Plant Cell Rep (2015) 34:23–35
DOI 10.1007/s00299-014-1684-6

ORIGINAL PAPER

The *Brachypodium distachyon BdWRKY36* gene confers tolerance to drought stress in transgenic tobacco plants

Jiutong Sun · Wei Hu · Run Zhou · Lianzhe Wang · Xiatian Wang · Qiong Wang · Zhijuan Feng · Yaping Li · Ding Qiu · Guangyuan He · Guangxiao Yang

Received: 4 July 2014 / Revised: 3 September 2014 / Accepted: 5 September 2014 / Published online: 16 September 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract

Key message **The expression of *BdWRKY36* was upregulated by drought treatment. *BdWRKY36*-overexpressing transgenic tobacco increased drought tolerance by controlling ROS homeostasis and regulating transcription of stress related genes.**

Abstract WRKY transcription factor plays important roles in plant growth, development and stress response. However, the function of group IIe WRKYs is less known. In this study, we cloned and characterized a gene of group IIe *WRKY*, designated as *BdWRKY36*, from *Brachypodium distachyon*. Transient expression of *BdWRKY36* in onion epidermal cell suggested its localization in the nucleus. Transactivation assay revealed that the C-terminal region, instead of full length BdWRKY36, had transcriptional activity. *BdWRKY36* expression was upregulated by drought. Overexpression of *BdWRKY36* in transgenic tobacco plants resulted in enhanced tolerance to drought stress. Physiological–biochemical indices analyses showed that *BdWRKY36*-overexpressing tobacco lines had lesser ion leakage (IL) and reactive oxygen species (ROS) accumulation, but higher contents of chlorophyll, relative water content (RWC) and activities of antioxidant enzyme than that in control plants under drought condition. Meanwhile expression levels of some ROS-scavenging and stress-responsive genes were upregulated in *BdWRKY36*-overexpressing tobacco lines under drought stress. These results demonstrate that BdWRKY36 functions as a positive regulator of drought stress response by controlling ROS homeostasis and regulating transcription of stress related genes.

Communicated by Leandro Peña.

J. Sun and W. Hu contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-014-1684-6) contains supplementary material, which is available to authorized users.

J. Sun · W. Hu · R. Zhou · L. Wang · X. Wang · Q. Wang · Z. Feng · Y. Li · D. Qiu · G. He (✉) · G. Yang (✉)
The Genetic Engineering International Cooperation Base of Chinese Ministry of Science and Technology, Key Laboratory of Molecular Biophysics of Chinese Ministry of Education, College of Life Science and Technology, Huazhong University of Science & Technology, Wuhan 430074, China
e-mail: hegy@mail.hust.edu.cn; hegy@hust.edu.cn

G. Yang
e-mail: ygx@hust.edu.cn

J. Sun
e-mail: sunjiutong@126.com

W. Hu
e-mail: huwei2010916@126.com

R. Zhou
e-mail: 1844268653@qq.com

L. Wang
e-mail: lzwang@hust.edu.cn

X. Wang
e-mail: wangxiatiancwz@163.com

Q. Wang
e-mail: 754631109@qq.com

Z. Feng
e-mail: zhijuanke@163.com

Y. Li
e-mail: 397510388@qq.com

D. Qiu
e-mail: 296589797@qq.com

Keywords *Brachypodium distachyon* · *BdWRKY36* · Drought stress tolerance · Reactive oxygen species (ROS) · Tobacco

Abbreviations

ABA	Abscisic acid
CaMV	Cauliflower mosaic virus
CAT	Catalase
DAB	3,3′-diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
DW	Dry weight
FW	Fresh weight
GAL4BD	GAL4 DNA binding domain
GFP	Green fluorescent protein
IL	Ion leakage
LEA	Late embryogenesis abundant
MS	Murashige and skoog
NBT	Nitroblue tetrazolium
NCED	9-cis-epoxycarotenoid dioxygenase
OE	Overexpression
ORF	Open reading frame
POD	Peroxidase
qRT-PCR	Quantitative RT-PCR
ROS	Reactive oxygen species
RT–PCR	Reverse transcription–PCR
RWC	Relative water content
SOD	Superoxide dismutase
TW	Turgid weight
VC	Vector control
WT	Wild type

Introduction

Plants are unable to escape unfavorable environmental conditions thus have to endure these environmental challenges such as soil salinity, drought and cold temperature during the course of their life. To maintain growth, development and productivity, plants have evolved elaborate mechanisms to tolerate and adapt to those environmental stresses (Zhu 2001; Ross et al. 2007; Hirayama and Shinozaki 2010; Xiang et al. 2013). When plants are exposed to these conditions, multiple stress-responsive genes, multiple complicated and precisely-regulated physiological and molecular networks and signaling pathways are involved in coping with these abiotic stresses to protect plants from further injury (Mittler 2006; Tang et al. 2012). Transcription factors including WRKY expanded and evolved in plants that play a central role in coordinating gene expression to maintain growth, development and productivity during the changing environments (Ross et al. 2007). The WRKY transcription factors contain one or two domains composed of the conserved amino acid sequence WRKYGQK at the N-terminus and a zinc-finger motif at the C-terminus (Eulgem et al. 2000; Rushton et al. 2012). The conserved WRKY domain plays important roles in recognizing and binding to the W-box in the promoter regions of target genes (Chen et al. 2012). The WRKY superfamily is classified into three distinct groups (I, II and III) based on the number of the WRKY domains and zinc-fingers feature. Group II can be further subdivided into five subgroups (IIa to IIe) depending on the presence of additional short and conserved structural motifs (Eulgem et al. 2000). WRKY proteins are able to activate or repress transcription of the target genes through directly binding to the W-box containing a core sequence (T)(T)TGAC(C/T) in the promoter (Eulgem et al. 2000). Previous studies revealed that WRKY transcription factors function in many processes including plant growth and development (Johnson et al. 2002; Zhang et al. 2011), senescence (Miao et al. 2004), response to abiotic stresses (Zhou et al. 2008; Niu et al. 2012; Luo et al. 2013), as well as plant defense (Xu et al. 2006; Zheng et al. 2006). As one of the largest transcription factor families in plants, there are 74 *WRKY* genes in *Arabidopsis thaliana*, more than 100 members in *Oryza sativa* (Ross et al. 2007; Rushton et al. 2010) and at least 43 putative *WRKY* genes that have been identified in *Triticum aestivum* (Niu et al. 2012).

Some *WRKY* genes responding to abiotic stress had been studied. In Arabidopsis, *AtWRKY25* plays a role in thermotolerance by regulating heat shock proteins-related signaling pathway (Li et al. 2009). *AtWRKY33* gene conferring salt stress tolerance and the *AtWRKY63* (*ABO3*) gene involved in drought stress response are mediated by ABA signaling pathway (Jiang and Deyholos 2009; Ren et al. 2010). Arabidopsis *WRKY18*, *WRKY40* and *WRKY60* respond to abscisic acid and abiotic stress (Chen et al. 2010). *AtWRKY70* and *AtWRKY54* modulate osmotic stress tolerance by regulating stomatal aperture (Li et al. 2013). *AtWRKY6* modulates *PHOSPHATE1* expression in response to low Pi stress (Chen et al. 2009). *AtWRKY57* confers drought tolerance by elevation of ABA level (Jiang et al. 2012). *AtWRKY39* mediates response to heat stress by regulating the cooperation between the SA- and JA-activated signaling pathways in Arabidopsis (Li et al. 2010). The rice *WRKY* genes, *OsWRKY24, -45, -72* and *-77*, are involved in ABA signaling (Xie et al. 2005). Rice *OsWRKY11* enhances heat and drought tolerance in rice seedlings (Wu et al. 2009). Overexpression of *OsWRKY30* results in increased drought tolerance by activation of MAP kinases and *OsWRKY45* overexpression improves rice tolerance to drought and cold stresses (Tao et al. 2011; Shen et al. 2012). *OsWRKY89* enhances ultraviolet B tolerance in rice (Wang et al. 2007). *OsWRKY08* improves

osmotic stress tolerance in Arabidopsis (Song et al. 2009). All these stress responsive *WRKY* genes are distributed mainly in group I, III, IIa, IIb, IIc and IId, whereas whether group IIe *WRKY* genes are involved in abiotic stress tolerance is still unknown.

As one of the major staple food crops, wheat (*T. aestivum* L.) genome consisting of the A, B and D genomes with about 16,000 Mb, is 40-fold larger than rice (Bhalla 2006; Tripathi et al. 2012) and is not sequenced completely, which hinders the identification and isolation of *WRKY* genes. In wheat, only a few *WRKY* genes involved in abiotic stress have been functionally characterized. Recently, four wheat *WRKY* genes (*TaWRKY1, -2, -17 and -19*) were reported to be induced by four or five stress treatments (salt, drought, cold, wounding and ABA) (Niu et al. 2012). Overexpression of *TaWRKY2* exhibits tolerance to salt and drought stresses and *TaWRKY19* confers tolerance to salt, drought and freezing stresses by regulating downstream genes in transgenic Arabidopsis (Niu et al. 2012). In addition, wheat *TaWRKY10* confers tolerance to multiple abiotic stresses by regulating the osmotic balance, ROS scavenging and transcription of stress related genes in transgenic tobacco and *TaWRKY79* enhances tolerance to both salinity and ionic stresses in Arabidopsis (Qin et al. 2013; Wang et al. 2013). Fortunately, the genome of *Brachypodium distachyon* is fully sequenced which is a phylogenetic sister group to wheat and has been used as a new model organism for wheat (Li et al. 2012a; Wen et al. 2014). Recently, 86 *WRKY* genes have been identified in *B. distachyon* (Tripathi et al. 2012; Wen et al. 2014). Studies of WRKY transcription factors from *B. distachyon* therefore will provide useful reference for their ortholog in wheat. However, the function of *WRKY* gene from *B. distachyon* is largely unknown. In this study, a group IIe *WRKY* gene designated as *BdWRKY36* from *B. distachyon* was characterized and analyzed in regulating drought stress tolerance by activating the antioxidant system and the expression of stress-responsive genes.

Materials and methods

Plant materials, growth conditions and stress treatments

B. distachyon (Bd 21) seeds were surface-sterilized using 75 % (v/v) ethanol for 1 min and 1 % (v/v) mercury chloride for 5 min, and then were washed 3–5 times with sterile distilled water. Sterilized seeds were germinated on the filter paper in Petri dishes with sterile water under dark conditions. After germination, the Petri dishes were transferred to greenhouse under normal conditions with 16 h/light, 8 h/dark and 24 °C temperature for 14 days. For drought treatment, 14-day-old seedlings were dehydrated on filter paper at 24 °C with 30 % relative humidity. For NaCl treatment, seedlings were incubated in 200 mM NaCl solution. For treatments with signaling molecules, seedlings were sprayed with 100 µM ABA and 10 mM H_2O_2, respectively. For cold or heat treatment, seedlings were grown at 4 or 40 °C conditions, respectively. Leaves from control or treated plants were harvested after 0, 1, 3, 6, 12 and 24 h of treatments, and frozen in liquid nitrogen, and then stored at −70 °C for RNA preparation. For the organs differential expression analysis, roots, leaves, stems and spikes were collected from 3-month-old plants grown under greenhouse condition as described above.

Cloning and identification of *BdWRKY36* gene

Total RNA from leaves of *B. distachyon* seedlings was extracted with Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The first strand cDNAs were synthesized using the first strand cDNA Synthesis kit (Fermentas, USA). Polymerase chain reaction (PCR) was performed with gene-specific primer pairs (P1; Table S1) designed by the software Primer Premier 5.0 based on the open reading frame (ORF) of *BdWRKY36* from databases http://www.igece.org/WRKY/BrachyWRKY/BrachyWRKYIndex.html and http://plants.ensembl.org/Multi/blastview. Amplified products were cloned into pMD18-T vectors (TaKaRa, Japan) and were subsequently subjected to sequencing analysis. To verify the reliability of the results, the putative protein sequence was assessed with SMART (http://smart.embl-heidelberg.de/) and UniProt (http://www.uniprot.org/) databases. Multiple sequence alignment was performed using the putative protein sequence of BdWRKY36 and WRKY protein sequences from other species retrieved from GenBank by DNAMAN software. Phylogenetic tree was constructed using software MEGA 5.0 (Tamura et al. 2011).

Semi-quantitative RT-PCR

Reverse transcription-PCR (RT-PCR) was used to analyze the expression levels of *BdWRKY36* in different organs of *B. distachyon* plants, at different time intervals (0, 1, 3, 6, 12 and 24 h) from *B. distachyon* seedlings and in the transgenic tobacco lines. PCR amplifications were performed as follows: 95 °C for 5 min, 28 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, and the final extension at 72 °C for 5 min. Expression levels of target genes were normalized using *BdUBC18* or *NtUbiquitin* as internal controls. The relative amount of the transcription was analyzed using Image Lab 4.1 analyzer software. Specific primers (P3–P5; Table S1) were designed using software Primer Premier 5.0.

Gene expression analysis by qRT-PCR

Expression of *BdWRKY36* in *B. distachyon* seedlings with various treatments as well as relative transcription levels of ROS-scavenging and stress-responsive genes including *NtPOX2*, *NtCAT*, *NtSOD*, *NtLEA5*, *NtNCED1* and *NtDREB3* in transgenic tobacco lines were measured by quantitative RT-PCR (qRT-PCR) using SYBR Green qPCR Mix (ToYoBo, Japan). Specific primers (P2, P4–P5 and P13–P18; Table S1) used in qRT-PCR were designed to avoid the highly conserved sequences. The efficiency and specificity of the primer pairs were examined by melting curve, agarose gel electrophoresis, and sequencing analysis. The PCR parameters were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 10 s, 59 °C for 10 s and 72 °C for 30 s according to the manufacturer's protocol. Three biological replicates were performed. *BdUBC18* and *NtUbiquitin* were used as the internal controls for *B. distachyon* and tobacco, respectively, which served as a benchmark to normalize tested genes. Expression levels for all the candidate genes were calculated and normalized using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Plasmid construction and subcellular localization analysis

The *BdWRKY36* coding sequence without the stop codon was amplified using primers (P6; Table S1) containing *Bgl*II/*Spe*I restriction sites. The PCR products were purified and digested with *Bgl*II/*Spe*I restriction enzymes and then were inserted into the plant binary vector pCAMBIA1304 under the control of *Cauliflower mosaic virus* 35S (CaMV 35S) promoter to generate a fusion construct pCAMBIA1304-*BdWRKY36*-*GFP*. For protein localization assays, the recombinant plasmid pCAMBIA1304-*BdWRKY36*-*GFP* and the vector pCAMBIA1304-*GFP* as a control were transformed into onion epidermal cells, respectively, by particle bombardment methods using a gene gun (BIO-RAD, USA). After transformation, the onion epidermal cells were incubated on MS medium at 25 °C under dark condition for 16 h. Subsequently, nuclei were stained with 100 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Solarbio, China) in phosphate-buffered saline for 10 min, and then the localization of green fluorescent proteins in transformed cells was observed by fluorescence microscope (Olympus, Japan).

Analysis of transcriptional activation in yeast cells

To examine the transcriptional activation activity, the complete coding sequence of *BdWRKY36*, N-terminal (from the 1st to the 132th aa), N-terminal-WRKY domain (from the 1st to the 199th aa) and C-terminal (from 200th aa to the end of C-terminus) fragments were amplified using specific primers (P9–P12; Table S2) containing *Eco*RI/*Bam*HI restriction sites, respectively. The PCR products digested by restriction enzymes *Eco*RI/*Bam*HI were cloned into the pGBKT7 vector digested with the same restriction enzymes to create pGBKT7-*BdWRKY36*, pGBKT7-*BdWRKY36*-N and pGBKT7-*BdWRKY36*-N-WRKY, and pGBKT7-*BdWRKY36*-C, respectively. These constructs and the pGBKT7 vector as a control were transformed into yeast strain AH109 according to the manufacturer's protocol (Clontech, USA). The transformants were screened on the SD/Trp^- and SD/Trp^-/His^- mediums, respectively. Transcriptional activation was evaluated based on the growth status of yeast cells after incubating SD/Trp^-/His^- medium with X-α-gal at 30 °C for 3 days.

Tobacco transformation and generation of transgenic lines

The recombinant plasmid pCAMBIA1304-*BdWRKY36*-*GFP* and the vector pCAMBIA1304-*GFP* as a control were introduced into *Agrobacterium tumefaciens* strain EHA105 and then transformed into tobacco (*Nicotiana tabacum* L. cv Samsun) by *Agrobacterium*-mediated leaf disk transformation method according to Horsch et al. (1985). The seeds from transgenic plants were screened on MS medium with 30 mg/L of hygromycin. The T_1 *BdWRKY36*-overexpressing tobacco plants were confirmed by PCR assay with specific primers (P6–P8; Table S1) for *BdWRKY36*, *GFP* and *HptII* genes, respectively. The control plants transformed with *GFP* only containing vector were confirmed by PCR analysis using specific primers for *GFP* and *HptII* genes. Three independent transgenic T_2 lines (OE-6, OE-14, OE-18), which almost all survived on MS medium with 30 mg/L of hygromycin were used for further experiments. *BdWRKY36* expression in the three independent T_2 lines was analyzed with semi-quantitative RT-PCR using primers (P3; Table S1) with *NtUbiquitin* as an internal control.

Drought stress tolerance assay for transgenic tobacco

For drought stress tolerance assay, the seeds of wild type (WT), vector control (VC) and transgenic lines(OE-6,OE-14, OE-18)were surface-sterilized using 75 % (v/v) ethanol for 1 min and 10 % (v/v) H_2O_2 for 15 min and washed 3–5 times with sterile water. The sterilized seeds grew on MS medium for 10 days and then were transplanted into plastic pots filled with soil and sand (3:1) under normal conditions for 20 days. Some plants were still cultured under normal conditions and the others were subjected to water depriving for 20 days and then were rewatered for 10 days to evaluate the survival rate.

Measurements of physiological–biochemical parameters

For drought treatment, groups of 30-day-old transgenic and control plants were withheld water for 20 days. The control groups were watered regularly. Then the leaves from control and transgenic plants under normal or drought treatments were sampled to detect the physiological–biochemical parameters.

For measurement of relative water content (RWC), the leaves were detached immediately and weighed as fresh weight (FW) followed by incubating in distilled water for 4 h at room temperature under normal light, and then the turgid weight (TW) was recorded. The samples were then transferred into an oven at 80 °C for 48 h and the dry weights (DW) were recorded. RWC was calculated as follows: RWC (%) = [(FW − DW)/(TW − DW)] × 100 according to Hu et al. (2013).

For measurement of ion leakage (IL), about 0.5 g tobacco leaves were cut into strips and incubated in 15 mL tubes with distilled water for 12 h at 26 °C. After incubation, initial electrolyte conductivity (C1) was measured with a conductivity meter (DDBJ-350, China) followed with the samples boiled for 30 min. We did not measure the electrolyte conductivity (C2) until the samples were cooled to room temperature. IL was calculated according to the formula: IL (%) = C1/C2 × 100 based on the method described by Jiang and Zhang (2001).

For measurement of chlorophyll content, the chlorophyll of tobacco leaves was determined with UV spectrophotometry method according to Yang et al. (2009). ROS accumulation of O_2^- and H_2O_2 in situ was detected based on histochemical staining by nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB), respectively, as described by Pan et al. (2012). H_2O_2 content was assayed using Detection Kit (A064, Jiancheng, China) according to the instruction of manufacturer. The activities of peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) were measured by a spectrophotometer. About 0.5 g tobacco leaves was homogenized in 5 mL extraction buffer with 0.05 M phosphate buffer (pH 7.8), then the samples were centrifuged at 10,000 g at 4 °C for 15 min to collect the supernatant that was used to determine enzyme activities of POD, CAT and SOD using Detection Kit (A084, A007 and A001, Jiancheng, China) according to the instruction of manufacturers.

Statistical analysis

Statistical analyses were performed using the software in Excel and Photoshop CS5 (Microsoft and Adobe, USA). Mean values ± SD are calculated from at least two replicates, and significant differences between transgenic and control plants in measured parameters are given at $P < 0.01$ and $P < 0.05$ based on Student's t test.

Results

Cloning and sequence analysis of *BdWRKY36*

The coding sequence of the *BdWRKY36* gene was cloned from the leaves of *B. distachyon* seedlings by RT-PCR. The ORF of *BdWRKY36* is 1,200 bp, encoding a protein with 399 amino acid residues. Its predicted relative molecular mass is 42.3 kDa with isoelectric point of 9.01. Blast analysis showed that BdWRKY36 shared a high degree of sequence identity with WRKYs from other plant species: 72 % sequence identity with OsWRKY37 and 58 % with OsWRKY66 from *O. sativa*, 50 % with AtWRKY35 and 47 % with AtWRKY14 from *A. thaliana*. Amino acid sequence alignment by homology search from the GenBank database showed that BdWRKY36 was homologous to WRKY transcription factors, especially with regards to the WRKY domains. BdWRKY36 contains a single WRKYGQK motif, a C2H2-type zinc finger and a nuclear localization signal "KRRK" sequence (Fig. S1). Phylogenetic analysis revealed that BdWRKY36 was more closely related to OsWRKY37 and both of them belong to the group IIe WRKY (Fig. S2).

Expression analysis of *BdWRKY36* in response to abiotic stresses and signaling molecules

To determine the expression profile of *BdWRKY36* in different organs, we performed semi-quantitative RT-PCR with the total RNA from different organs of *B. distachyon*. *BdWRKY36* was ubiquitously expressed in all organs examined, including roots, stems, leaves and spikes, and exhibited higher expression levels in stems and leaves (Fig. 1a). In addition, we also determined the expression pattern of *BdWRKY36* at different time intervals (0, 1, 3, 6, 12 and 24 h) by RT-PCR using the total RNA extracted from leaves of 2-week-old *B. distachyon* seedlings. The results showed that there were no expression changes of *BdWRKY36* at different time intervals under normal conditions (Fig. S3). Therefore, the sample at 0 h was used as a control in the following expression assay. To explore the response of *BdWRKY36* to various abiotic stress conditions and signaling molecules, we carried out qRT-PCR using the total RNA extracted from 2-week-old *B. distachyon* seedlings subjected to various treatments at different time intervals (0, 1, 3, 6, 12 and 24). Under drought stress, the expression of *BdWRKY36* was increased 5.4-fold at 1 h and 2-fold at 6 h followed by a decrease (Fig. 1b-i). Cold (4 °C) treatment enhanced *BdWRKY36* transcripts about

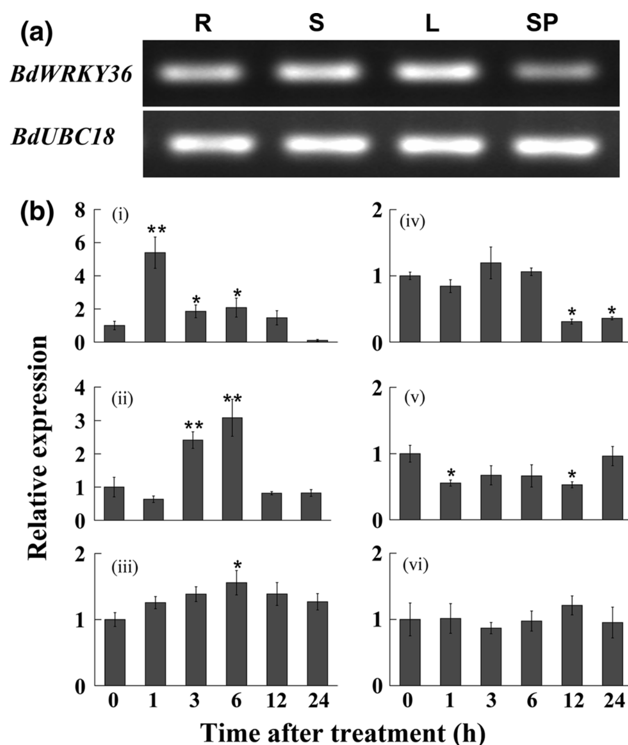

Fig. 1 RT-PCR and qRT-PCR analysis of expression profiles of *BdWRKY36*. **a** The expression analysis of *BdWRKY36* in different organs by semi-quantitative RT-PCR. Total RNA was isolated from roots (R), stems (S), leaves (L) and spikes (SP), respectively. *BdUBC18* gene was used as an internal control. **b** The expression analysis of *BdWRKY36* under different treatments by real-time RT-PCR analysis. Total RNA was isolated from leaves of *B. distachyon* seedlings treated with (i) drought, (ii) cold (4 °C), (iii) heat (40 °C), (iv) 200 mM NaCl, (v) 100 µM ABA, (vi) 10 mM H_2O_2 at indicated times. Data are mean ± SD calculated from three replicates. *Asterisks* indicate significant difference between the control (0 h) and the treatments (*$P < 0.05$; **$P < 0.01$). Three biological experiments were performed, which produced similar results

2.5-fold at 3 h and 3-fold at 6 h followed by a recovery (Fig. 1b-ii). *BdWRKY36* transcripts were also slightly upregulated by heat treatment (Fig. 1b-iii). When treated with 200 mM NaCl, the expression of *BdWRKY36* was not decreased until 6 h treatment (Fig. 1b-iv). In addition, under the treatment of ABA, the expression levels of *BdWRKY36* was decreased slightly after 1 h and then remained at low level until 12 h (Fig. 1b-v). In case of treatment with H_2O_2, there was not significant change for *BdWRKY36* expression from 1 to 24 h (Fig. 1b-vi). These results suggested that *BdWRKY36* expression could be upregulated by drought, cold and heat treatments and downregulated by NaCl and ABA treatments.

BdWRKY36 is localized in nucleus

To clarify the subcellular localization of the BdWRKY36, the ORF of *BdWRKY36* was fused to the N-terminus of the green fluorescent protein (GFP) reporter gene under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. We performed transient expression assays using onion epidermal cells with the construct BdWRKY36-GFP fusion and GFP alone by particle bombardment methods. As shown in Fig. 2, GFP alone distributed throughout the whole cell, whereas BdWRKY36-GFP fusion protein accumulated in the nucleus only. Additionally, the green

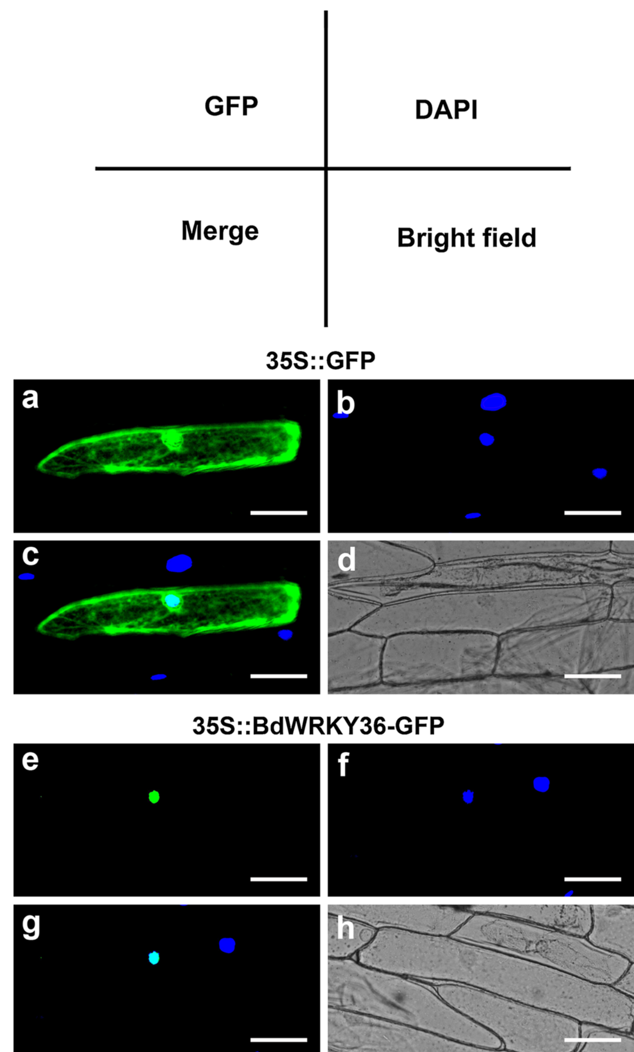

Fig. 2 Subcellular localization of BdWRKY36-GFP The fusion protein 35S-BdWRKY36-GFP (pCAMBIA1304-*BdWRKY36-GFP*) and 35S-GFP (pCAMBIA1304-*GFP* as control) were transiently expressed in onion epidermal cells and visualized under bright and fluorescence field before or after DAPI staining with fluorescence microscopy after bombardment of 16 h. **a** Fluorescence image of GFP control (**b**) the corresponding DAPI staining for the nucleus (**c**) merged fluorescence and DAPI image (**d**) the corresponding bright field (**e**) fluorescence image of BdWRKY36-GFP, (**f**) the corresponding DAPI staining for the nucleus, (**g**) merged fluorescence and DAPI image, (**h**) the corresponding bright field. *Scale bars* = 100 µm. Three biological experiments were performed, which produced similar results

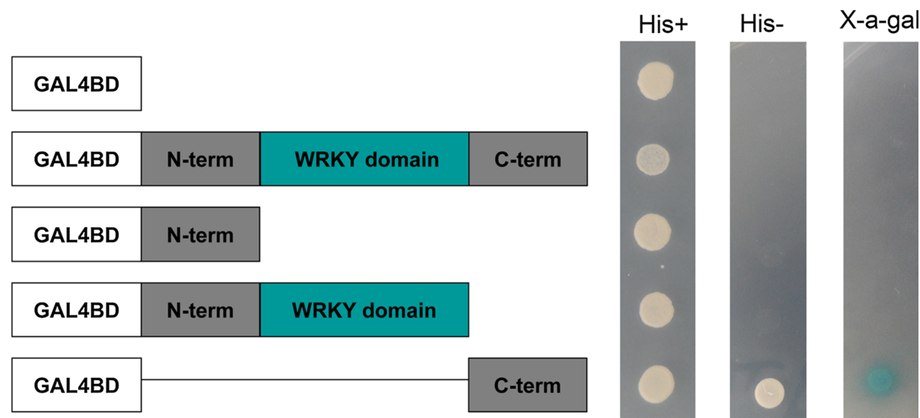

Fig. 3 Transactivation activity of the BdWRKY36 protein in yeast. **a** The schematic diagram demonstrating the *BdWRKY36* cDNA fragments encoding different portions of BdWRKY36 that were fused to the yeast vector pGBKT7. **b** Transactivation activity analysis of BdWRKY36 was performed using yeast strain AH109. The transformants were screened on the SD/Trp^- and SD/Trp^-/His^- mediums. The transformants were examined for growth in the presence of X-α-gal. Three biological experiments were performed, which produced similar results

fluorescence and blue (DAPI staining) were both confined to the nucleus in BdWRKY36-GFP-expressing cells, suggesting the nucleus location of BdWRKY36. These results clearly demonstrated that BdWRKY36 is localized in the nucleus, which is consistent with its predicted function as a transcription factor.

C-terminal domain of BdWRKY36 shows transcriptional activity

To investigate whether BdWRKY36 possesses transcriptional activation activity, the ORF of *BdWRKY36* was fused to the GAL4 DNA binding domain (GAL4BD) in the vector pGBKT7 and the construct was transformed into the AH109 yeast strain. As shown in Fig. 3, the reporter genes *His* and *LacZ* were not expressed in the yeast cell transformed with the GAL4BD-*BdWRKY36* construct as well as GAL4BD as a control, indicating that the full length ORF of *BdWRKY36* had no transcriptional activity or was repressed by some repression region in itself in the yeast cell.

To further check the transactivation region of BdWRKY36, GAL4BD-N-terminus, GAL4BD-N-WRKY domain and GAL4BD-C-terminus were constructed and transformed into the yeast strain AH109. The yeast cell growth on the His^- medium and LacZ staining indicated that the transactivation region of BdWRKY36 is located at the C-terminal region of BdWRKY36 (beginning from 200th aa to the end of C-terminus (Fig. S1) only, whereas its full length, N-terminal domain and N-term-WRKY domain had no transcriptional activity (Fig. 3).

Generation of transgenic tobacco overexpressing *BdWRKY36*

To further investigate the biological function of *BdWRKY36*, transgenic tobacco plants overexpressing *BdWRKY36* under the control of CaMV 35S promoter were generated. A total of 26 independent transgenic lines (T_1) were obtained by 30 mg/L hygromycin resistance selection and confirmation by genomic PCR using primers specific to *BdWRKY36*, *HptII* and *GFP*. Among the T_1 lines, three (OE-6, OE-14 and OE-18) lines segregated at a ratio of ∼ 3:1 based on 30 mg/L hygromycin resistance and showed higher expression levels of *BdWRKY36* by RT-PCR analysis (Fig. 4a). In this experiment, tobacco plants transformed with the empty vector alone were also subjected to similar analysis.

Overexpression of the *BdWRKY36* gene enhances drought tolerance in transgenic tobacco

The expression of *BdWRKY36* was upregulated under drought stress in *B. distachyon* (Fig. 1), indicating that *BdWRKY36* may play a role in drought stress response. Thus, 30-day-old tobacco plants were used to test drought tolerance. After water withholding for 20 days, all the leaves of WT and VC plants severely wilted and curled, whereas some leaves of transgenic plants remained expanded (Fig. 4b). When rewatered for 10 days, only 10 % of the WT and 12.5 % of the VC plants survived, while the survival rate of the transgenic lines OE-6, OE-14 and OE-18 was 45, 68.75 and 36.25 %, respectively (Fig. 4c). These results suggested that overexpression of *BdWRKY36* enhances drought stress tolerance in transgenic tobacco plants.

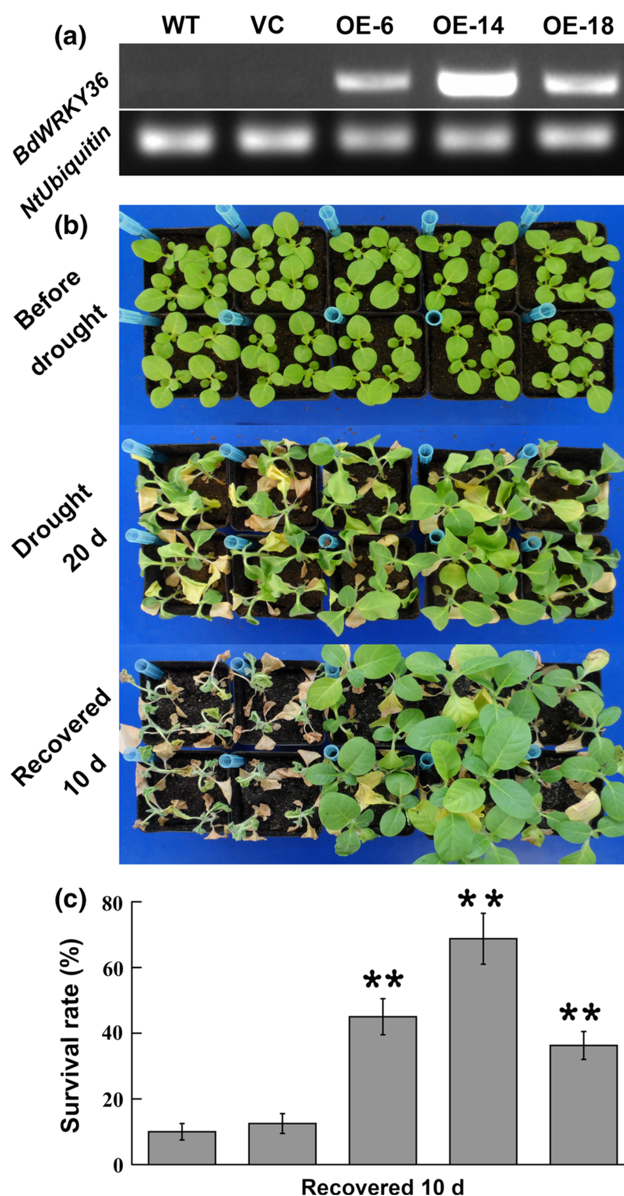

Fig. 4 Analysis of the enhanced drought tolerance in transgenic lines. **a** The expression of *BdWRKY36* in transgenic lines (OE-6, OE-14, OE-18). The tobacco *NtUbiquitin* gene was used as internal reference. *WT* wild type, *VC* vector control; OE-6, OE-14 and OE-18, three independent transgenic tobacco lines overexpressing *BdWRKY36*. **b** The photographs of WT, VC and transgenic lines before drought, after water withholding for 20 days, and after rewatering for 10 days. **c** The survival rates of WT, VC and transgenic lines after rewatering for 10 days. Data are mean ± SD calculated from three replicates. *Asterisks* indicate significant difference between WT and three transgenic lines (**$P < 0.01$). Two biological experiments were performed, which produced similar results

Overexpression of *BdWRKY36* improves RWC and chlorophyll level, decreases IL and H_2O_2 in transgenic tobacco under drought stress

Enhanced drought tolerance in transgenic plants promoted us to analyze the physiological-biochemical differences between transgenic plants and controls. Relative water content (RWC) that gives a credible evaluation of the plant water status is a relevant tool for the measurement of drought tolerance. Transgenic lines showed higher RWC than WT plants after 20 days drought treatment (Fig. 5a). In addition, chlorophyll content which is an indicator to plant living status was measured in transgenic and WT plants. As shown in Fig. 5b, transgenic lines presented significantly higher chlorophyll content than WT plants under drought treatment. IL, also an important indicator of membrane injury, was significantly lower in transgenic lines compared with WT plants under drought stress (Fig. 5c), indicating that transgenic plants suffered less membrane damage than WT plants. Furthermore, H_2O_2 content was lower in transgenic lines than that in WT plants under drought treatment (Fig. 5d). These results suggested that *BdWRKY36*-overexpressing plants can keep more RWC and were less injured under drought stress condition.

Overexpression of *BdWRKY36* reduces ROS accumulation and increases SOD, POD and CAT activities under drought stress

To confirm the function of *BdWRKY36* in reducing ROS levels in transgenic tobacco plants under drought stress, we examined the accumulation of endogenous H_2O_2 and superoxide radical anions (O_2^-) in transgenic plants and controls using DAB and NBT staining, respectively. As shown in Fig. 6, overexpression of *BdWRKY36* decreased the accumulation of brown (DAB staining) and blue (NBT staining) pigments compared with WT and VC after 20 days drought stress. Since the antioxidant enzymes play vital roles in ROS scavenging and influence cellular ROS balance in the antioxidant defense system. Therefore, the activities of three important antioxidant enzymes SOD, POD and CAT were also measured in the leaves of transgenic and WT plants. As shown in Fig. 7, higher SOD, POD and CAT activities were observed in transgenic plants than that in WT plants under drought conditions. These results suggested that overexpression of *BdWRKY36* increases SOD, POD and CAT activities under drought stress.

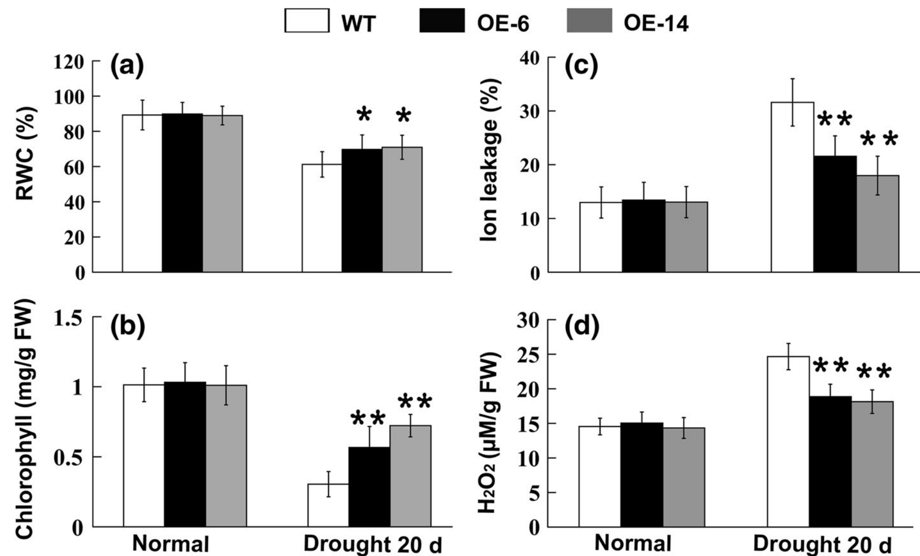

Fig. 5 Analysis of RWC (**a**), chlorophyll content (**b**), IL (**c**) and H_2O_2 content (**d**) in WT and transgenic lines (OE-6, OE-14) under normal and drought conditions. Thirty-day-old tobacco plants were deprived of water for 20 days, and then leaves were collected to measure RWC, IL, chlorophyll and H_2O_2. Data are mean ± SD calculated from three replicates. *Asterisks* indicate significant difference between the WT and the two transgenic lines (*$P < 0.05$, **$P < 0.01$). Two biological experiments were performed, which produced similar results

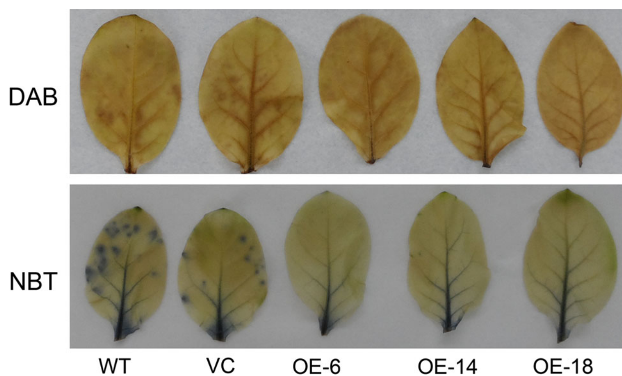

Fig. 6 Analysis of ROS accumulation in the WT, VC and transgenic lines (OE-6, OE-14 and OE-18) under drought conditions. Thirty-day-old tobacco plants were deprived of water for 20 days and then leaves were collected to detect H_2O_2 and O_2^- by DAB (*upper*) and NBT (*lower*) staining, respectively. Two biological experiments were performed, which produced similar results

Overexpression of *BdWRKY36* affects the expression of ROS-scavenging and stress-responsive genes under drought stress

To further understand the molecular mechanism underlying the drought resistance conferred by *BdWRKY36*, the expression levels of ROS-scavenging and stress-responsive genes were detected in the transgenic (OE-14) and WT plants by qRT-PCR under normal and drought stress. Genes including *NtSOD*, *NtPOX2* and *NtCAT* involved in ROS scavenging, *NtLEA5* related to stress defense, *NtNCED1* involved in ABA biosynthesis and the modulatory gene *NtDREB3* were selected for this assay. Under normal conditions, there was no obvious difference for the expression of tested genes between transgenic lines and WT. Under drought conditions, transcription levels of ROS-scavenging genes *NtSOD*, *NtPOX* and *NtCAT* as well as stress-responsive genes *NtLEA5*, *NtNCED1* and *NtDREB3* were higher in the transgenic lines compared with WT. These results indicated that overexpression of *BdWRKY36* enhances the expression of ROS-scavenging and stress-responsive genes under drought conditions.

Discussion

Lots of WRKY transcription factor genes have been identified and the function of some has been characterized from numerous higher plant species since the first *WRKY* gene was identified from sweet potato by Ishiguro and Nakamura (1994). However, there is no report about functional characterization of WRKY transcription factor from *B. distachyon*, which is now used as a model for the grasses especially for the wheat research recently. In the present study, we cloned a *WRKY* gene designated as *BdWRKY36* from *B. distachyon*. Sequence analysis indicated that BdWRKY36 contains WRKYGQK and C2H2-type zinc finger motifs and belongs to group IIe WRKY. The result of subcellular localization of BdWRKY36-GFP indicated that BdWRKY36 is located in the nucleus (Fig. 2), which is consistent with previous studies on WRKY transcription factors from other species (Luo et al. 2013; Wang et al. 2013). Transcriptional activation analysis showed that the transcriptional activity is in its C-terminal region of BdWRKY36 only, whereas its full length, N-terminal domain and N-term-WRKY domain have no transcriptional activity (Fig. 3), which is similar with previous report on OsbZIP46, the full length of which also has no transactivation activity while its D domain

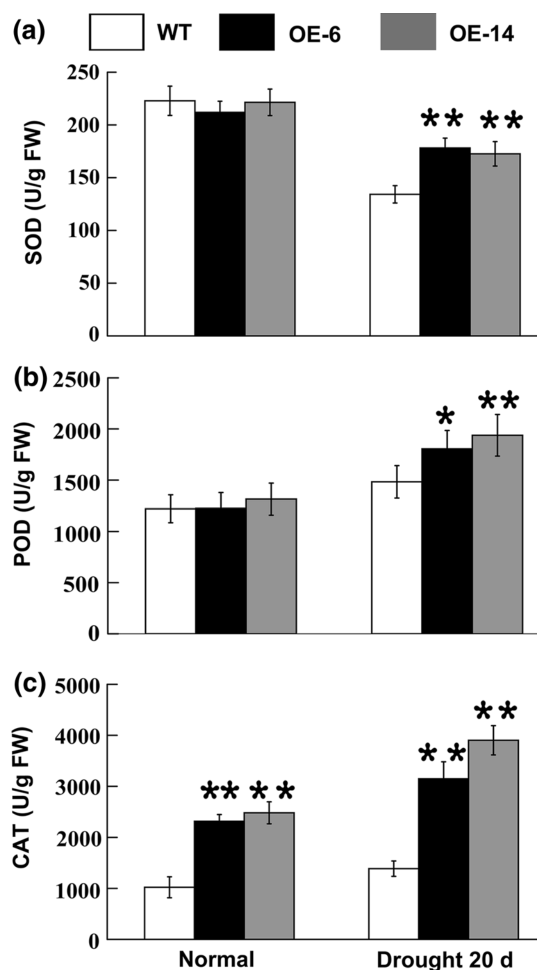

Fig. 7 Analysis of SOD (**a**), POD (**b**) and CAT (**c**) activities in the WT and transgenic lines (OE-6, OE-14) under normal and drought conditions. Thirty-day-old tobacco plants were deprived of water for 20 days and then leaves were collected to detect SOD, POD and CAT activities. Data are mean ± SD calculated from three replicates. *Asterisks* indicate significant difference between the WT and the two transgenic lines (* $P < 0.05$, ** $P < 0.01$). Two biological experiments were performed, which produced similar results

deletion mutant has the activity (Tang et al. 2012). It seems that N-terminal domain or N-term-WRKY domain may have an important role in the regulation of the transactivation activity of BdWRKY36 and that the activation of BdWRKY36 may need posttranslational level modifications or need to be activated by some unknown upstream proteins. For instance, Arabidopsis ABO3 (AtWRKY63) needs some ABA-triggered post-translational modification, or need to cooperate with other ABA-related proteins for its capacity to mediate plant responses to abscisic acid and drought tolerance (Ren et al. 2010). Phosphorylation of OsWRKY33 by OsBWMK1 increases its W-box binding activity to mediate SA-dependent defense responses (Koo et al. 2009). The senescence-related AtWRKY53 protein needs MEKK1 for its W-box binding activity (Miao et al. 2007). AtWRKY33 functions in the MPK4-mediated JA/ET-activating signaling pathways (Zheng et al. 2006). These data suggest that the regulation of BdWRKY36 transactivation activity is important for its functionality.

qRT-PCR results showed that *BdWRKY36* expression was downregulated by NaCl and ABA, but not changed by H_2O_2 treatment (Fig. 1b). However, transcription levels of *BdWRKY36* were upregulated by drought, cold and heat, in particular the drought stress, suggesting that *BdWRKY36* may play a role in abiotic stress responses. The stronger upregulation of the *BdWRKY36* by drought stress treatment promoted us to analyze its function in drought tolerance by overexpressing *BdWRKY36* in transgenic tobacco. Phenotype analysis of *BdWRKY36*-overexpressing transgenic lines showed that expression of *BdWRKY36* in tobacco greatly enhances their ability to survive a very strong drought stress. (Fig. 4b). This result is agreement with previous studies on the functions of some *WRKY* genes. For instance, overexpressions of wheat *TaWKRY2* and *TaWRKY19* in transgenic Arabidopsis enhanced drought tolerance by regulating downstream genes (Niu et al. 2012). In addition, expression of soybean *WRKY20* in Arabidopsis enhanced drought tolerance by regulating ABA signaling (Luo et al. 2013). Overexpression of *TaWRKY10* in transgenic tobacco resulted in enhanced drought stress tolerance by improving proline and soluble sugar contents and reducing ROS accumulation (Wang et al. 2013). Although functional assays using water withheld not necessarily indicate that a plant has tolerance to moderate drought (Skirycz et al. 2011), it is still a beneficial trait of choice in agricultural practice especially in arid regions.

The deteriorative effects of drought on plants may include leaf wilting, reduction in leaf area, leaf abscission, changes in chlorophyll content, RWC and IL, generation of ROS, and accumulation of free radicals which disrupt cellular homeostasis by reacting with lipids, proteins, pigments and nucleic acids resulting in lipid peroxidation, membrane damage, and inactivation of enzymes, thus affecting cell viability (Roychoudhury et al. 2013). Thus, we carried out a set of experiments to analyze physiological and biochemical parameters of transgenic plants, and the results showed that the enhanced tolerance of transgenic lines to drought stress is related to higher contents of chlorophyll and RWC, but lesser IL and H_2O_2 (Fig. 5). Furthermore, transgenic plants accumulated lower levels of H_2O_2 and O_2^- under drought stress compared with controls (Fig. 6). These results indicate that *BdWRKY36* enhancing plant tolerance to drought is likely through reduction of membrane injury by inhibiting ROS accumulation or scavenging ROS production. To minimize cellular damage caused by oxidative stress, plants have to maintain their ROS pools at low levels (Foyer and Shigeoka 2011). To regulate ROS homeostasis, plants have evolved a complex

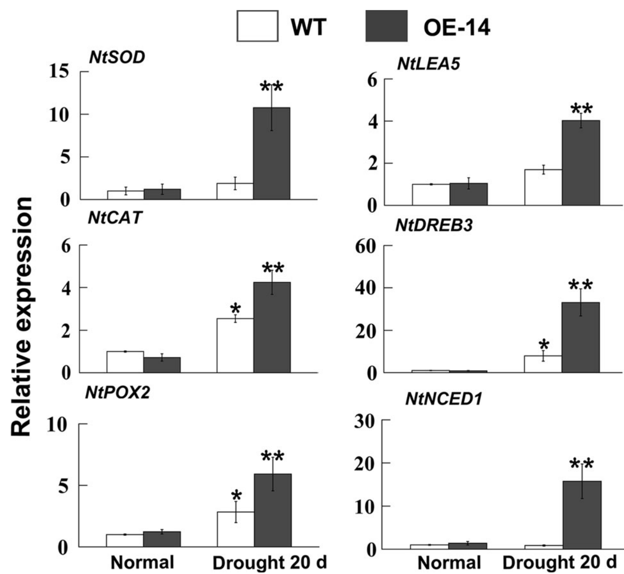

Fig. 8 Analysis of expression levels of ROS-related and stress-responsive genes in the WT and the transgenic line (OE-14) by qRT-PCR under normal and drought conditions. Thirty-day-old tobacco plants were deprived of water for 20 days and then leaves were used to detect gene expression. Data are mean ± SD of three replicates. *Asterisks* indicate significant difference between normal and drought treatment (*$P < 0.05$, **$P < 0.01$). Two biological experiments were performed, which produced similar results

antioxidant system that includes several enzymes, which scavenge ROS and protect cells against oxidative stress (Huang et al. 2010; Miller et al. 2010). Thus, the levels of antioxidant enzyme activity including SOD, POD and CAT were analyzed, and the results showed that the transgenic plants have higher SOD, POD and CAT activity than that in WT under drought stress (Fig. 7). This implies that ROS scavenging systems in transgenic plants might work more effectively compared to WT. Taken together, these data demonstrate that overexpression of *BdWRKY36* gene may function in activation of antioxidant defense system, which results in transgenic plants suffering from less ROS-mediated injury under drought stress. These results are consistent with previous studies on some *WRKY* genes in other plants, which function in activation of antioxidant system under abiotic stress. For example, in a recent study, overexpression of the *DgWRKY3* gene in tobacco confers tolerance to salt stress by increasing SOD, POD and CAT activities of antioxidant system (Liu et al. 2013). In addition, overexpression of the *SlWRKY* gene in tobacco has been shown tolerance to salt and drought stresses due to the elevation of SOD and POD activity (Li et al. 2012b) (Fig. 8).

To further understand the regulatory roles of *BdWRKY36* at transcriptional levels under drought condition, the expression patterns of some ROS-scavenging and stress-responsive genes were investigated. It was found that the transcript levels of the genes encoding ROS-scavenging enzymes including *NtSOD*, *NtPOX* and *NtCAT* were upregulated in the transgenic lines under drought condition, which may contribute to the improved antioxidant system. Moreover, ABA plays crucial roles in adaptive responses to environmental stress including drought. Biochemical and genetic evidence revealed that ABA biosynthesis under stress is regulated by the rate-limiting enzyme, 9-cis-epoxycarotenoid dioxygenase (NCED) (Huang et al. 2010). The expression of *NtNCED1* was enhanced in transgenic plants compared with the WT under drought condition, indicating that ABA biosynthesis may be enhanced by *BdWRKY36* overexpression. *NtLEA5* encodes group five late embryogenesis abundant (LEA) proteins, which functions in dehydration tolerance by binding water, stabilizing labile enzymes, and protecting cellular and macromolecular structures (Hundertmark and Hincha 2008). Higher level transcription of *NtLEA5* in the transgenic plants under drought stress implied that more LEA proteins might be synthesized in transgenic lines, which contributes to transgenic plants maintaining higher RWC and less membrane damage.

Taken together, under drought condition, the expression levels of ROS-scavenging and stress-responsive genes were significantly upregulated in transgenic plants than that in WT plants. However, no significant difference in the expression of these genes was found between the *BdWRKY36*-overexpressing and WT tobacco plants under normal condition, despite the fact that the constitutive promoter (35S) was used to drive *BdWRKY36*. Similar results were reported such as *DgWRKY3* (Liu et al. 2013). The possible explanation is that *BdWRKY36* may mediate the activation of such genes accompanied by other stress-responsive regulators (Sun et al. 2010; Yang et al. 2012; Liu et al. 2013).

In conclusion, the findings of this study demonstrate a role for *BdWRKY36*, group IIe *WRKY* in enhancing drought stress tolerance. *BdWRKY36* confers tolerance to drought stress through improving antioxidant system and upregulating the transcriptional levels of ROS-scavenging and stress-responsive genes. These findings will help our understanding of the mechanisms of environmental stress on plants and highlight the role of WRKY transcription factor in the activation of antioxidant system and transcriptional regulation. In the future, exploring the direct targets of *BdWRKY36* involved in drought stress tolerance will shed light on the molecular mechanism of *BdWRKY36*-mediated stress tolerance.

Acknowledgments This work was supported by International S & T Cooperation Key Projects of MoST (Grant no. 2009DFB30340), National Genetically Modified New Varieties of Major Projects of China (Grant no. 2014ZX08010004), Research Fund for the Doctoral Program of Higher Education of China (Grant no. 2012014211075)

and Open Research Fund of State Key Laboratory of Hybrid Rice in Wuhan University (Grant no. KF201302). We thank Wuhan Botanic Garden of Chinese Academy of Sciences for supplying seeds of *Brachypodium distachyon* (Bd21).

Conflict of interest The authors declare no conflict of interest.

References

Bhalla PL (2006) Genetic engineering of wheat-current challenges and opportunities. Trends Biotechnol 24:305–311

Chen YF, Li LQ, Xu Q, Kong YH, Wang H, Wu WH (2009) The WRKY6 transcription factor modulates phosphate1 expression in response to low Pi stress in Arabidopsis. Plant Cell 21:3554–3566

Chen H, Lai ZB, Shi JW, Xiao Y, Chen ZX, Xu XP (2010) Roles of arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. BMC Plant Biol 10:281

Chen LG, Song Y, Li SJ, Zhang LP, Zou CS, Yu DQ (2012) The role of WRKY transcription factors in plant abiotic stresses. Biochim Biophys Acta 1819:120–128

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci 5:199–206

Foyer CH, Shigeoka S (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol 155:93–100

Hirayama T, Shinozaki K (2010) Research on plant abiotic stress responses in the post-genome era: past, present and future. Plant J 61:1041–1052

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SC, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Hu W, Huang C, Deng XM, Zhou SY, Chen LH, Li Y, Wang C, Ma ZB, Yuan QQ, Wang Y, Cai R, Liang XY, Yang GX, He GY (2013) TaASR1, a transcription factor gene in wheat, confers drought stress tolerance in transgenic tobacco. Plant Cell Environ 36:1449–1464

Huang XS, Liu JH, Chen XJ (2010) Overexpression of *PtrABF* gene, a bZIP transcription factor isolated from *Poncirus trifoliata*, enhances dehydration and drought tolerance in tobacco via scavenging ROS and modulating expression of stress-responsive genes. BMC Plant Biol 10:230

Hundertmark M, Hincha DK (2008) LEA (late embryogenesis abundant) proteins and their encoding genes in Arabidopsis thaliana. BMC Genom 9:118

Ishiguro S, Nakamura K (1994) Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 5′upstream regions of genes coding for sporamin and β-amylase from sweet potato. Mol Gen Genet 244:563–571

Jiang Y, Deyholos MK (2009) Functional characterization of Arabidopsis NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. Plant Mol Biol 69:91–105

Jiang MY, Zhang JH (2001) Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. Plant Cell Physiol 42:1265–1273

Jiang YJ, Liang G, Yu DQ (2012) Activated expression of *WRKY57* confers drought tolerance in Arabidopsis. Mol Plant 5:1375–1388

Johnson CS, Kolevski B, Smyth DR (2002) Transparent TESTA GLABRA2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor. Plant Cell 14:1359–1375

Koo SC, Moon BC, Kim JK, Kim CY, Sung SJ, Kim MC, Cho MJ, Cheong YH (2009) OsBWMK1 mediates SA-dependent defense responses by activating the transcription factor OsWRKY33. Biochem Biophys Res Commun 387:365–370

Li SJ, Fu QT, Huang WD, Yu DQ (2009) Functional analysis of an Arabidopsis transcription factor WRKY25 in heat stress. Plant Cell Rep 28:683–693

Li SJ, Zhou X, Chen LG, Huang WD, Yu DQ (2010) Functional characterization of *Arabidopsis thaliana* WRKY39 in heat stress. Mol Cells 29:475–483

Li C, Rudi H, Stockinger EJ, Cheng H, Cao M, Fox SE, Mockler TC, Westereng B, Fjellheim S, Rognli OA, Sandve SR (2012a) Comparative analyses reveal potential uses of *Brachypodium distachyon* as a model for cold stress responses in temperate grasses. BMC Plant Biol 12:65

Li JB, Luan YS, Jin H (2012b) The tomato *SlWRKY* gene plays an important role in the regulation of defense responses in tobacco. Biochem Biophys Res Commun 427:671–676

Li J, Besseau S, Törönen P, Sipari N, Kollist H, Holm L, Palva ET (2013) Defense-related transcription factors WRKY70 and WRKY54 modulate osmotic stress tolerance by regulating stomatal aperture in Arabidopsis. New Phytol 200:457–472

Liu QL, Zhong M, Li S, Pan YZ, Jiang BB, Jia Y, Zhang HQ (2013) Overexpression of a chrysanthemum transcription factor gene, *DgWRKY3*, in tobacco enhances tolerance to salt stress. Plant Physiol Biochem 69:27–33

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25:402–408

Luo X, Bai X, Sun XL, Zhu D, Liu BH, Ji W, Cai H, Cao L, Wu J, Hu MR, Liu X, Tang LL, Zhu YM (2013) Expression of wild soybean *WRKY20* in Arabidopsis enhances drought tolerance and regulates ABA signaling. J Exp Bot 64:2155–2169

Miao Y, Laun T, Zimmermann P, Zentgraf U (2004) Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis. Plant Mol Biol 55:853–867

Miao Y, Laun TM, Smykowski A, Zentgraf U (2007) Arabidopsis MEKK1 can take a short cut: it can directly interact with senescence-related WRKY53 transcription factor on the protein level and can bind to its promoter. Plant Mol Biol 65:63–76

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 33:453–467

Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends Plant Sci 11:15–19

Niu CF, Wei W, Zhou QY, Tian AG, Hao YJ, Zhang WK, Ma B, Lin Q, Zhang ZB, Zhang JS, Chen SY (2012) Wheat *WRKY* genes *TaWRKY2* and *TaWRKY19* regulate abiotic stress tolerance in transgenic Arabidopsis plants. Plant Cell Environ 35:1156–1170

Pan JW, Zhang MY, Kong XP, Xing X, Liu YK, Zhou Y, Liu Y, Sun LP, Li DQ (2012) *ZmMPK17*, a novel maize group D MAP kinase gene, is involved in multiple stress responses. Planta 235:661–676

Qin YX, Tian YC, Han L, Yang XC (2013) Constitutive expression of a salinity-induced wheat WRKY transcription factor enhances salinity and ionic stress tolerance in transgenic *Arabidopsis thaliana*. Biochem Biophys Res Commun 441:476–481

Ren X, Chen Z, Liu Y, Zhang H, Zhang M, Liu Q, Hong X, Zhu JK, Gong Z (2010) ABO3, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in Arabidopsis. Plant J 63:417–429

Ross CA, Liu Y, Shen QJ (2007) The WRKY gene family in rice (*Oryza sativa*). J Integr Plant Biol 49:827–842

Roychoudhury A, Paul S, Basu S (2013) Cross-talk between abscisic acid-dependent and abscisic acid-independent pathways during abiotic stress. Plant Cell Rep 32:985–1006

Rushton PJ, Somssich IE, Ringler P, Shen QJ (2010) WRKY transcription factors. Trends Plant Sci 15:247–258

Rushton DL, Tripathi P, Rabara RC, Lin J, Ringler P, Boken AK, Langum TJ, Smidt L, Boomsma DD, Emme NJ, Chen X, Finer JJ, Shen QJ, Rushton PJ (2012) WRKY transcription factors: key components in abscisic acid signaling. Plant Biotechnol J 10:2–11

Shen HS, Liu CT, Zhang Y, Meng XP, Zhou X, Chu CC, Wang XP (2012) OsWRKY30 is activated by MAP kinases to confer drought tolerance in rice. Plant Mol Biol 80:241–253

Skirycz A, Vandenbroucke K, Clauw P, Maleux K, De Meyer B, Dhondt S, Pucci A, Gonzalez N, Hoeberichts F, Tognetti VB, Galbiati M, Tonelli C, Van Breusegem F, Vuylsteke M, Inzé D (2011) Survival and growth of Arabidopsis plants given limited water are not equal. Nat Biotechnol 29:212–214

Song Y, Jing SJ, YU DQ (2009) Overexpression of the stress-induced *OsWRKY08* improves osmotic stress tolerance in Arabidopsis. Chinese Sci Bull 54:4671–4678

Sun SJ, Guo SQ, Yang X, Bao YM, Tang HJ, Sun H, Huang J, Zhang HS (2010) Functional analysis of a novel Cys2/His2-type zinc finger protein involved in salt tolerance in rice. J Exp Bot 61:2807–2818

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tang N, Zhang H, Li XH, Xiao JH, Xiong LZ (2012) Constitutive activation of transcription factor OsbZIP46 improves drought tolerance in rice. Plant Physiol 158:1755–1768

Tao Z, Kou YJ, Liu HB, Li XH, Xiao JH, Wang SP (2011) OsWRKY45 alleles play different roles in abscisic acid signalling and salt stress tolerance but similar roles in drought and cold tolerance in rice. J Exp Bot 62:4863–4874

Tripathi P, Rabara RC, Langum TJ, Boken AK, Rushton DL, Boomsma DD, Rinerson CI, Rabara J, Reese RN, Chen X, Rohila JS, Rushton PJ (2012) The WRKY transcription factor family in *Brachypodium distachyon*. BMC Genom 13:270

Wang HH, Hao JJ, Chen XJ, Hao ZN, Wang X, Lou YG, Peng YL, Guo ZJ (2007) Overexpression of rice WRKY89 enhances ultraviolet B tolerance and disease resistance in rice plants. Plant Mol Biol 65:799–815

Wang C, Deng PY, Chen LL, Wang XT, Ma H, Hu W, Yao NC, Feng Y, Chai RH, Yang GX, He GY (2013) A wheat WRKY transcription factor TaWRKY10 confers tolerance to multiple abiotic stresses in transgenic tobacco. PLoS One 8:e65120

Wen F, Zhu H, Li P, Jiang M, Mao WQ, Ong C, Chu ZQ (2014) Genome-wide evolutionary characterization and expression analyses of *WRKY* family genes in *Brachypodium distachyon*. DNA Res 21:327–339

Wu XL, Shiroto Y, Kishitani S, Ito Y, Toriyama K (2009) Enhanced heat and drought tolerance in transgenic rice seedlings overexpressing *OsWRKY11* under the control of *HSP101* promoter. Plant Cell Rep 28(1):21–30

Xiang JX, Ran J, Zou J, Zhou XY, Liu AL, Zhang XW, Peng Y, Tang N, Luo GY, Chen XB (2013) Heat shock factor OsHsfB2b negatively regulates drought and salt tolerance in rice. Plant Cell Rep 32:1795–1806

Xie Z, Zhang ZL, Zou XL, Huang J, Ruas P, Thompson D, Shen QJ (2005) Annotations and functional analyses of the rice *WRKY* gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. Plant Physiol 137:176–189

Xu XP, Chen CH, Fan BF, Chen ZX (2006) Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors. Plant Cell 18:1310–1326

Yang Q, Chen ZZ, Zhou XF, Yin HB, Li X, Xin XF, Hong XH, Zhu JK, Gong Z (2009) Overexpression of *SOS* (*salt overly sensitive*) genes increases salt tolerance in transgenic Arabidopsis. Mol Plant 2:22–31

Yang A, Dai XY, Zhang WH (2012) A R2R3-type MYB gene, *OsMYB2*, is involved in salt, cold, and dehydration tolerance in rice. J Exp Bot 63:2541–2556

Zhang CQ, Xu Y, Lu Y, Yu HX, Gu MH, Liu QQ (2011) The WRKY transcription factor OsWRKY78 regulates stem elongation and seed development in rice. Planta 234:541–554

Zheng Z, Qamar SA, Chen Z, Mengiste T (2006) Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J 48:592–605

Zhou QY, Tian AG, Zou HF, Xie ZM, Lei G, Huang J, Wang CM, Wang HW, Zhang JS, Chen SY (2008) Soybean WRKY-type transcription factor genes, *GmWRKY13, GmWRKY21,* and *GmWRKY54*, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. Plant Biotechnol J 6:486–503

Zhu JK (2001) Cell signaling under salt, water and cold stresses. Curr Opin Plant Biol 4:401–406