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Chrysanthemum WRKY gene *CmWRKY17* negatively regulates salt stress tolerance in transgenic chrysanthemum and Arabidopsis plants

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Abstract

Key message **CmWRKY17 was induced by salinity in chrysanthemum, and it might negatively regulate salt stress in transgenic plants as a transcriptional repressor.**

Abstract WRKY transcription factors play roles as positive or negative regulators in response to various stresses in plants. In this study, *CmWRKY17* was isolated from chrysanthemum (*Chrysanthemum morifolium*). The gene encodes a 227-amino acid protein and belongs to the group II WRKY family, but has an atypical WRKY domain with the sequence WKKYGEK. Our data indicated that CmWRKY17 was localized to the nucleus in onion epidermal cells. CmWRKY17 showed no transcriptional activation in yeast; furthermore, luminescence assay clearly suggested that CmWRKY17 functions as a transcriptional repressor. DNA-binding assay showed that CmWRKY17 can bind to W-box. The expression of *CmWRKY17* was induced by salinity in chrysanthemum, and a higher expression level was observed in the stem and leaf compared with that in the root, disk florets, and ray florets. Overexpression of *CmWRKY17* in chrysanthemum and Arabidopsis increased the sensitivity to salinity stress. The activities of superoxide dismutase and peroxidase and proline content in the leaf were significantly lower in transgenic chrysanthemum than those in the wild type under salinity stress, whereas electrical conductivity was increased in transgenic plants. Expression of the stress-related genes *AtRD29*, *AtDREB2B*, *AtSOS1*, *AtSOS2*, *AtSOS3*, and *AtNHX1* was reduced in the *CmWRKY17* transgenic Arabidopsis compared with that in the wild-type Col-0. Collectively, these data suggest that *CmWRKY17* may increase the salinity sensitivity in plants as a transcriptional repressor.

Keywords *Chrysanthemum morifolium* · Salinity stress · WRKY transcription factor

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Introduction

The WRKY family is among the largest families of transcription factors in plants and is named after the highly conserved sequence motif WRKYGQK (Eulgem et al. 2000). Since Ishiguro and Nakamura isolated the first WRKY gene, *SPF1*, from *Ipomoea batatas* in 1994 (Ishiguro and Nakamura 1994), an increasing number of WRKY genes have been identified from a diverse variety of plants (Abbruscato et al. 2012; Atamian et al. 2012; Borrone et al. 2004; Guo et al. 2011; Zheng et al. 2011; Lagacé and Matton 2004; Li et al. 2013; Liu et al. 2012; Marè et al. 2004; Niu et al. 2012; Pan et al. 2009; Park et al. 2006). Over 70 and 100 members of the family are known in Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), respectively (Agarwal et al. 2011; Kalde

et al. 2003; Wang et al. 2011). On the basis of the primary structure, WRKY proteins have been classified into three groups (I, II, and II) and various subgroups (Eulgem et al. 2000). Group II members contain a single C_2H_2 zinc finger with one conserved WRKY (WRKYGQK) domain, group III proteins contain one C_2HC zinc finger and the conserved WRKY domain as in group II, whereas group I proteins contain two C_2H_2 zinc fingers and two WRKY domains. The WRKY proteins can specifically bind to the W-box [TTGAC(C/T)], a common *cis*-element in the promoters of target genes (Maeo et al. 2001).

The WRKY proteins play various roles as positive or negative regulators in response to biotic and abiotic stresses (Agarwal et al. 2011; Rushton et al. 2010). In Arabidopsis, overexpression of *WRKY25* can improve the salinity tolerance of the transgenic plants (Jiang and Deyholos 2006), constitutive expression of *WRKY33* enhances resistance to heat stress (Li et al. 2011), while *WRKY8* plays a role in response to salt stress (Hu et al. 2013). *GmWKRY21*-transgenic Arabidopsis plants are tolerant to cold stress, and moreover *GmWRKY54* confers salt and drought tolerance. However, overexpression of *GmWRKY13* causes increased sensitivity to salt stress (Zhou et al. 2008). *ZmWRKY33* (Li et al. 2013) and *TaWRKY2/TaWRKY19* (Niu et al. 2012) are similarly induced by abiotic stresses, such as drought, salt, and abscisic acid (ABA) treatments. Transgenic Arabidopsis plants overexpressing *TaWRKY2* exhibit enhanced salt and drought tolerance, whereas overexpression of *TaWRKY19* confers increased tolerance to salt, drought, and freezing stresses. *AtWRKY17* acts as a negative regulator of basal resistance to *Pseudomonas syringae* pv. *Tomato* (Journot-Catalino et al. 2006). *OsWRKY28* (Chujo et al. 2013) from rice and *CaWRKY58* (Wang et al. 2013) from *Capsicum annuum* negatively regulate resistance to the rice blast fungus *Magnaporthe oryzae* and *Ralstonia solanacearum*, respectively. *NaWRKY3* and *NaWRKY6* from the native tobacco *Nicotiana attenuata* can be significantly upregulated in plants attacked by *Manduca sexta* (Hui et al. 2003). Besides abiotic and biotic stress response, WRKY proteins also play important roles in plant growth and development processes, such as seed coat development (Johnson et al. 2002), embryonic development (Lagacé and Matton 2004), lateral root growth (Zhang et al. 2008; Zhou et al. 2008), fruit ripening (Devaiah et al. 2007), and senescence (Hinderhofer and Zentgraf 2001; Ülker et al. 2007).

Chrysanthemum (*Chrysanthemum morifolium*), a widely grown ornamental plant, is sensitive to salinity, which induces extensive leaf chlorosis, retards growth, and in some cases even kills the plant. To our knowledge, little information on the isolation and functional identification of WRKY transcription factors in chrysanthemum is available. We have previously isolated 15 WRKY genes, which are induced by various stresses (Song et al. 2014a). Overexpression of *DgWRKY3*, which was isolated from chrysanthemum, increased salt stress tolerance in tobacco (Liu et al. 2013). Here, we identified a novel group II WRKY transcription factor, *CmWRKY17*, from chrysanthemum 'Zhongshanzigui', and showed that it was upregulated by salinity stress. Overexpression of *CmWRKY17* in chrysanthemum and Arabidopsis caused increased sensitivity to salinity stress compared with the wild-type (WT) plants.

Materials and methods

Plants materials and growth conditions

The chrysanthemum cultivars 'Zhongshanzigui' and 'Jinba' were obtained from the Chrysanthemum Germplasm Resource Conservation Centre, Nanjing Agricultural University, China. The plants were potted in a 1:1 (v/v) mixture of soil and vermiculite, and grown in a greenhouse. *A. thaliana* ecotype Columbia (Col-0) plants were grown in a 3:1 (v/v) mixture of vermiculite and soilrite in a growth chamber (SANYO, MLR-359H, Japan) with a 16-h light period (80–100 µmol/m^2/s illumination) at 23 °C, followed by an 8-h dark period at 18 °C, with a relative humidity of 70 %.

Cloning of *CmWRKY17* and sequence analyses

Total RNA was isolated from 'Zhongshanzigui' leaves using the RNAiso reagent (TaKaRa, Japan), following the manufacturer's instructions, and then treated with RNase-free DNase I (TaKaRa, Japan) to remove potential genomic DNA contamination. The first-strand cDNA was synthesized with the SuperScript III reverse transcriptase (Invitrogen). On the basis of chrysanthemum expressed sequence tags (Chen et al. 2009), a primer pair (CmWRKY17-F/R) was designed to amplify a fragment of *CmWRKY17*, then random amplification of cDNA ends (RACE) PCR was used to obtain the full-length cDNA. For 3′ RACE, the first-strand cDNA was synthesized using an oligo (dT) primer incorporating the sequence of the adaptor primer, followed by a nested PCR using the primers CmWRKY17-3-F1/F2 and the adaptor primer dT-R (Table 1). For 5′ RACE, the primers AAP and AUAP, and specific primers (CmWRKY17-5-R1/R2) were used in the nested PCR with the 5′ RACE System Kit v2.0 (Invitrogen). The PCR products were purified with the Biospin Gel Extraction Kit (BioFlux, China) and cloned into the pMD19-T easy vector (TaKaRa) for sequencing. Consequently, a pair of primers (CmWRKY17-ORF-F/R) was designed to amplify the

Table 1 Primer names and sequences used in this study

Prime name	Sequence (5′ to 3′)
CmWRKY17-F	ATCCCTTCTCATAACCAAAACCC
CmWRKY17-R	CGTCGTATGTCACCACAAGCA
CmWRKY17-3-F1	GCTACAGCGACGGAATCACC
CmWRKY17-3-F2	TTAGAAGAGTGCCGATTATTGGAT
Oligo (dT)	AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTT
dT-R	AAGCAGTGGTATCAACGCAGAGTAC
CmWRKY17-5-R1	GGCAACGGCGGTTTCCTC
CmWRKY17-5-R2	CGGTGATTCCGTCGCTGTA
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
AUAP	GGCCACGCGTCGACTAGTAC
CmWRKY17-ORF-F	GCTTCCTTGCAGGGCATCC
CmWRKY17-ORF-R	GCAAAGTAATCAATCAATAATGGACC
CmWRKY17-RT-F	CTTGTGGTGACATACGACGGG
CmWRKY17-RT-R	CCAACGAAACACTACTTGTTGACCGTAG
CmEF1α-F	TTTTGGTATCTGGTCCTGGAG
CmEF1α-R	CCATTCAAGCGACAGACTCA
CmWRKY17-BD-F	GGAATTCATGGCGGTCGAT
CmWRKY17-BD-R	CGGGATCCTACCACAACACTGGTCAA
CmWRKY17-GATE-F	CGGGATCCGGATGGCGGTCGAT
CmWRKY17-GATE-R	GAGCGGCCGCGATACCACAACACTGGTCAA
pHis-W–F	AATTCTTCCAT<u>TTGACT</u>AGTGTATTCCAT<u>TTGACT</u>AGTGTATTCCAT<u>TTGACT</u>AGTGTAT
pHis-W-R	CTAGATACACT<u>AGTCAA</u>ATGGAATACACT<u>AGTCAA</u>ATGGAATACACT<u>AGTCAA</u>ATGGAAG
pHis-mW-F	AATTCTTCCAT<u>TAATATA</u>GTGTATTCCAT<u>TAATATA</u>GTGTATTCCAT<u>TAATATA</u>GTGTAT
pHis-mW-R	CTAGATACACT<u>ATATTA</u>ATGGAATACACT<u>ATATTA</u>ATGGAATACACT<u>ATATTA</u>ATGGAAGAG
CmWRKY17-1301-F	CGGGATCCATCAATGGCGGT
CmWRKY17-1301-R	CGAGCTCCTTTGACTTATACCACAA
AtRD29A-F	AGGATTAAAGGATGGTTCGGTG
AtRD29A-R	TCATATTCTTAAAGCTCCTTCTGCA
AtDREB2B-F	CATCAGAGCCAAGACCAAAACC
AtDREB2B-R	TGTAGGACCATTGCCTCAGAAC
AtSOS1-F	TCGGCAGCATGGTTAATGTG
AtSOS1-R	TTGGCTGAAACGAGACCTTGA
AtSOS2-F	GAAACTGGAAAACATAATATGGAGGG
AtSOS2-R	TCGCAGGACAAGTTTTGAGAAGTA
AtSOS3-F	AATCCATCGCTCATCAAGAACA
AtSOS3-R	CGGTTTATTTCCAAATCCTAGCTTAC
AtNHX1-F	GACTCCTTCATGCGACCCG
AtNHX1-R	CCACGTTACCCTCAAGCCTTAC
AtAct2-F	TTCGTTTTTGCGTTTTAGTCCC
AtAct2-R	GGGAACAAAAGGAATAAAGAGGC

complete *CmWRKY17* open reading frame (ORF) with the first-strand cDNA as the template.

The CmWRKY17 amino acid sequence was aligned with its homologs using the DNAman 5.2.2 software and BLAST software online (http://www.ncbi.nlm.gov/blast). A phylogenetic tree was constructed using the neighbor-joining method with BioEdit and MEGA 5.2.2.

Assay of *CmWRKY17* expression in different tissues and under salinity stress

For salinity treatment, 'Zhongshanzigui' plants at the six-to eight-leaf stage were used. The plants were watered with 200 mM NaCl. Each experiment contained three biological replicates. Leaves of NaCl-treated plants were harvested

after 0, 1, 2, 4, 8, 12, and 24 h, frozen in liquid nitrogen, and stored at −80 °C for RNA extraction.

Total RNA was extracted from different tissues (root, stem, leaf, disk florets, and ray florets) in chrysanthemum and salinity-stressed plants at the above-mentioned time points using the RNAiso reagent (TaKaRa, Japan) following the manufacturer's instructions. The RNA was treated with RNase-free DNase I (TaKaRa) to remove genomic DNA contamination. The first cDNA strand was synthesized from 1 μg total RNA using M-MLV reverse transcriptase (TaKaRa) in accordance with the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa) was used to detect the expression of *CmWRKY17*. Each 20 μL qRT-PCR contained 10 μL SYBR Green PCR master mix, 0.4 μL of each primer (10 μM), 4.2 μL H$_2$O, and 5 μL cDNA template. The PCR cycling regime comprised an initial denaturation (95 °C for 2 min), followed by 40 cycles of 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 20 s. The resulting data were represented by the mean ± SD of three biological replicates. The primer pair CmWRKY17-RT-F/R was used and the *CmEF1α* gene (KF305681) acted as a reference sequence after sequencing of the amplified fragment by primer pairs. Relative transcript abundances were calculated using the $2^{-\Delta\Delta CT}$ method. The data were scaled by setting the expression of *CmWRKY17* in untreated leaves at 0 h as 1.

Transcriptional activity analysis of CmWRKY17

CmWRKY17 was examined for transactivation activity firstly using a yeast assay system. The ORF of *CmWRKY17* without the termination codon was amplified using the Phusion HighFidelity PCR Kit (New England Biolabs) with the primer pair CmWRKY17-BD-F/R (Table 1). The PCR products were subcloned into the pMD19-T vector (TaKaRa) and confirmed by sequencing. Both pMD19-T harboring *CmWRKY17* and pGBKT7 were digested with *Eco*R I and *Bam*H I, then the corresponding bands were recovered and ligated into pGBKT7 to yield the expression vector pGBKT7-CmWRKY17. The pGBKT7-CmWRKY17 construct, pCL1 (positive control), and pGBKT7 (negative control) were introduced into *Saccharomyces cerevisiae* strain Y2HGold (Clontech) following the manufacturer's protocol. Selection for transformants carrying either pGBKT7-CmWRKY17 or pGBKT7 employed an SD/-Trp medium, whereas pCL1 was selected on SD/-Leu medium. As a positive control, Y2H cells carrying pCL1 can grow on SD/-His-Ade medium, whereas Y2H cells carrying pGBKT7, the negative control, cannot grow on the medium.

Then the luminescence assay of CmWRKY17 was taken to further understand its transcriptional activity. To generate 35S::GAL4DB-CmWRKY17 fusion construct, the *CmWRKY17* ORF was amplified by PCR using the primer set CmWRKY17-GATE-F/R (Table 1) harboring the *Bam*H I and *Not* I sites. The amplified DNA fragment was directly cloned into the pENTR™1A dual selection vector (Invitrogen) to obtain pENTR™1A-CmWRKY17 by sequencing. The plasmid was subjected to the vector 35S::GAL4DB to obtain the construction of 35S::GAL4DB-CmWRKY17 using the LR reaction (Invitrogen). Protoplasts of Arabidopsis were prepared and transfected based on the protocol as described by Yoo et al. (2007). For the transfection, 7.5 μg of 35S::GAL4DB-AtARF5, 35S::GAL4DB, or 35S::GAL4DB-CmWRKY17 was transfected, respectively, with additional 7.5 μg 5×GAL4-LUC as reporter plasmid which has a reporter gene for luciferase driven by five copies of GAL4-binding elements. The luciferase activities were measured as previously reported (Song et al. 2013). Three independent experiments were carried out.

DNA-binding assay of CmWRKY17 using a yeast one-hybrid system

To examine whether CmWRKY17 has DNA-binding ability to W-box, a yeast one-hybrid analysis was performed using a Clontech system (Clontech, Mountain View, CA, USA). A W-box-related cis-acting element from *RD29A* in *A. thaliana* and its corresponding W-box mutant (mW-box) element were synthesized. The nucleotide primer sequences of W-box were pHis-W–F/R (Table 1), which contains three TTGACT repeats. The primer sequences of mW-box were pHis-mW-F/R (Table 1), in which the TTGACT core sequence of each W-box is changed to TAATAT. The two elements were obtained following the manufacturer's instruction (Clontech), and then inserted into the *Eco*R I and *Xba* I of pHis vector to generate pW-box-His and pmW-box-His constructs. The coding region of *CmWRKY17* was cloned into the yeast expression vector pGADT7 by LR reaction from pENTR™1A-CmWRKY17 to obtain pGADT7-CmWRKY17. All the constructs were transferred into yeast strain Y1H (Clontech). The empty vector pGADT7 was transformed as a negative control. The growth status of transformed yeast cells was tested on SD/-His-Ura-Leu medium containing 50 mM 3-aminotrizole (3-AT).

Subcellular localization of CmWRKY17

To generate the green fluorescent protein (GFP)-*CmWRKY17* fusion construct, the plasmid pENTR™1A-CmWRKY17 previously was subjected to the LR reaction to obtain a GFP-fused construct using the binary vector pMDC43, resulting in the plasmid 35S::GFP-

CmWRKY17. Onion (*Allium cepa*) epidermal strips were placed on Murashige and Skoog (MS) medium with the inner cell surface oriented upward. Both the 35S::GFP-CmWRKY17 plasmid and the vector 35 s::GFP were transiently introduced into the onion epidermal cells by a helium-driven particle accelerator (PDS-1000; Bio-Rad). After bombardment, the cells were incubated in the same petri dishes for 16 h at 22 °C in the dark before observation. An AX80T microscope (Olympus, Japan) was used to monitor the expression of GFP.

Plasmid construction and transformation of chrysanthemum and Arabidopsis

To further investigate the function of *CmWRKY17*, *CmWRKY17*-overexpressing chrysanthemum and Arabidopsis transformants were obtained. The *CmWRKY17* coding sequence was amplified with the primer pair CmWRKY17-1301-F/R harboring the *Bam*H I and *Sac* I sites (Table 1). The amplicons digested by *Bam*H I and *Sac* I were directly cloned into the pCAMBIA1301 cassette, containing the CaMV 35S promoter, to generate the plasmid 35S::*CmWRKY17*. This plasmid was transformed into *Agrobacterium tumefaciens* strain EHA105 using the freeze–thaw transformation method.

Transformation of chrysanthemum was performed as described previously (Cui et al. 2009). Leaf disks (5 mm diameter) taken from young leaves of 'Jinba' cultured in vitro were used as explants (Song et al. 2014b). The transformants were selected on culture medium containing 8 mg/L hygromycin. After regeneration, RNA was extracted from the putative transgenic chrysanthemum and WT plants, treated with RNase-free DNase I (TaKaRa), and reverse transcribed using Reverse Transcription M-MLV (TaKaRa). For qRT-PCR analysis, SYBR® Premix Ex Taq^TM II (Tli RNaseH Plus) (TaKaRa) was employed to detect *CmWRKY17* expression using the primer pair CmWRKY17-RT-F/R. The primer pair CmEF1α-F/R was used to amplify the reference gene *CmEF1α*. The transcription data (with three biological replicates) were calculated using the $2^{-\Delta\Delta Ct}$ method.

Arabidopsis transformants were generated by introducing 35S::*CmWRKY17* into the Col-0 ecotype using the floral dip method (Clough and Bent 1998). Transformants were selected by germination of seeds on MS medium containing 50 μg mL^{-1} hygromycin. The T$_3$ generations were obtained using RT-PCR with the primer pair CmWRKY17-ORF-F/R.

Salinity treatment of transgenic chrysanthemum and Arabidopsis plants

For the salinity tolerance test, plants of the transgenic chrysanthemum (OX plants) and the non-transgenic chrysanthemum 'Jinba' (WT) at the six- to eight-leaf stage were irrigated with 200 mM NaCl for 2 weeks. After treatment, the roots were washed with distilled water and the plants were repotted in fresh soil [1:1 (v/v) mixture of soil and vermiculite] and allowed to recover for 2 weeks (An et al. 2014), after which the survival rate was calculated. The median leaves (fourth to fifth leaves counted from the base of the stem) harvested before the NaCl treatment (control) and at 48 h after NaCl treatment were used for physiological analysis.

For salinity tolerance evaluation, 3-week-old Col-0 and CmWRKY17-transgenic Arabidopsis plants with 90 of each lines were watered for 12 days at 4-day intervals, initially with 100 mM NaCl, then 200 mM NaCl, and finally with 300 mM NaCl before being scored and photographed following Gao et al. (2012).

Physiological changes in transgenic chrysanthemum

Physiological traits of OX-3, OX-7 and WT plants were measured at 0 h and 48 h of the salinity test. The activities of superoxide dismutase (SOD) and peroxidase (POD), leaf proline content, and the electrical conductivity (EC) were investigated. Activities of SOD and POD were assessed as described by Pan et al. (2006). One unit of SOD activity was defined as the amount of enzyme required to cause a 50 % inhibition and expressed as enzyme units per g of protein, while a unit of POD activity was expressed as the change in absorbance per minute at 470 nm and specific activity as enzyme units per g soluble protein, respectively. Protein concentration was measured based on the method of Bradford (1976), using bovine serum albumin as a standard. Leaf proline content was estimated following the method of Liu et al. (2005). The EC of the leaf tissue was determined using a Mode DDS-320 conductivity meter as described previously (Xu and Chen 2008).

Expression analysis of salinity stress-related genes in CmWRKY17-transformed Arabidopsis

To analyze the expression of salinity stress-responsive genes, cDNA was synthesized using RNA from the leaves of Col-0 and transgenic Arabidopsis subjected to salinity stress as template by qRT-PCR. The stress-related genes monitored were *AtRD29A* (AT5G52310.1), *AtDREB2B* (AT3G11020.1), *AtSOS1* (AT2G01980.1), *AtSOS2* (AT5G35410.1), *AtSOS3* (AT5G24270.1), and *AtNHX1* (AT5G27150.1). *AtAct2* (AT3G18780.2) was used as the reference gene. The sequences of all relevant primers are listed in Table 1.

Statistical analysis

A one-way analysis of variance, followed using Tukey's multiple range test ($P = 0.05$), was employed to identify

treatment means that differed statistically. SPSS v17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Cloning and sequence analysis of *CmWRKY17*

The WRKY transcription factor *CmWRKY17* (KM359572) was isolated from chrysanthemum. The gene consisted of 904 bp with a 681 bp ORF encoding a 227-amino acid protein. CmWRKY17 contained one WRKY domain of WKKYGEK, which differed from the typical WRKYGQK domain, and one C_2H_2 zinc finger motif (C-X5-C-X23-H–X1-H) (Fig. 1a). Therefore, we assigned the cloned *CmWRKY17* to group II of the WRKY family based on the characteristics of the conserved domains. Phylogenetic analysis showed that CmWRKY17 showed a higher similarity with AtWRKY17 and was most closely related to PqWRKY10 from *Panax quinquefolius* (Fig. 1b). Furthermore, except for the conserved WRKY domain, CmWRKY17 shared few similarities between different WRKYs from a variety of plant species (data not shown).

Expression profiles of *CmWRKY17* in different tissues of chrysanthemum and in response to salinity stress

Expression patterns of *CmWRKY17* in different tissues of chrysanthemum plants were investigated using qRT-PCR. Transcripts of *CmWRKY17* were detected in all tissues analyzed. The highest expression level was observed in the stem and leaf, whereas the lowest level was observed in ray florets (Fig. 2a). Under treatment with 200 mM NaCl, *CmWRKY17* transcript expression was induced at an early stage, increasing rapidly to be 82-fold higher than that of the control 4 h after treatment (Fig. 2b). However, thereafter the *CmWRKY17* relative expression level decreased, but was maintained at a higher level compared with that of the untreated control at 24 h after treatment. It was inferred that *CmWRKY17* might regulate the plant's response to salinity stress.

Transrepression assay of CmWRKY17

Yeast one-hybrid expression and luminescence assay were used to investigate the transcriptional activation of CmWRKY17. Yeast cells harboring the negative control pGBKT7 or pGBKT7-CmWRKY17 construct were unable to grow on SD/-His-Ade medium, whereas the positive control pCL1 grew extremely well (Fig. 3a). CmWRKY17 showed no transcriptional activation in yeast cells. To further analyze the transactivation function of CmWRKY17,

the plasmid encoding CmWRKY17 was used to transfect Arabidopsis protoplasts together with reporter plasmid. The relative LUC units (RLU) of 35S::GAL4DB-CmWRKY17 were significantly lower than that of 35S::GAL4DB-AtARF5 in Arabidopsis protoplasts ($P < 0.01$), while the RLU of 35S::GAL4DB-CmWRKY17 were lower than that of 35S::GAL4DB ($P < 0.05$) (Fig. 3b), suggesting that CmWRKY17 was a repressor of transcription.

DNA-binding assay of CmWRKY17

All the yeast cells harboring the two kinds of plasmids grew well on SD/-His-Ura-Leu medium (Fig. 3c). On SD/-His-Ura-Leu medium plus 50 mM 3-AT, the cells containing pGADT7-CmWRKY17 plus pW-box-His vectors grew well, and pGADT7-CmWRKY17 plus pmW-box-His grew slowly (Fig. 3c), suggesting that interaction between CmWRKY17 and the W-box element activates transcription of the reporter gene *HIS3*. In contrast, yeast cells with both pW-box-His or pmW-box-His and pGADT7 vectors did not grow on SD/-His-Ura-Leu medium supplemented with 50 mM 3-AT (Fig. 3c), as the negative control. These results suggested that CmWRKY17 binds to TTGACT strongly, and to the mutant TAATAT slightly.

Subcellular localization of CmWRKY17

The construct 35 s::GFP-CmWRKY17 and a positive vector harboring only 35S::GFP were introduced into onion epidermal cells via particle bombardment. Onion epidermal cells expressing 35S::GFP showed GFP fluorescence throughout the cells (Fig. 3d). In contrast, GFP fluorescence localized solely in nuclei of the onion epidermal cells transformed with the 35 s::GFP-CmWRKY17 fusion protein (Fig. 3d). These results indicated that CmWRKY17 was localized to the nucleus in vivo.

CmWRKY17 overexpression increased sensitivity of chrysanthemum to salinity stress

Transgenic chrysanthemum lines overexpressing *CmWRKY17* were regenerated. The transcript levels of *CmWRKY17* were measured by qRT-PCR. In the overexpressing (OX) plants, the *CmWRKY17* transcript levels in OX-3 and OX-7 were significantly higher than that in WT plants (Fig. 4a). Therefore, the lines OX-3 and OX-7 were selected for further salinity tolerance evaluation.

With NaCl treatment for 2 weeks, the leaves of both OX-3 and OX-7 plants became severely wilted, withered, and some plants even died (Fig. 4c). In contrast, the WT plants were less affected by salinity stress and showed less severe withering of the leaves, and most plants survived the
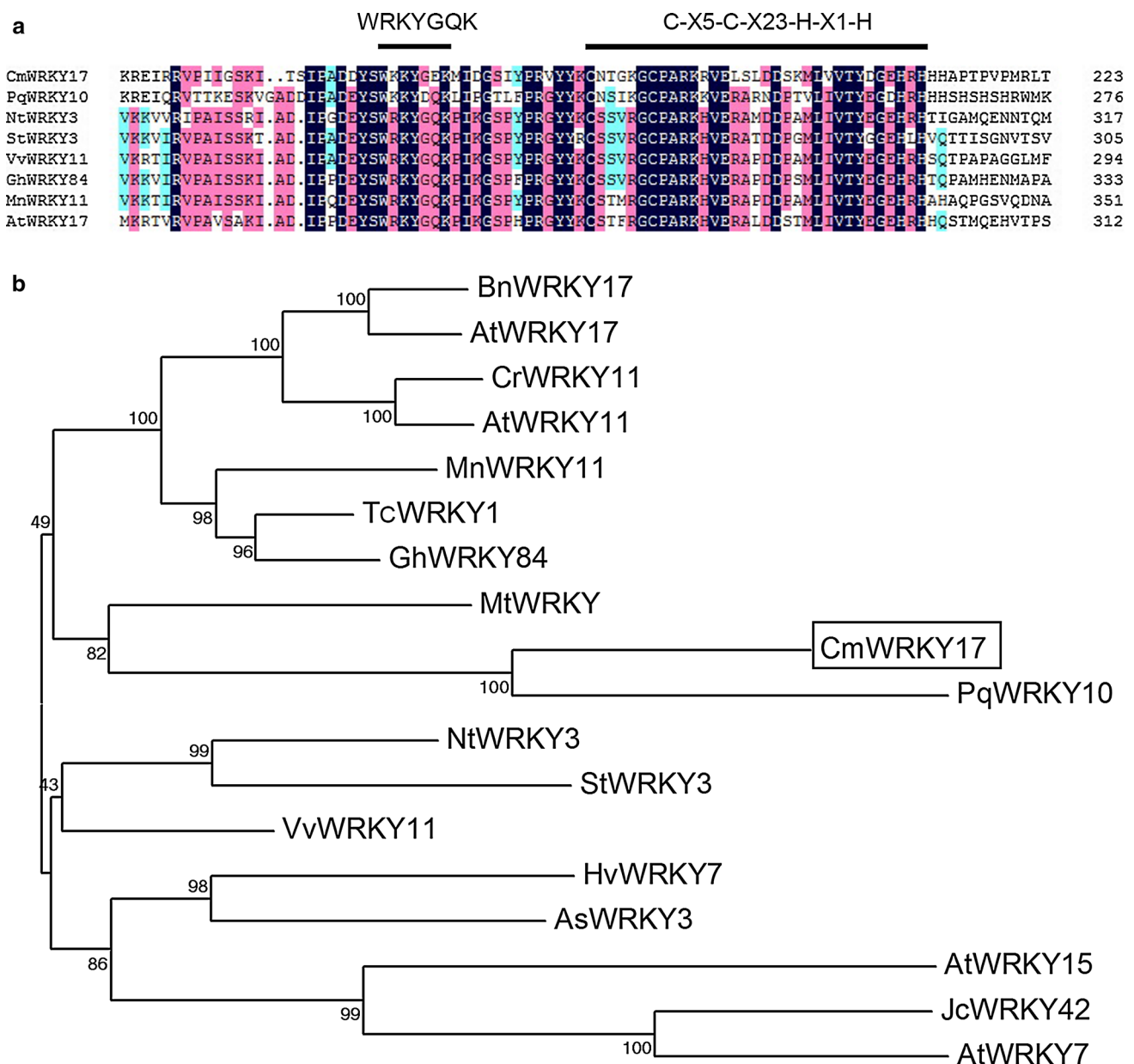
a

b

Fig. 1 Deduced peptide sequences of CmWRKY17 and other WRKY proteins. **a** Alignment of the putative amino acid sequence of CmWRKY17 with homologous proteins. Features of the sequence include a WRKY domain (WRKYGQK) and C₂H₂ zinc finger (both highlighted by *lines* above the alignment). **b** Phylogenetic analysis of relationships between CmWRKY17 and WRKY proteins from other plant species. The amino acid sequences were aligned with Clustal W and the phylogenetic tree was constructed using the neighbor-joining method with MEGA 5.0. The sequence details are as follows:

BnWRKY17 (ACQ76799.1), AtWRKY17 (AAL13049.1), CrWRKY11 (AAS66779.1), AtWRKY11 (AAK96194.1), MnWRKY11 (EXC32736.1), TcWRKY1 (XP_007011614.1), GhWRKY84 (AIE43859.1), MtWRKY (XP_003610020.1), PqWRKY10 (AEQ29023.1), NtWRKY3 (BAA77358.1), StWRKY3 (NP_001274938.1), VvWRKY11 (XP_002266188.1), HvWRKY7 (ABI13373.1), AsWRKY3 (AAD32676.1), AtWRKY15 (NP_179913.1), JcWRKY42 (AGQ04232.1), AtWRKY7 (AEE84877.1). CmWRKY17 is *boxed*

stress. The percentage survival of OX-3 and OX-7 plants was 28.6 % and 18.6 %, respectively, whereas that of WT plants was 50.0 % (Fig. 4b).

After 48 h of salinity stress, SOD and POD activities, proline content, and electrolyte leakage (EL) in leaves were measured in both WT and OX plants. Activities of SOD and POD and proline content were significantly higher in

WT than in OX-3 or OX-7 plants (Fig. 5a–c). In contrast, EL was significantly higher in OX-3 and OX-7 relative to WT plants (Fig. 5d). These results indicated that the transgenic plants suffered oxidative damage much more severely than did WT plants. This suggested that *CmWRKY17* overexpression increased the salinity sensitivity in chrysanthemum.
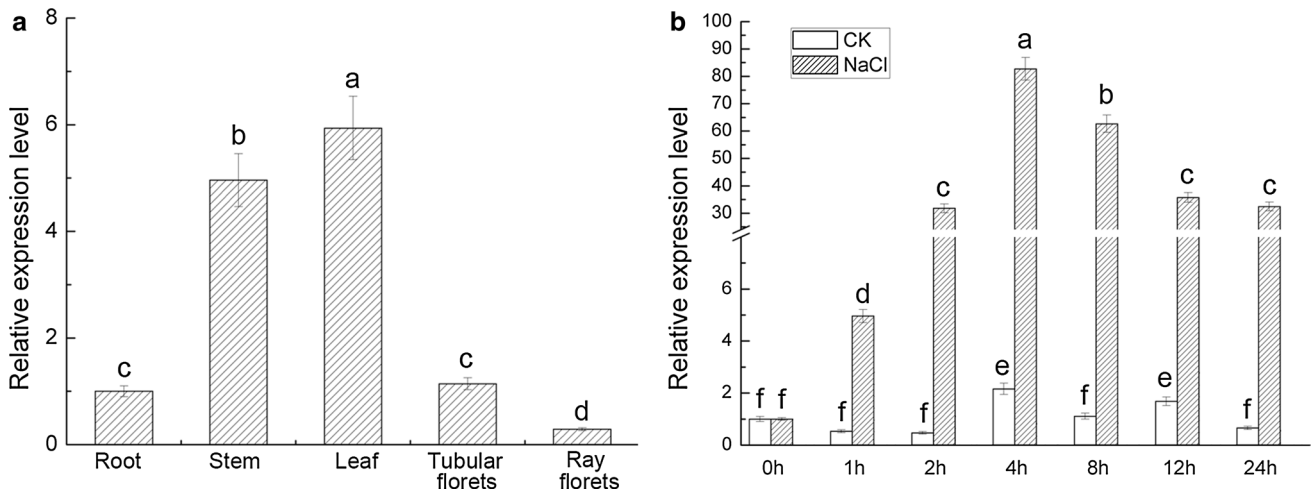
Fig. 2 Expression of *CmWRKY17* in chrysanthemum plants in different organs and under salinity treatment as revealed by quantitative real-time PCR. **a** Expression patterns of *CmWRKY17* in the root, stem, leaf, disk florets, and ray florets under non-stress conditions. **b** Expression of *CmWRKY17* in response to 200 mM NaCl treatment
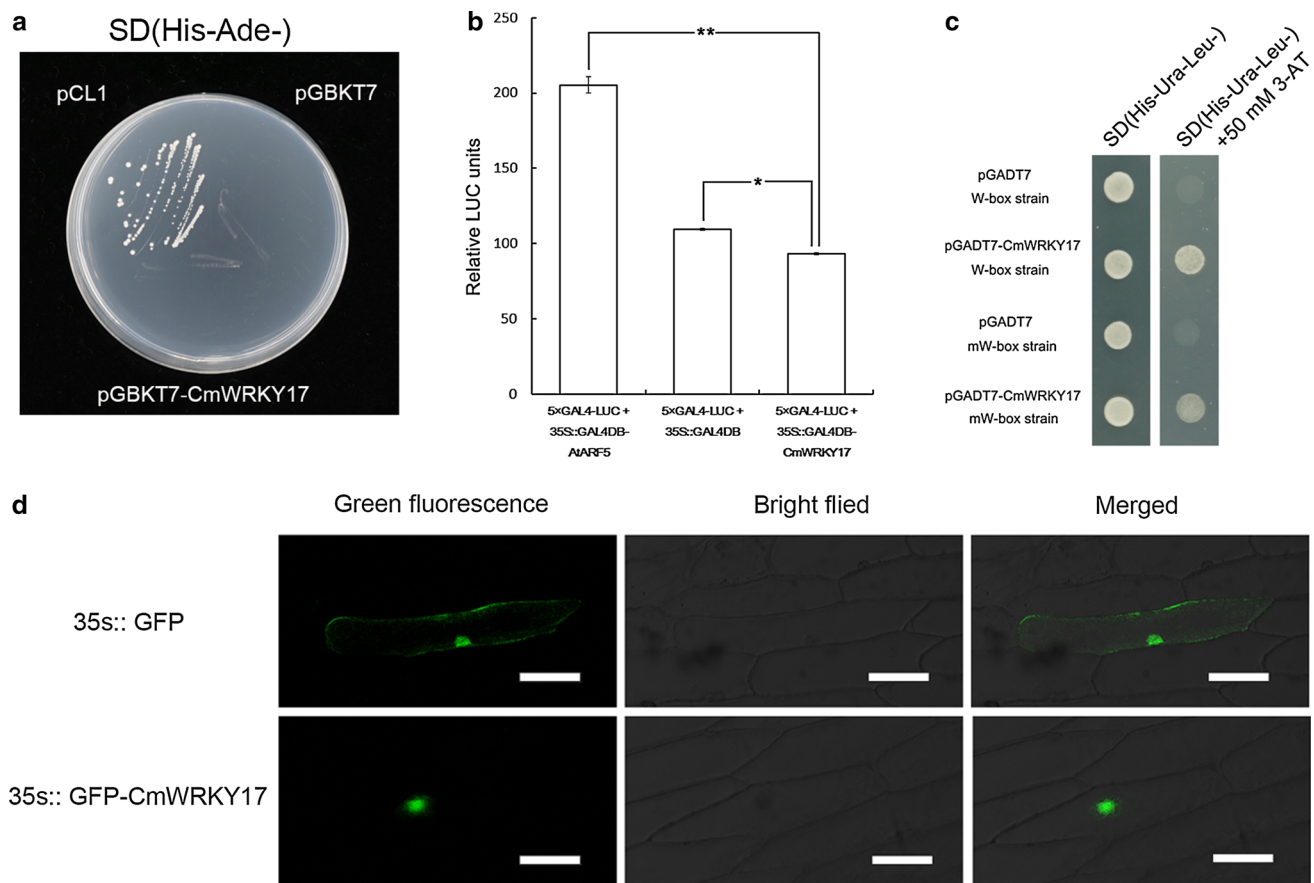

Fig. 3 Transactivation analysis and subcellular localization of CmWRKY17. **a** The transcriptional activity analysis of CmWRKY17 in a yeast assay system, in which pCL1 acts as a positive control, pGBKT7 as a negative control. **b** Relative luciferase activities in Arabidopsis mesophyll protoplasts after transfection with 35S::GAL4DB-CmWRKY17. **c** Assay of CmWRKY17 binding to W-box using a yeast system. **d** Subcellular localization of the CmWRKY17. *Bar* 100 μm

Fig. 4 Salinity tolerance of wild-type 'Jinba' and transgenic chrysanthemum plants overexpressing *CmWRKY17*. **a** Transcript abundance of *CmWRKY17* in wild-type 'Jinba' and transgenic chrysanthemum plants overexpressing *CmWRKY17*. WT, Wild type; OX-3, OX-7, OX-8, and OX-9, transgenic plants overexpressing *CmWRKY17*. **b** Plant survival measured at the end of the recovery period. **c** Phenotypic effect of watering with 200 mM NaCl for 2 weeks, followed by a 2-week recovery period
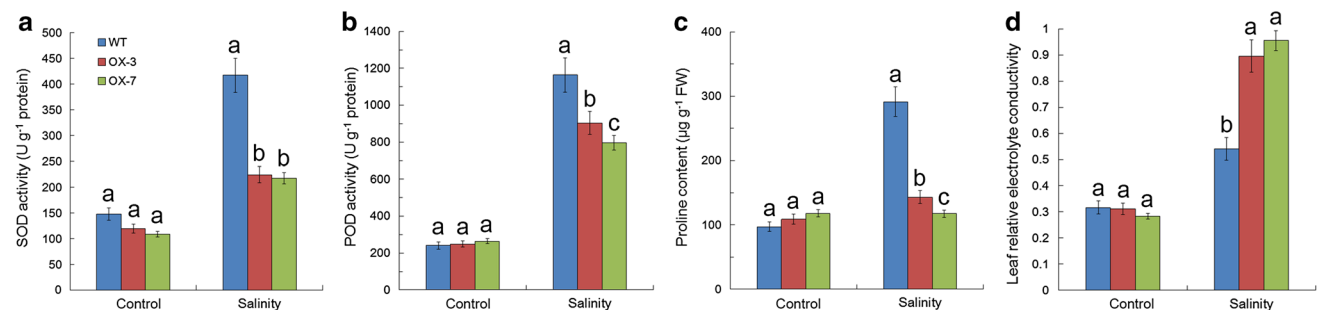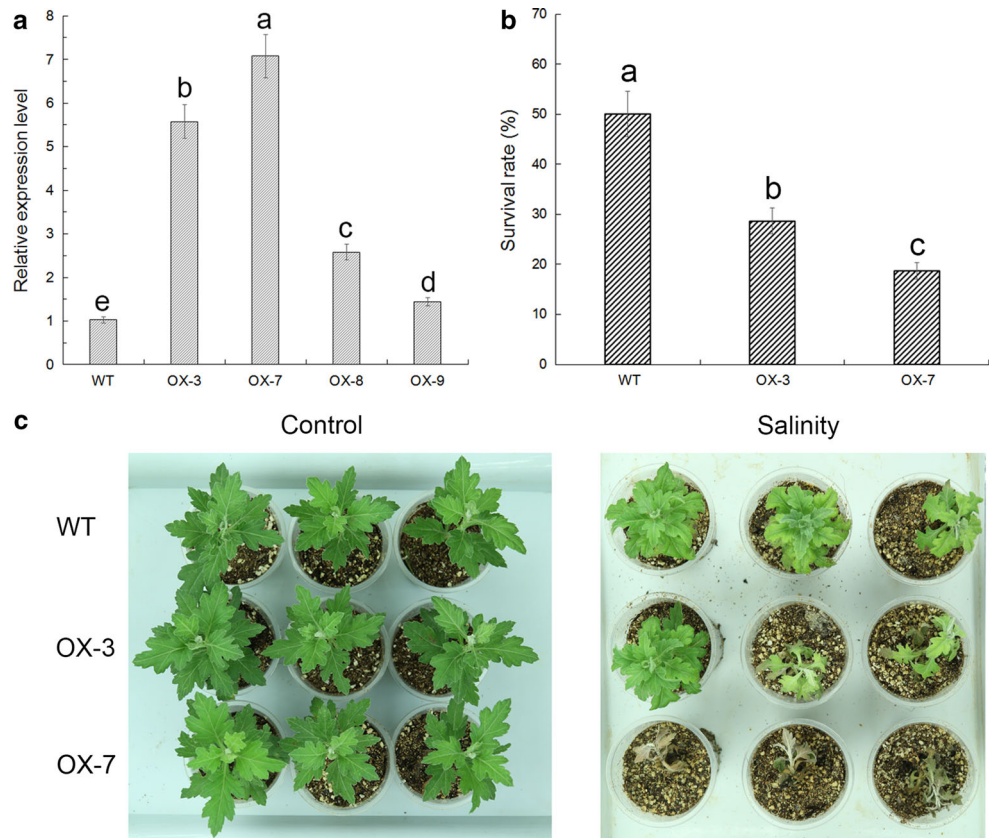

Fig. 5 Physiological effects of salinity stress on wild-type 'Jinba' and transgenic chrysanthemum plants overexpressing *CmWRKY17*. **a** Leaf superoxide dismutase (SOD) activity, **b** leaf peroxidase (POD) activity, **c** Leaf proline content, **d** leaf electrical conductivity (EC). Data represent means and standard errors of three replicates

Overexpression of *CmWRKY17* reduced salinity tolerance in transgenic Arabidopsis

Transgenic Arabidopsis plants overexpressing *CmWRKY17* were successfully generated. Exogenous *CmWRKY17* was expressed in transgenic Arabidopsis 17-5, 17-6, 17-8, and 17-9 lines but not in the Col-0 wild type (Fig. 6a). We selected the lines 17-5 and 17-6, which showed a high expression level of *CmWRKY17*, for further study. No difference in phenotypes was observed between transgenic and Col-0 plants under non-stress conditions. However, after salinity treatment, the transgenic lines were severely withered, whereas Col-0 plants were less affected by salinity (Fig. 6b). The percentage survival of the transgenic lines 17-5 and 17-6 was 8.9 and 7.8 %, respectively, compared with 24.4 % for Col-0 (Fig. 6c). Thus, CmWRKY17 was indicated to increase the salinity sensitivity of the transgenic Arabidopsis plants.

To explain the possible regulatory mechanisms of CmWRKY17 in response to salinity stress, the expression levels of four genes involved in Na^+ homeostasis, comprising *AtSOS1*, *AtSOS2*, *AtSOS3*, and *AtNHX1*, as well as *AtRD29A* and *AtDREB2B* were compared between transgenic and Col-0 plants (Fig. 7). Under non-stress
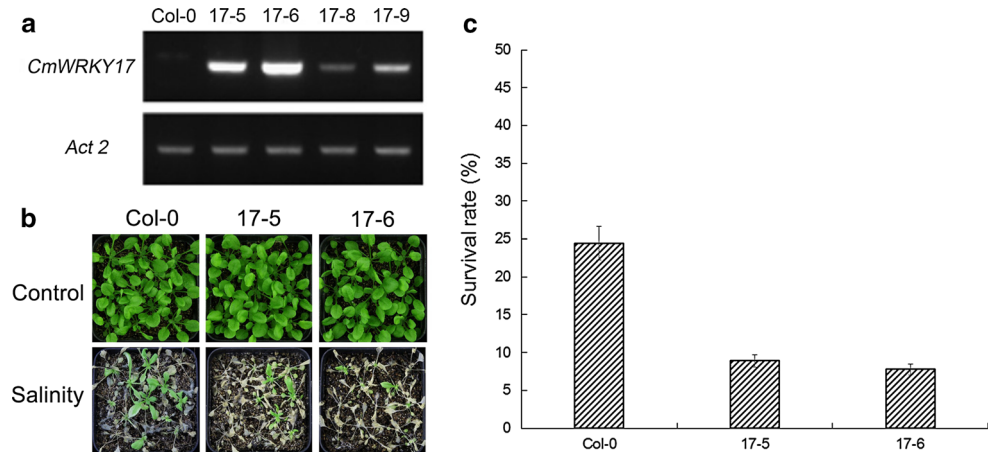
conditions, the expression of *AtRD29A*, *AtDREB2B,*
AtSOS1, *AtSOS2*, *AtSOS3*, and *AtNHX1* was inhibited in
CmWRKY17-overexpressing plants. Consistent with the
finding that the background level of *AtRD29A* expression
was inhibited in CmWRKY17-overexpressing plants, in-
duction of *AtRD29A* in response to salinity treatment was
reduced in CmWRKY17-overexpressing plants compared
with that in Col-0 (Fig. 7a). More specifically, the early
phase of induction (at 1 h) was reduced by 74 %. An ap-
proximately twofold reduction in relative expression level
was observed at 2 and 4 h. Similarly, the expression levels
of *AtSOS1*, *AtSOS2*, and *AtSOS3* in the early phase of in-
duction were reduced by 44 % (at 1 h), 60 % (at 2 h), and
48 % (at 1 h), respectively, compared with that of Col-0.
Subsequently, a twofold, twofold, and threefold reduction
were observed at 12 h for *AtSOS1*, 24 h for *AtSOS2,* and
4 h for *AtSOS3*, respectively (Fig. 7c–e). For clarity, we
divided the stress gene mRNAs in *CmWRKY17*-overex-
pressing plants into two pools: "constitutive" and "in-
duced". To assess the change in the induced pool, we
subtracted the mRNA level at 0 h from the mRNA level at
the other time points. We found that *AtDREB2B* and
AtNHX1 gene expression in the *CmWRKY17*-overexpress-
ing plants remained at the "basal" level and did not in-
crease during the first 2 h of induction, whereas induction
in Col-0 was clearly detected within 1 h (Fig. 7b, f). Thus,
CmWRKY17 may increase salinity sensitivity in Ara-
bidopsis via inhibition of the expression of salinity-related
genes.

Discussion

WRKY proteins positively or negatively regulate the re-
sponse to various stresses and development in plants
(Agarwal et al. 2011; Rushton et al. 2010). In the present

study, we identified a group II WRKY gene, *CmWRKY17*,
from chrysanthemum. Sequence analysis showed that it
contains a special WRKY domain characterized by the
sequence WKKYGEK, which differs from the classical
conserved WRKY domain of WRKYGQK (Fig. 1a), and
one C_2H_2 zinc finger motif (C-X5-C-X23-H-X-H). The
protein containing the WRKYGKK motif is unable to bind
to the W-box (Zhou et al. 2008), but binds to other ele-
ments (van Verk et al. 2008). Our result showed that
CmWRKY17 can bind to W-box strongly, and slightly
bind to the mutant W-box (Fig. 3c), suggesting that
CmWRKY17 might bind to the multiple promoter ele-
ments of the target genes. The amino acid of *CmWRKY17*
was similar to *AtWRKY17* in Arabidopsis, which negatively
regulated the basal resistance in *A. thaliana* (Journot-Cat-
alino et al. 2006).

CmWRKY17 was significantly induced by salinity stress,
which was similar to that of *AtWRKY25*, *AtWRKY33* in *A.
thaliana* (Jiang and Deyholos 2006), *TcWRKY53* in *Thlaspi
caerulescens* (Wei et al. 2008), and *GmWRKY13, 27, 54* in
soybean (Zhou et al. 2008). In addition, *CmWRKY17* did
not show significant change under cold, heat, PEG, or ABA
treatments (Fig. S1). CmWRK17 was supposed to be lo-
calized to the nucleus on the basis of the subcellular lo-
calization, which was in consistent with all the WRKY
proteins ever reported before. The pGBKT7-CmWRKY17
cannot grow on the SD/His-Ade-, which suggested that
CmWRKY17 might have no transcription activity in yeast
cells (Fig. 3a). Further study of luminescence assay
showed that it might act as a transcription repressor in
Arabidopsis proroplasts (Fig. 3b). As we know, AtWRKY7
functions as a transcriptional repressor in plant cells (Kim
et al. 2006), with higher similarity to the amino acid of
CmWRKY17.

To further understand the function of *CmWRKY17* in
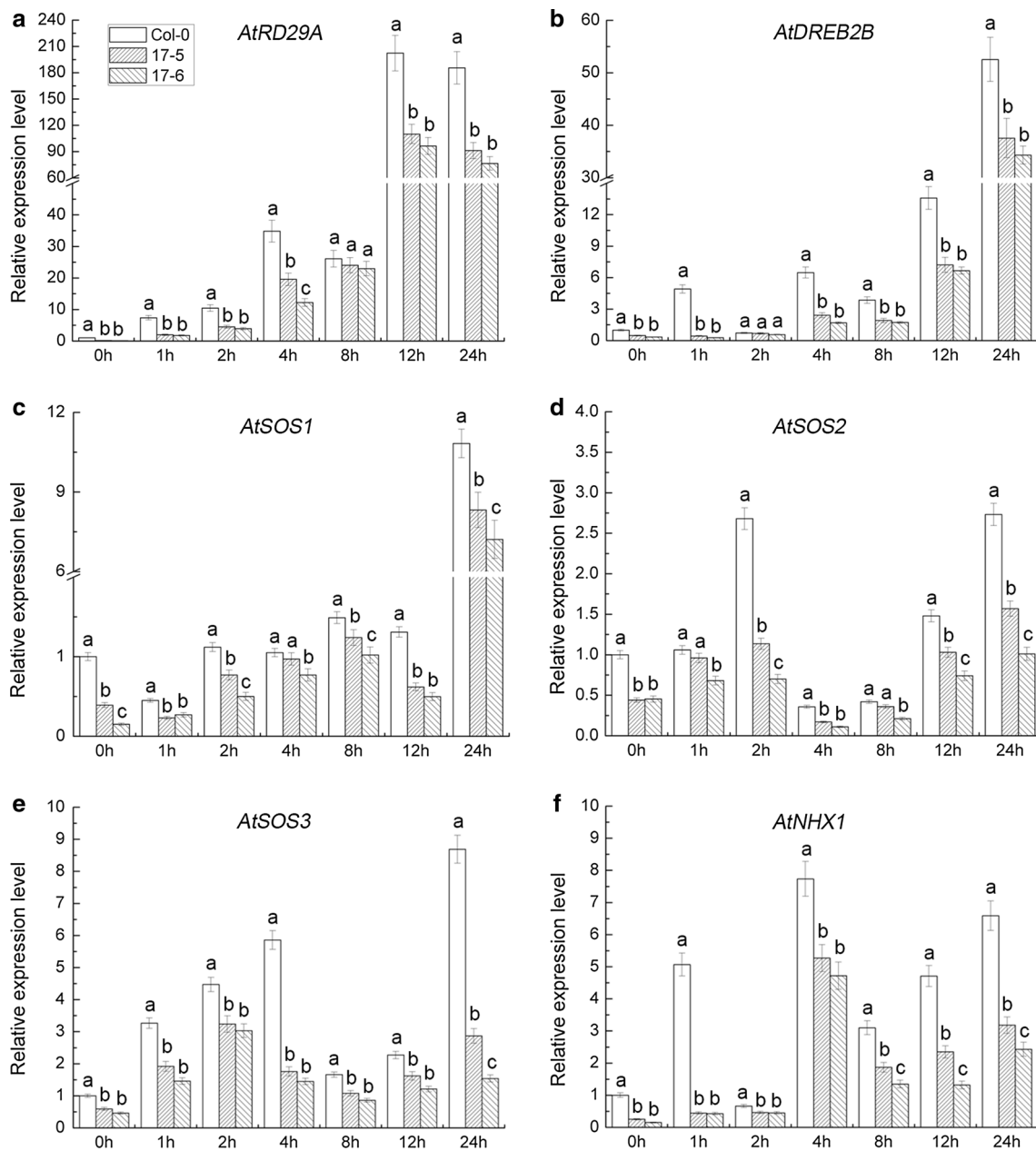response to salinity stress, we generated several transgenic

Fig. 7 Expression of stress-related genes in Col-0 and the 35 s::*CmWRKY17* transgenic lines (17-5 and 17-6). Data represent means and standard errors of three replicates

chrysanthemum (Fig. 4a) and Arabidopsis lines (Fig. 6a), respectively. There were two transgenic lines to exclude the effect of the insertion site on the characteristics of the offspring. Additionally, there was no significant difference between the empty vector lines and wild plants before or after the salt treatment as descried previously (Gao et al. 2012; Shan et al. 2012; Song et al. 2014b).

Salinity stress causes the accumulation of reactive oxygen species (ROS), leading to a marked increase in oxidative damage to plants (Hasegawa et al. 2000). Enzymes such as SOD and POD are involved in oxidative

protection; SOD is a major scavenger of O_2^-, and it catalyzes the dismutation reaction of superoxide radical anions into O_2 and H_2O_2. CAT dismutates H_2O_2 into water and molecular oxygen, whereas POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Pan et al. 2006). The proline content would strangely congest in response to a variety of stresses in plants, while electrolyte leakage reflects membrane injury caused by stresses. In the present study, no difference in the activities of ROS-scavenging enzymes (SOD and POD) was observed in either OX or WT plants under non-

stress conditions, whereas the activities in OX plants were significantly lower than those in WT plants after salinity stress (Fig. 5a, b). There was no obvious difference of CAT before or after the salt treatment between WT and transgenic plants (Data not shown). Higher quantities of proline were accumulated in the leaves of WT plants compared with those in OX plants under salinity stress (Fig. 5c). However, leaf EC was much higher in OX plants than in WT plants (Fig. 5d). These results indicated that overexpression of *CmWRKY17* in chrysanthemum increased the salinity sensitivity because of a decline in ROS scavenging and that salinity caused ROS damage in chrysanthemum. Low activity of ROS-scavenging enzymes and low proline contents in transgenic chrysanthemum might underlie the increased sensitivity to salinity in *CmWRKY17*-overexpressing plants, thereby indicating that CmWRKY17 alters the salinity sensitivity via regulation of ROS levels.

Similarly, *AtWRKY17* (Journot-Catalino et al. 2006) and *OsWRKY28* (Chujo et al. 2013) negatively regulate the basal resistance to *Pseudomonas syringae* pv. *tomato* and *Magnaporthe oryzae* (strain Ina86-137), respectively. Transgenic Arabidopsis overexpressing *GmWRKY13*, a group II WRKY, showAQ increased sensitivity to salt stress, while it was strongly induced by salinity (Zhou et al. 2008). In the current study, heterologous expression of *CmWRKY17* increased the sensitivity to salinity stress in Arabidopsis (Figs. 6, 7), which was significantly induced by salt stress, showing similar function. As a well-known stress marker gene and a lea-type stress protein, *RD29A* has been proposed to function in the detoxification or alleviation of stress-related damage (Zhu 2001). The *DREB* transcription factor mediates the expression of some stress-related genes in response to secondary stresses caused by high salinity (Zhu 2001). Genes such as *AtSOS1*, *AtSOS2*, *AtSOS3*, and *AtNHX1* are involved in a regulatory pathway for ionic homeostasis under salt stress. Recently, the *SOS1* gene was shown to encode a putative plasma membrane Na^+-H^+ antiporter (Shi et al. 2000). SOS3 interacts physically with SOS2, which is a serine/threonine protein kinase (Halfter et al. 2000; Liu et al. 2000). One downstream target of the SOS3–SOS2 kinase complex is SOS1, which exports Na^+ from the cell (Shi et al. 2000). Overexpression of *AtNHX1* appears to improve plant salt tolerance substantially (Apse et al. 1999). The present data showed that salinity-induced expression of *AtRD29A* was inhibited by overexpression of *CmWRKY17* (Fig. 7a). Similarly, under salinity stress, the induction of the genes *AtDREB2B*, *AtSOS1*, *AtSOS2*, *AtSOS3*, *and AtNHX1* was reduced in *CmWRKY17*-overexpressing plants compared with that in the control (Col-0) (Fig. 7b–f). These results indicated that *CmWRKY17* might reduce the expression of SOS pathway genes to increase the salinity sensitivity in Arabidopsis. The complemented line shows lower induction of all genes compared with that in Col-0, which is consistent with the earlier observation that higher levels of *CmWRKY17* expression inhibited salt induction.

WRKY transcription factors are able to bind to the W-box in the promoter of the target genes. Although *CmWRKY17* contained a WKKYGEK domain, which is quite different from the typical WRKYGQK motif, and a yeast one-hybrid system showed that CmWRKY17 can bind to the W-box, and it might bind to the mutant W-box, further regulation of expression of the genes in the salinity stress-response pathway remains unknown. Increased expression of *CmWRKY17* reduced salt-induced expression of stress-responsive genes such as *AtRD29A* and *AtDREB2B*, as well as *AtSOS1*, *AtSOS2*, *AtSOS3*, and *AtNHX1*. In this study, the data showed that CmWRKY17 acted as a transcription repressor. Collectively, these results suggest that *CmWRKY17* may serve as a negative regulator of the salinity response pathway through inhibiting the expression of stress-related gene.

In summary, a novel group II WRKY gene, *CmWRKY17*, a transcriptional repressor, was induced by salinity stress. *CmWRKY17*-overexpressing transgenic chrysanthemum and Arabidopsis plants showed increased salinity sensitivity compared with the wild-type plants. These results indicated that *CmWRKY17* might directly or indirectly negatively regulate the stress-related genes, at least partially through regulation of the SOS pathway, to increase the sensitivity to salinity.

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