology and transformed it into *Arabidopsis* plants. Over expression of *TaWRKY10-1* gene increases the sensitivity of transgenic plants to NaCl, PEG and mannitol, indicating *TaWRKY10-1* might act as a negative regulator in response to the salinity and osmostresses. This work provides the key basis for further functional analysis of wheat *TaWRKY10-1* gene.

Keywords: Wheat; *TaWRKY10-1*; Inducible expression plasmid; NaCl and PEG; Arabidopsis.
Abbreviations: Ampicillin resistance; CDS-Coding sequence; DEX- Dexamethasone.

Introduction

WRKY proteins belong to a superfamily of transcription factors with one or two WRKY domains containing zinc finger motifs, and their name is derived from the seven highly conserved amino acids "WRKYGQK" found at its N-terminus end. Since Ishiguro and Nakamura (1994) identified the first WRKY protein in sweet potato (*Ipomoea batatas*), many members of this family have been cloned (Eulgem et al., 2000) from various species, including *Arabidopsis*, wild oats (*Avena fatua*), orchardgrass (*Dactylis glomerata*), barley (*Hordeum vulgare*), tobacco (*Nicotiana tabacum*), chamomile (*Matricaria chamomilla*), rice (*Oryza sativa*), parsley (*Petroselinum crispum*), a desert legume (*Retama raetam*), sugarcane (*Saccharum hybrid cultivar*), bittersweet nightshade (*Solanum dulcamara*), potato (*Solanum tuberosum*), and wheat (*Triticum aestivum*) (Wu et al., 2008; Niu et al., 2012). According to the NCBI database, there are more than 1000 RefSeq records of WRKY proteins from different spices so far. WRKY transcription factors show high binding affinity for the DNA sequence (C/T)TGAC(T/C) (also known as W box). It has been suggested that WRKY are involved in responses to wounding, pathogen infection, abiotic stresses, seed development and senescence in numerous plant species (Eulgem et al., 2000; Rushton et al., 2010; Hyun et al., 2011). Wheat is one of the most important food crops in the world. But the information related to wheat *WRKY* genes is very limited. In 2008, Wu *et al.* (2008) reported the cloning of 15*WRKY* genes including *TaWRKY10* gene from wheat (*Triticum aestivum* L.) varieties (CAU 3338). In their study, *TaWRKY10* was shown to be up-regulated after treatment with NaCl and PEG by semi-quantitative RT-PCR (Wu et al., 2008), but it remain elusive how plants overexpressing TaWRKY10 would respond to these abiotic stresses. In this study, we cloned new allele of TaWRKY10 gene, TaWRKY10-1, from wheat accession Yangmai 158, constructed an inducible TaWRKY10-1 overexpression vector using the Gateway technology, transformed it into Arabidopsis plants and clarify the responses of plants overexpressing TaWRKY10-1 to abiotic stresses. This study will provide a basis for studying the regulatory mechanisms of TaWRKY10-1 gene in wheat in detail in the future.

Results

Cloning of TaWRKY10 gene

Total RNA from wheat (Yangmai 158) seedlings was reverse-transcribed and PCR amplification was performed with primers (TaWRKY10-1F and TaWRKY10-1R). A PCR product of about 800bp (Fig. 1) was identified and subsequently cloned into a pGEM-T Vector (TaKaRa). Recombinant plasmids were transformed into DH5α competent cells and screened by blue-white screening. By bacteria colony PCR reactions, three positive colonies were identified (Fig. 2) and recombinant plasmids were extracted for further sequence analysis.

Sequence analysis of TaWRKY10-1 gene

Sequencing results showed that the PCR product was 783 bp

with a 669 bp coding region and a 114 bp 5' and 3' UTR. The alignment of the CDS of cloned gene indicated 96% identity to the known *TaWRKY10* CDS (Genbank: EF368361) in nucleotide sequences. There were 15 amino acid differences within the ORF region of the obtained target fragment, suggesting that the cloned gene is likely a new allele of *TaWRKY10* gene and thus designated as *TaWRKY10-1*. The computational search for conserved domains within the TaWRKY10-1 amino acid sequence identified a Q to K change within the conserved "WRKYGQK" region and a novel zinc-finger-like motif $Cx_4Cx_{23}HxH$ (Fig. 3). These results suggest that the *TaWRKY10-1* gene could be a novel member of the group II WRKY transcription factor superfamily.

Physico-chemical properties of TaWRKY10-1 protein were predicted by ProtParam tool (*Gasteiger* et al., 2005). The parameters computed by ProtParam listed as follows: the molecular weight: 23735.3 Daltons, theoretical pI: 6.52, amino acid composition (Table 1), Formula: $C_{1041}H_{1571}N_{297}O_{323}S_{10}$, total number of negatively charged residues (Asp + Glu): 23, total number of positively charged residues (Arg + Lys): 22, the instability index 45.51. These data revealed that TaWRKY10-1 is an unstable transcription factor.

TaWRKY10-1 is stimulated by the treatments of NaCl and PEG

TaWRKY10 gene was regulated by abiotic stresses as previously reported (Wu et al., 2008). To determine whether *TaWRKY10-1* would respond to stimuli as *TaWRKY10* did, we conducted semi quantitative RT-PCR using wheat seedling challenged by NaCl and PEG. The results showed that *TaWRKY10-1* mRNA level was very low at 1h after challenged by NaCl or PEG-6000, but increased a lot in 2h and 3h under these stresses treatments, indicating TaWRKY10-1 was involved in plant responses to these environmental stimuli (Fig. 4).

Construction of Entry Clones Using the BP Recombination Reaction

A crucial step for Gateway cloning technology is to construct an entry clone by BP recombination reaction. Plasmid pGEM-TaWRKY10-1 was used as a template for PCR amplification with *attB*-containing primers. We then generated *TaWRKY10-1* with *attB*sites on two ends and purified the target 760bp PCR products (Fig. 5) BP reaction between *att*B-containing PCR products and the pDONR (Amp^r) vector with *att*P sites were conducted to create entry clones.

We transformed the reaction mixture into DH5α competent cells and screened for positive colonies. Colonies with ampicillin resistance were selected for overnight culture and plasmid extraction. Sequencing results from recombinant plasmids showed *TaWRKY10-1*coding sequence had been correctly inserted into the pDONR vector and new entry clones were named as pDONR-TaWRKY10-1 (Fig. 6).

Construction of Expression Clones Using the LR Recombination Reaction

After generating an entry clone, we performed the LR recombination reaction between a pDONR-TaWRKY10-1 entry clone and the pKIGW destination vector. We then transformed the reaction mixture into DH5α competent cells and screened the positive expression clones using the LB medium containing 75μg/ml kanamycin. Through sequencing, we confirmed that the *TaWRKY10-1* gene was inserted into pKIGW vector (Fig. 7). The recombinant expression vector was named as pKIGW-TaWRKY10-1. *Agrobacterium*transformation was performed by electroporation using the *Agrobacterium tumefaciens*strain GV3101. The transgenic agrobacterium colonies were further confirmed by PCR with the *att*B-TaWRKY10-1(ORF)-F and *att*B-TaWRKY10-1(ORF)-R primers. The target PCR product was 760bp (Fig. 8), indicating the pKIGW-TaWRKY10-1 plasmid was successfully transformed into *Agrobacterium*. Transgenic *Agrobacterium* can be used for

Table 1. Number and percentage of various amino acids in TaWRKY10-1

Amino Acid	Number	Mol %	Amino Acid	Number	Mol %
Ala (A)	35	15.7	Asn (N)	5	2.24
Cys (C)	3	1.35	Pro (P)	14	6.28
Asp (D)	11	4.93	Gln (Q)	2	0.9
Glu (E)	12	5.38	Arg (R)	16	7.17
Phe (F)	8	3.59	Ser (S)	22	9.87
Gly (G)	23	10.31	Thr (T)	12	5.38
His (H)	5	2.24	Val (V)	11	4.93
Ile (I)	2	0.9	Trp (W)	4	1.79
Lys (K)	6	2.69	Tyr (Y)	13	5.83
Leu (L)	11	4.93	Pyl (O)	0	0.0
Met (M)	7	3.14	Sec (U)	0	0.0

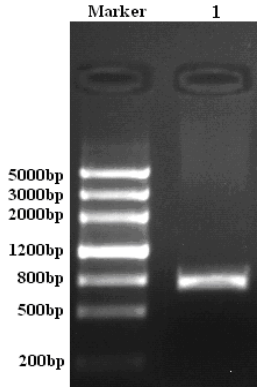

Fig 1. Amplification of the target gene. Marker: DNA Marker IV(Biomed); 1:Target gene(783bp)

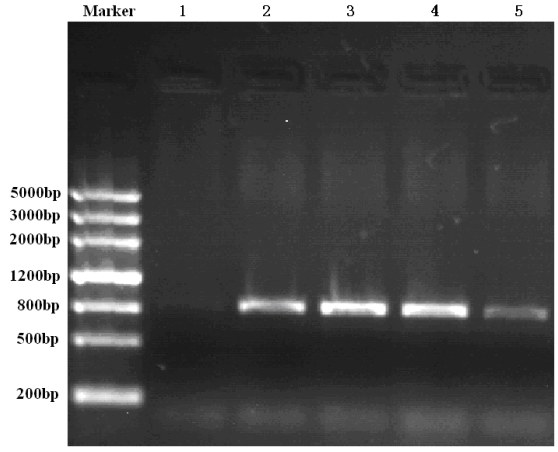

Fig 2. PCR result for identifying recombinant clones. Marker:DNA Marker IV(Biomed); 1:Negative Control ; 2,3,4: Colony PCR; 5.Positive Control

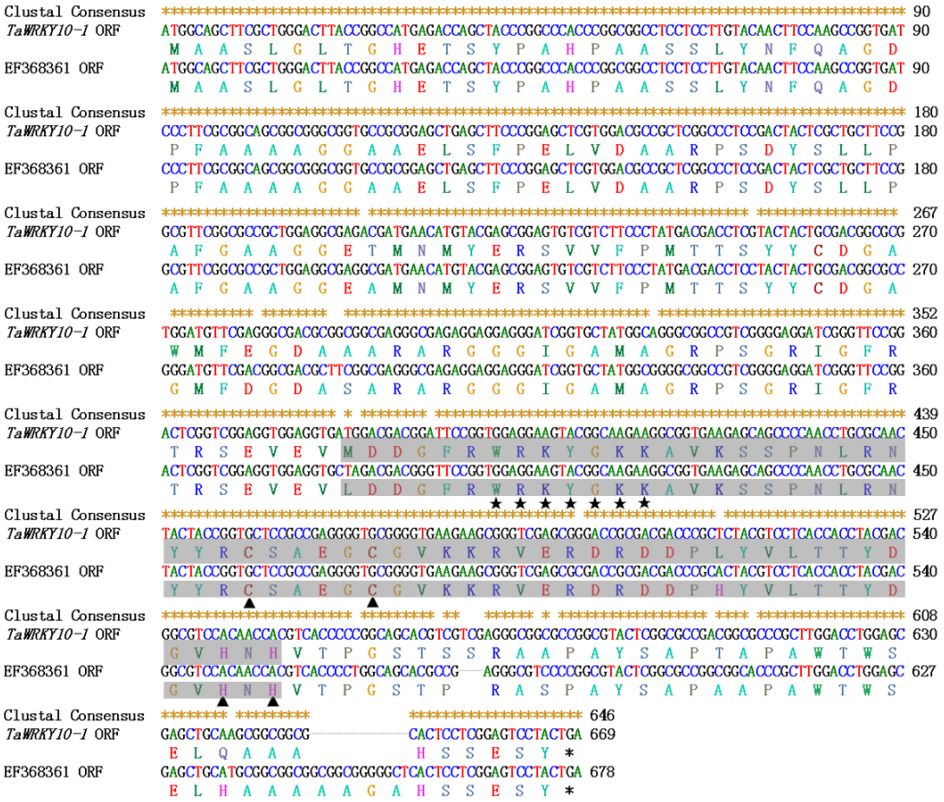

Fig 3. The amino acid sequence for *TaWRKY10-1* CDS. The shaded region indicates the conserved domains; ★ indicates conserved amino acids; ▲ indicates zinc finger motifs（C2H2）

the subsequent plant transformation and overexpression analysis.

Transgenic plants overexpressing TaWRKY10-1 are sensitive to NaCl and mannitol

Our results showed *TaWRKY10-1* would be induced by NaCl and PEG in wheat (Fig. 4). To determine the role of TaWRKY10-1 in plant responses to NaCl and PEG, we transformed the cassette with inducibly overexpressing TaWRKY10-1 into *Arabidopsis* wild type plant, Col-0. Transgenic plants resistant to kanamycin were screened and then subjected to treatments of NaCl and PEG. *TaWRKY10-1* was induced in transgenic plants after DEX application on media, while there was no *TaWRKY10-1* was detected from transgenic plants grown on media without DEX, indicating this inducible overexpression cassette functions as expected (Fig. 9). Transgenic plants without DEX application would grow as normal as wild type plants (plants 1 in Fig.10), but became very sensitive to NaCl, PEG and mannitol once *TaWRKY10-1* was over expressed after DEX application (plants 3 in Fig.10). Wild type plants showed no phenotype changes after DEX treatment(plants 2 and 4 in Fig.10) . These results implied that TaWRKY10-1 possibly acted as a negative regulator in responses to salinity and osmostress.

Discussion

WRKY transcription factors are one of the largest families of transcriptional regulators in plants and form integral parts of signalling webs that modulate many plant processes (Rushton et al., 2010). Numerous members of the three major WRKY groups are reported and most of them differ in the number of WRKY domains and the pattern of the zinc-finger motif (Eulgem et al., 2000; Wu et al., 2005; Dong et al., 2003). Evidence has emerged to show that WRKY proteins do not exclusively exist in higher plants and are identified from mosses (*Physcomitrella patens*) and the unicellular protist (*Giardia lamblia*) (Ulker and Somssich, 2004; Zhang et al., 2005) as well. In *Giardia lamblia*, the *WRKY* gene contains group I-like sequences and group III members have not been found in *Physcomitrella patens* (Ulker and Somssich, 2004). These findings imply that group I *WRKY* genes may represent the ancestral form, and the evolution process of *WRKY* genes is likely to be from group I to group II, and III. In this study, the newly cloned *TaWRKY10-1* gene belongs to group II, but the conserved amino acid sequence was changed to "WRKYGKK". This sequence variation within the conserved domain suggests that *WRKY* allelic genes might play a variety-specific role in biological processes in wheat.

To elucidate the functions of TaWRKY10-1 more precisely, we constructed an inducible overexpression vector to reduce the ectopic effects of genes caused by constitutive promoters. The expression vector pKIGW contains components of pOp6/LhGR system,used for DEX controlled transgene expression in plants (Fig. 7). The pOp6/LhGR system(Craft et al., 2005) is constructed by the fusion of ligand-binding domains of a rat glucocorticoid receptor (GR LBD) to the amino terminus of the synthetic transcription factor LhGR. The pOp/LhGR system has been successfully used to induce a cytokinin biosynthetic gene (Jasinski et al., 2005) and RNA interference construct (Venugopala and Meyerowitz, 2005; Wielopolska et al., 2005). The pOp6 promoter in *Arabidopsis* can give a high induction efficiency and will not hinder the plant growth and development even at high levels of DEX or LhGR expression (Craft et al., 2005). Our studies demonstrated that the expression of *TaWRKY10-1* gene can only start in the presence of the inducer DEX (Fig. 9) and confirmed the accuracy of this system in controlling the expression of the target gene in

transgenic *Arabidopsis* plants. A previous study (Wu et al., 2008) revealed that the *TaWRKY10* gene was up-regulated after treatment with high salinity and PEG. But there is no evidence about whether TaWRKY10 transgenic plant would be tolerant or sensitive to these environmental stresses because no expression vector of TaWRKY10 had been constructed (Wu et al., 2008). In this study, we cloned the *TaWRKY10-1* gene and showed *TaWRKY10-1* expression was also enhanced by these stresses in wheat young seedlings as expected. To determine the functions of TaWRKY10-1 gene further, we constructed an inducible overexpression plasmid and obtained transgenic Arabidopsis plants. The results showed that *Arabidopsis* plants overexpressing *TaWRKY10-1* are more vulnerable to NaCl, PEG and mannitol, supporting our proposition that TaWRKY10-1 functions as a negative regulator in response to the salinity and osmostresses. WRKY transcript factors as negative regulators were also reported by other studies. *GmWRKY13* overexpression resulted in increased sensitivity to salt and mannitol (Zhou et al., 2008) and OsWRKY24and OsWRKY45 were found to act as repressors of an ABA-inducible promoter in a transient expression study using aleurone cells (Xie et al., 2005). In the other hand, a latest study showed overexpression of another wheat two genes, *TaWRKY2* and *TaWRKY19*, in *Arabidopsis* plants would enhance plant tolerance to abiotic stresses (Niu et al., 2012). These results provided further evidences on diversity and specificity of *WRKY* genes functions. Our studies established a solid basis for dissecting the function of the*TaWRKY10-1* gene. However, comprehensive studies are still needed for the elucidation of the regulatory mechanisms of the *TaWRKY10-1* gene and for genetic engineering of stress responses in wheat in the future.

Material and Methods

Wheat seedlings

For gene cloning experiments, wheat (Yangmai 158) seeds were surface sterilized with 1% sodium hypochloride for 5min and rinsed with distilled water, before being stratified at 4 ^{o}C for 7 days. Stratified seeds were then grown in a growth chamber under a controlled environment of 26/20 ^{o}C day/night temperature, relative humidity of 75%, lighting intensity of 3000lx and photoperiods of 12h·d^{-1}. Five-week-old mature leaves of Yangmai 158 were frozen in liquid nitrogen and stored at -80^{o}C for use.

For abiotic stress treatments, wheat (Yangmai 158) seeds were surface sterilized with 75% ethanol for 2min , rinsed with distilled water three times, incubated in distilled water in the darkness for 12h. then put in a 22℃ incubator for 24h. Germinated seeds then were transferred into sand pots and watered 50ml distilled water daily in a growth chamber as above. 12-day-old wheat seedlings were divided into three groups and challenged with 15% NaCl, 25% PEG-6000 and water (control), respectively. Seedlings leaves at 1h, 2h and 3h after treatments were sampled as described above.

Total RNA extraction and cDNA synthesis

Total RNAs was isolated using Total RNA Isolation System (TIANGEN Co., Ltd), and cDNAs were synthesized using SuperScript First-Strand Synthesis System (Invitrogen) for RT-PCR. Chemicals were obtained from TaKaRa Biotechnology (Dalian) Co., Ltd, such as Taq Polymerase, pGEM-T Vector Kit, DNA purification kit. The DNA sequencing was conducted by BIOMED Co., Ltd.

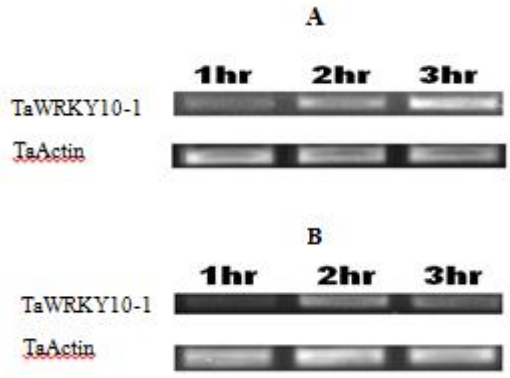

Fig 4. RT-PCR of TaWRKY10-1 in wheat seedlings treated with NaCl and PEG-6000
A. TaWRKY10-1 expression at 1hr, 2hr and 3hr after NaCl treatment.
B. TaWRKY10-1 expression at 1hr, 2hr and 3hr after PEG-6000 treatment.

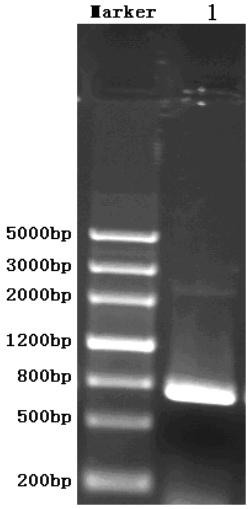

Fig 5. PCR result for attB1- TaWRKY10-1(ORF)-attB2.
Marker: DNA Marker IV; 1: attB1-TaWRKY10-1(ORF)-attB2.

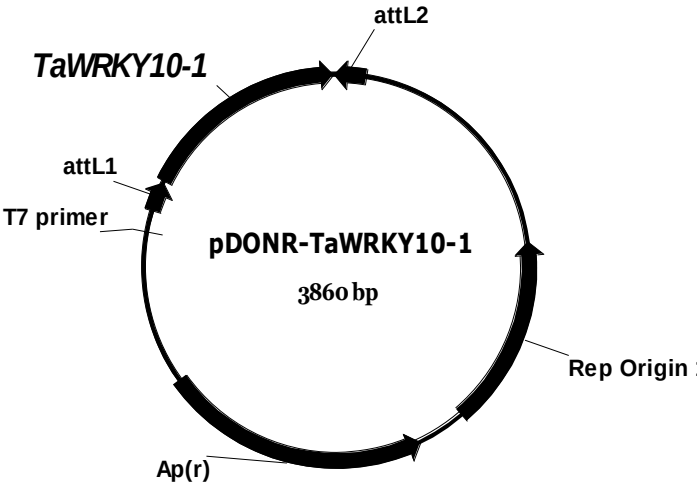

Fig 6. Map for plasmid pDONR-TaWRKY10-1

Primer design and synthesis

Primers were designed according to the annotated *TaWRKY10* mRNA（GenBank: EF368361）with a full-length open reading frame. Two oligonucleotides based on the EF368361 were synthesized, including forward primer (TaWRKY10-1F）and reverse primer（TaWRKY10-1R）.
TaWRKY10-1F：5′-AGCTCGTCTGTGCAGTGCACTTAT-3′
TaWRKY10-1R：5′-TCGTGTACATGCATCCGTGAGATT-3′

Plasmid construction

TaWRKY10 gene was amplified with Phusion High-Fidelity DNA polymerase (BioLab) from cDNA using TaWRKY10-1F and TaWRKY10-1R. The reaction was denatured at 98°C for 30 s, followed by 98 °C for 10 s, 58 °C for 20 s, 72 °C for 1 min, for 35 cycles and a final 5 min extension at 72 °C. The final PCR product was purified using the Agarose Gel DNA Purification Kit (Takara), cloned into pGEM-T Vector (Takara) and transformed into DH5α competent cells by heat shock method. The recombinant clones were screened by "white-blue plaque selection". By colony PCR method, positive clones were identified and then sequenced (BIOMED).

Sequence analysis

Database searches were carried out by Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). Open reading frame (ORF) analysis was carried with the NCBI ORF finder online tool. Protein sequence analysis was conducted with DNAMAN software (http://www.lynnon.com/dowload/index/html/). Conserved domains were identified by CD-Search (Marchler-Bauer and Bauer, 2004; Marchler-Bauer et al., 2009, 2011). Physical and chemical properties of proteins were analyzed using ProtParam tool（http://www.expasy.ch/tools/protparam.html）.

Construction of expression vector by Gateway Technology

To generate PCR products suitable for use as substrates in a Gateway BP recombination reaction with a donor vector, we incorporated *att*B sites into the target gene. We designed the *att*B PCR primer at both ends of the coding region of the *TaWRKY10 -1* gene.
The forward *att*B site is indicated in bold and the ATG start codon for *TaWRKY10-1* gene is underlined. *att*B-TaWRKY10-1(ORF)-F : 5′-GGGG**ACAAGTTTGTACAAAAAAGCAGGCT** TAATGGCAGCTTCGCT-3′; The reverse *att*B2 site is indicated in bold and the stop codon for *TaWRKY10-1* gene is underlined. *att*B-TaWRKY10-1(ORF)-R : 5′-GGGG**ACCACTTTGTACA AGAAAGCTGGGT**CTCAGTAGGACTCCGA-3′
BP and LR reactions were conducted according to the invitrogen manual. BP Clonase™ enzyme and LR Clonase™ enzyme were ordered from invitrogen. pKIGW (GenBank：FN377812.1) and a modified pDONR221 with ampicillin resistance gene were used as destination vectors and pDNOR vectors, respectively. The *TaWRKY10-1*gene-specific primers （ 5′-ATGGCCCTGGACTCCGTCCC-3′ ） were used for sequencing the expression vectors to confirm the correct clones.

Application of abiotic stresses on transgenic Arabidopsis plants

Transgenic plants were generated according to Clouch et al. protocol (1998) with minor modifications. T1 seeds were

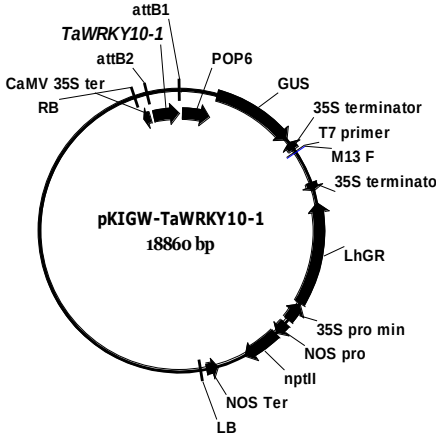

Fig 7. Map for plasmid pKIGW-TaWRKY10-1. Key components of the plasmid are indicated on the map.

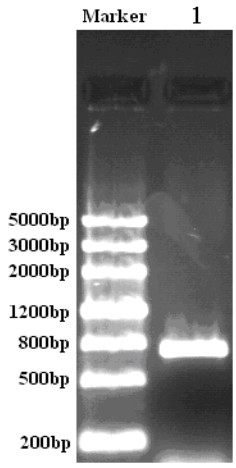

Fig 8. PCR validation of pKIGW-TaWRKY10-1
Marker: DNA Marker IV (Biomed); 1: *TaWRKY10-1*(760bp).

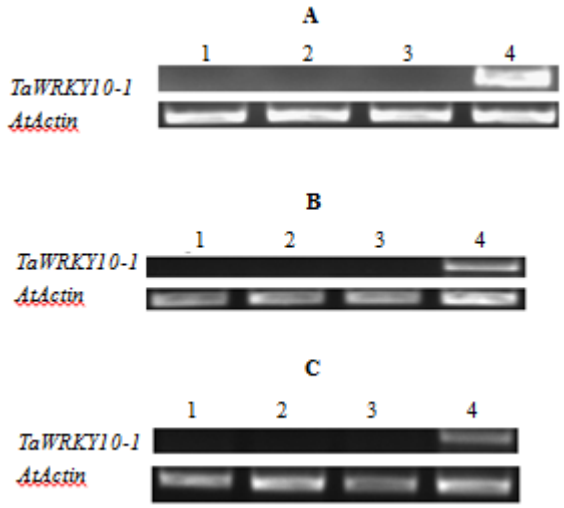

Fig 9. RT-PCR of *TaWRKY10-1* in transgenic Arabidopsis seedlings challenged by NaCl, PEG-6000 and mannitol
A. *TaWRKY10-1* mRNA levels in plants on NaCl (75mM) media; B. *TaWRKY10-1* mRNA levels in plants on PEG-6000 (2%) media; C. *TaWRKY10-1* mRNA levels in plants on mannitol (150mM) media Lane 1-2.Transgenic and wild type *Arabidopsis* on solid media without DEX; Lane 3-4. wild type and transgenic *Arabidopsis* on solid media with DEX.

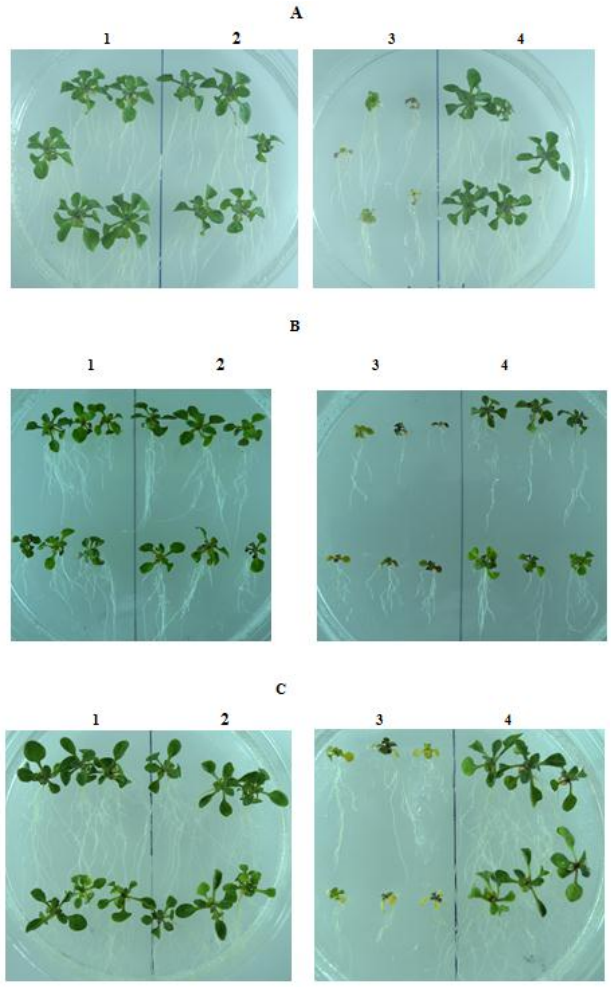

Fig 10. *TaWRKY10-1* transgenic Arabidopsis seedlings challenged by NaCl, PEG-6000 and mannitol. A. Transgenic plants on NaCl (75mM) media; B. Transgenic plants on PEG-6000 (2%) media; C. Transgenic plants on mannitol (150mM) media 1-2.Transgenic and wild type plants on solid media without DEX; 3-4. Transgenic and wild type plants on solid media with DEX.

surface sterilized by 75% ethanol for 1 min, sown on half MS solid media with kanamycin(75mg/l) and transferred into growth chamber(22℃、light period 12h/d) after stratification for 3d. Seven-day-old transgenic and wild type plants were divided into 4 groups and grown on abiotic solid media, including three treatments groups NaCl (75mM), PEG-6000 (2%) and mannitol (150mM) with DEX and three corresponding control groups without DEX. Morphological differences among transgenic plants with and without DEX treatments were determined two weeks after plants were transferred onto abiotic solid media.

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