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ORIGINAL PAPER

Activated expression of AtWRKY53 negatively regulates drought tolerance by mediating stomatal movement

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Abstract

Key message **AtWRKY53 is an early factor in drought response and activated expression of AtWRKY53 regulates stomatal movement via reduction of H_2O_2 content and promotion of starch metabolism in guard cells.**

Abstract Drought is one of the most serious environmental factors limiting the productivity of agricultural crops worldwide. However, the mechanisms underlying drought tolerance in plants remain unclear. AtWRKY53 belongs to the group III of WRKY transcription factors. In this study, we observed both the mRNA and protein products of this gene are rapidly induced under drought conditions. Phenotypic analysis showed *AtWRKY53* overexpression lines were hypersensitive to drought stress compared with Col-0 plants. The results of stomatal movement assays and abscisic acid (ABA) content detection indicated that the impaired stomatal closure of 53OV lines was independent of ABA. Further analysis found that WRKY53 regulated stomatal movement via reducing the H_2O_2 content and promoting the starch metabolism in guard cells. The results of quantitative real-time reverse transcriptase PCR showed that the expression levels of *CAT2*, *CAT3* and *QQS* were up-regulated in 53OV lines. Chromatin immunoprecipitation assays demonstrated that AtWRKY53 can directly bind to the *QQS* promoter sequences, thus led to increased starch metabolism. In summary, our results indicated that the activated expression of AtWRKY53 inhibited stomatal closure by reducing H_2O_2 content and facilitated stomatal opening by promoting starch degradation.

Keywords Transcription factor · AtWRKY53 · Drought stress · Stomatal movement · Abscisic acid

Abbreviations

ABA	Abscisic acid
GUS	β-Glucuronidase
RT-PCR	Reverse transcription-PCR
qRT-PCR	Quantitative real-time reverse transcription-PCR
ChIP	Chromatin immunoprecipitation

Introduction

Plants are confronted with a series of biotic and abiotic stresses in their natural environment. Among these, drought is one of the most limiting and pervasive factors affecting plant growth, often with severe economic impacts in terms of agricultural production (Zhu 2002). Under drought conditions, the resulting decreased water uptake induces cellular dehydration. To respond to this stress, plants have

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evolved complex adaptive strategies, including morphological, physiological, and biochemical adaptations (Ingram and Bartels 1996; Xiong et al. 2002; Zhu 2002; Shinozaki et al. 2003; Bohnert et al. 2006). Some of these strategies aim to avoid dehydration stress by increasing water uptake or reducing water loss, whereas others seek to protect plant cells from the increased levels of H_2O_2 induced by dehydration (Verslues et al. 2006). These strategies correspond to different signaling pathways that, although specific, may partially crosstalk.

Abscisic acid (ABA) is an important phytohormone in osmotic stress perception and signaling (Ingram and Bartels 1996; Bray 1997). Drought causes increased biosynthesis and accumulation of ABA, which promotes stomatal closure and which regulates a large number of genes involved in dehydration response in both vegetative tissues and seeds (Leung and Giraudat 1998; Finkelstein 2013). Following relief from drought stress, ABA is rapidly catabolized (Taylor et al. 2000).

A pair of guard cells forms the stomatal pore, which serves as a major gateway for both CO_2 influx from the atmosphere and for water loss through transpiration (Kim et al. 2010). One of the key factors in drought tolerance is transpiration-based water loss through the stomata (Xiong et al. 2002). The closing and opening of the stomatal pores are mediated by turgor-driven change in the volume of the guard cells (Yu et al. 2008). This change can be influenced by many factors, including levels of light, phytohormones, potassium ions, calcium ions, malate, NO, and H_2O_2 (Assmann and Wang 2001; Schroeder et al. 2001; Nilson and Assmann 2007; Shimazaki et al. 2007). It is well known that starch degradation can promote stomatal opening under blue light by producing malate in the guard cells (Vavasseur and Raghavendra 2005); however, how starch is metabolized in guard cells remains unclear. What is known is that guard cells sense and integrate environmental signals to modulate stomatal apertures in response to drought stress.

The *WRKY* gene family is one of the major groups of plant-specific transcriptional factors containing a highly conserved WRKYGQK motif at the N-terminus, and a Cys_2His_2 or $Cys_2HisCys$ zinc-binding motif at the C-terminus (Eulgem et al. 2000; Rushton et al. 2010). In *Arabidopsis thaliana*, the WRKY family consists of 74 members and is classified into three groups on the basis of both the number of WRKY domains and the type of zinc-finger-like motifs present. WRKY transcription factors play several important roles in bio/abiotic stress responses (Eulgem and Somssich 2007; Miller et al. 2008; Chen et al. 2012). For example, AtWRKY63 mediates plant responses to ABA and drought tolerance in *Arabidopsis* (Ren et al. 2010). Overexpression of the stress-induced *OsWRKY45* and *LcWRKY5* enhance drought tolerance in *Arabidopsis* (Qiu and Yu 2008; Ma et al. 2014). Male gametophyte-specific AtWRKY34 mediates cold sensitivity of mature pollen in *Arabidopsis* (Zou et al. 2010), while AtWRKY25, AtWRKY26, AtWRKY33, and AtWRKY39 all play important roles in response to heat stress (Li et al. 2009a, 2010, 2011). AtWRKY57 confers drought tolerance in *Arabidopsis* by directly binding to the W-box of the *RD29A* and *NCED3* promoter sequences (Jiang et al. 2012). In addition, our recent studies showed that AtWRKY8 functions antagonistically with its interacting partner VQ9 to modulate salinity stress tolerance (Hu et al. 2013), and functions in the TMV-cg defense response in *Arabidopsis* by mediating both abscisic acid and ethylene signaling (Chen et al. 2013). Recent studies have indicated that WRKY transcription factors also play important roles in physiological and developmental programs, including senescence, seed germination and trichome development (Hinderhofer and Zentgraf 2001; Johnson et al. 2002; Singh et al. 2002). OsWRK23 enhances dark-induced leaf senescence in *Arabidopsis* (Jing et al. 2009), while AtWRKY22 participates in the dark-induced senescence signal-transduction pathway (Zhou et al. 2011). AtWRKY2 mediates seed germination and post-germination arrest of development by ABA (Jiang and Yu 2009), and AtWRKY57 functions as a node of convergence for jasmonic acid- and auxin-mediated signaling in jasmonic acid-induced leaf senescence (Jiang et al. 2014).

AtWRKY53 (AT4G23810), which belongs to the group III of WRKY transcription factors, has been reported to play a central role in senescence regulation (Hinderhofer and Zentgraf 2001; Miao and Zentgraf 2007; Zentgraf et al. 2010) and in basal resistance against *Pseudomonas syringae* (Murray et al. 2007). In the present study, we found that AtWRKY53 was rapidly induced by drought. Further analysis revealed that overexpression of AtWRKY53 led to hypersensitivity to drought. Overall, our data indicated that AtWRKY53 involves stomatal movement via reduction of H_2O_2 content and promotion of starch metabolism in guard cells.

Materials and methods

Plant materials and growth conditions

A. thaliana cv. Columbia was grown in soil or on half-strength Murashige and Skoog medium (1/2 MS) supplemented with 1 % (w/v) sucrose. Seeds were surface-sterilized [20 % (v/v) bleach for 15 min] before sowing on 1/2 MS medium and incubation at 4 °C for 3 days. Plants were then kept in a growth cabinet at 22 °C under short-day conditions (12 h light/12 h dark) and long-day conditions (16 h light/8 h dark). The T-DNA insertion mutants

wrky53 (SALK_034157) and *wrky46* (SALK_134310) were also obtained from Arabidopsis Biological Resource Center (ABRC; http://abrc.osu.edu). For *AtWRKY53* complementation and transgenic GUS lines, a 2461-bp length of native promoter was fused to *myc-WRKY53* cDNA or the GUS sequence, respectively, and then cloned into a binary vector (Poca28). Recombinant plasmids were transformed into *wrky53* mutant plants or Col-0 wild-type plants using the *Agrobacterium* EHA105. For *AtWRKY53* overexpression lines, the recombinant plasmid containing 35s-*myc-WRKY53* was transformed into Col-0 wild-type plants. The primers used are listed in Supplementary Table S1.

GUS staining

Histochemical detection of GUS activity was performed with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as the substrate. Plant tissues were first prefixed in ice-cold 90 % (v/v) acetone for 20 min and then washed three times with GUS staining buffer (without X-Gluc) before incubation in X-Gluc solution [1 mM X-Gluc, 50 mM $NaPO_4$ (pH 7), 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, and 0.05 % Triton X-100] under a vacuum for 10 min at room temperature, and then incubated overnight at 37 °C. Chlorophyll was removed using several changes of 70 % (v/v) ethanol and the tissues were then photographed.

Stress treatment and water loss assays

For expression analysis, 4-week-old plants were dehydrated on Whatman 3MM paper at 22 °C and 60 % humidity under dim light for drought treatment. For other stress treatments, 4-week-old plants were hydroponically subjected to 200 mM mannitol, 100 mM NaCl and 15 % PEG6000, respectively.

For drought tolerance assays, seedlings from wild-type Col-0, *wrky53*, *wrky53*C-1, *wrky53*C-7, *WRKY53*OV-3, *WRKY53*OV-5, *wrky46*, and *wrky46 wrky53*, were used. Seedlings grown for 7 days on 1/2 MS medium plates were transplanted into separate pots (7 cm in diameter, 6 cm height) under normal watering conditions. After 3 weeks growth, plants were subjected to drought (water was withheld). Following 11 days without watering, the drought-treated plants were re-watered and recovery was checked after 24 h. Drought experiments were repeated four times and at least 35 plants from each individual line were used in each repeat experiment and one representative photograph per line was obtained.

The rate of water loss by the leaves was measured. The aboveground shoots of 4-week-old plants were detached from the root system and immediately weighed. The shoots were placed in a plate on a laboratory bench and weighed at designated time intervals. The proportion of fresh weight lost was calculated on the basis of the initial weight of the plant. At least three biological replicates for each sample were used for drought tolerance assays.

Expression analysis

For RT-PCR analysis, total RNA was extracted using TRIzol reagent (Invitrogen) and treated with RNase-free DNaseI (Fermentas) in accordance with the manufacturer's instructions. Total RNA (~2 μg) was reverse-transcribed in a 20-μL reaction mixture using the Superscript II reverse transcriptase (Invitrogen). After the reaction, 1-μL aliquots were used as a template for PCR amplification. For qRT-PCR analysis, the PCR templates were obtained using the same procedure as for the RT-PCR. SYBR Green was used to monitor the kinetics of PCR product amplification in the qRT-PCR. As an internal control, the *ACTIN2* transcript was used to quantify the relative transcript level of each target gene in each tissue type. Three replicate biological experiments were conducted. Gene-specific primers are listed in Supplementary Table S1.

For western blot assay, proteins from 3-week-old transgenic seedlings expressing Pro*WRKY53-myc-WRKY53* under drought treatments were extracted in a lysis buffer (150 mM NaCl, 1.0 % Triton X-100, 0.5 % sodium deoxycholate, 10 % glycerol, 50 mM Tris, pH 8.0). Protein gel blotting analysis was performed as described previously (Ding et al. 2014). Crude antisera of anti-myc were used at dilutions of 1:5000. For relative protein expression level assay, the protein gel blotting analysis was repeated three times.

ABA content

Measurement of ABA content was conducted as described by Yang et al. (2001). Briefly, 3-week-old seedlings with or without drought treatment were used. One gram of seedling tissue was used for ABA quantification with the ABA immunoassay kit as described by Yang et al. (2001). At least three biological replicates per sample were used.

Stomatal movement assay response to ABA

Stomatal closure assays were conducted as described previously (Pei et al. 1997). Rosette leaves were floated in a solution containing 50 μM $CaCl_2$, 10 mM KCl, 10 mM MES (2-(*N*-morpholino)ethanesulfonic acid)-Tris, pH 6.15, and exposed to light for 2 h. Subsequently, ABA was added to the solution up to 5 μM. After ABA treatment for 2 h, stomatal apertures were measured. Three replicate biological experiments were conducted.

Determination of ROS accumulation

For the ROS accumulation assay in guard cells, prepared epidermal peels with or without ABA treatment were loaded with 50 μM 2,7-dichlorofluorescin diacetate (H_2DCF-DA; Sigma-Aldrich) for 15 min, as described previously (Pei et al. 2000). After staining, epidermal strips were briefly washed with the buffer containing 50 μM $CaCl_2$, 10 mM KCl, 10 mM MES, and photographed using a fluorescent stereoscope.

Isolation of guard cells and determination of starch and malate content

Guard cell protoplasts were isolated according to Pandey et al. (2002). In brief, about 100 leaves of 4-week-old plants were blended using a commercial Waring blender for 1 min in 100 mL cold tap water. The epidermal peels were purified through a 200 μm mesh by washing with deionized water. The peels were further processed by two-step enzyme digestion as described by Pandey et al. (2002). The released guard cells were filtered through a 20 μm mesh, centrifuged for 5 min at 200×*g*, and concentrated in a small volume of fresh basic solution. Starch content was measured according to the enzymatic method described in Rose et al. (1991). The malate concentrations were measured according to the enzymatic method described in Delhaize et al. (1993). Three replicate biological experiments were conducted.

ChIP assays

ChIP assays were performed essentially in accordance with previously described protocols (Saleh et al. 2008; Shang et al. 2010). Pro*WRKY53-myc-WRKY53* seedlings with or without dehydration treatment were fixed with 20 mL of 1.0 % formaldehyde under vacuum for 10 min. The chromatin DNA was extracted and sheared to 200–1000 bp fragments by sonication. The sheared DNA (100 μL) was immunoprecipitated with 3–5 μg anti-myc for 90 min at 50–100 rpm at room temperature. In addition, 1 μL of normal mouse IgG was used as a negative control. DNA fragments that specifically associated with *AtWRKY53* were released, purified, and used as templates for qRT-PCR with specific primers (Table S1).

Results

Expression patterns of *AtWRKY53*

It has previously been reported that AtWRKY53 expression can be induced by pathogen attack, hydrogen peroxide and salicylic acid (SA) (Miao et al. 2004; Miao and Zentgraf 2007; Murray et al. 2007). To further clarify the potential functions of WRKY53, we examined its expression profiles more precisely. First, we examined the basal expression of *AtWRKY53* by quantitative real-time reverse transcriptase PCR (qRT-PCR). As shown in Fig. 1a, while high levels of *WRKY53* transcripts were observed in the root, weaker expression was observed in the leaf, stem and silique. *WRKY53* transcripts were barely detected in the flowers.

To further investigate the tissue-specific expression of *AtWRKY53* in detail, we prepared transgenic pants harboring a Pro*WRKY53-GUS* construct and examined the *WRKY53* promoter activity by histochemical GUS staining. In accordance with the results of qRT-PCR, GUS activity was observed in leaf, root, silique and stem, while no GUS activity was detected in corolla, androecium and gynoecium (Fig. 1b).

We also measured the induced expression of *AtWRKY53* in response to certain abiotic stresses and the results showed that *AtWRKY53* expression was strongly up-regulated by dehydration and other osmotic stressors, including high salinity, PEG6000 and mannitol (Fig. 1c). To confirm whether the level of *AtWRKY53* protein expression corresponded to its mRNA abundance under drought stress, we generated the transgenic line (*wrky53* background) expressing AtWRKY53 fused to a c-myc tag under the regulation of the native promoter (2461 bp). A western blot assay demonstrated that AtWRKY53 protein was significantly induced within 1 h of drought treatment (Fig. 1d). These results indicated that AtWRKY53 is an early factor in drought response.

Phenotypic analysis of the *AtWRKY53* T-DNA insertion mutant

As *AtWRKY53* expression was up-regulated in response to mannitol, PEG6000, NaCl, and dehydration treatments (Fig. 1c), which implied that AtWRKY53 may play a role in abiotic stress response. To clarify the underlying role of AtWRKY53 in drought tolerance, a loss-of-function mutant, *wrky53* (SALK_034157), and two complemented lines were used (Supplementary Fig. S1). However, the *wrky53* mutant showed drought sensitivity levels similar to wild-type plants in soil culture (Fig. 2a). We propose that a homolog of AtWRKY53 exists that is able to compensate for the loss of function of AtWRKY53 in *Arabidopsis*. AtWRKY46 (AT2G46400), which belongs to group III of the WRKY transcription factors, also demonstrated up-regulated expression in response to drought treatment and *P. syringae* (Hu et al. 2012; Ding et al. 2014), and has previously been reported to play a

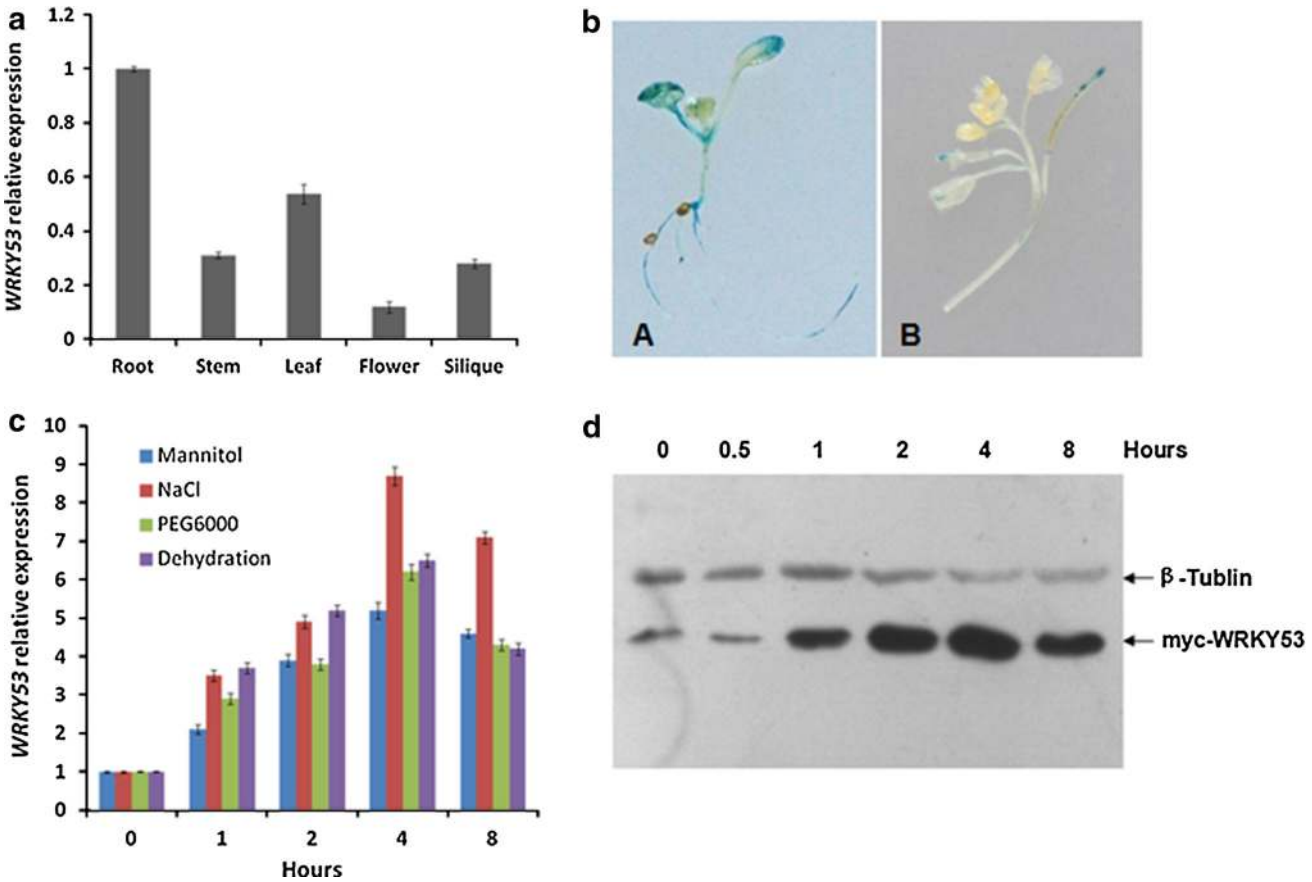

Fig. 1 Expression pattern of AtWRKY53. **a** Tissue specificity of *AtWRKY53* expression. RNA was isolated from various tissues of wild-type plants that were grown under normal conditions, and the *AtWRKY53* expression was determined by qRT-PCR. Relative expression levels compared with the *AtWRKY53* level in roots are presented. Three independent repeats were done with similar results. Data show one experiment. *Error bars* indicate SE ($n = 3$). **b** GUS staining of *AtWRKY53* localization in seedling (*A*), inflorescence and silique (*B*). **c** Expression of *AtWRKY53* in response to multiple treatments. RNAs were extracted from 4-week-old wild-type (Col-0) plants under control and multiple treatments for design time. Gene expression was determined by qRT-PCR using *ACTIN2* mRNA as internal reference. Three independent repeats were done with similar results. Data show one experiment. *Error bars* indicate SE ($n = 3$). **d** Accumulation of myc-WRKY53 fused protein under drought, protein expression was determined by western blot using β-tubulin as internal reference. Three independent repeats were performed with similar results

role in stomatal movement. To confirm whether AtWRKY46 can compensate for the functional loss of AtWRKY53, we obtained a homozygous *wrky46* mutant (Salk_134310, Supplementary Fig. S2), and generated the *wrky46 wrky53* double mutant. However, the single- and double-mutant plants showed no apparent differences in survival rate compared with Col-0 after drought treatment (Fig. 2b). The result was in agreement with that reported in the literature (Ding et al. 2014) which described that there was no significant difference in survival rate after drought treatment between AtWRKY46 T-DNA insertion mutant plants and wild-type plants even though the shoot biomass of mutant plants were more sensitive to drought and salt stress.

Overexpression of AtWRKY53 leads to hypersensitivity to drought

To further investigate the role of AtWRKY53 in dehydration tolerance, two *AtWRKY53* overexpression lines (53OV-3 and 53OV-5) driven by the CaMV35 promoter were used (Fig. 3a). The 53OV-3, 53OV-5 and wild-type (Col-0) seeds were germinated simultaneously and then planted in soil. Four weeks after germination, the plants were subjected to drought (water was withheld; Fig. 3b). After 9 days without watering, the 53OV plants exhibited severe dehydration symptoms, whereas the Col-0 plants showed only mild dehydration symptoms. After 11 days, all plants were re-watered. None of the 53OV plants

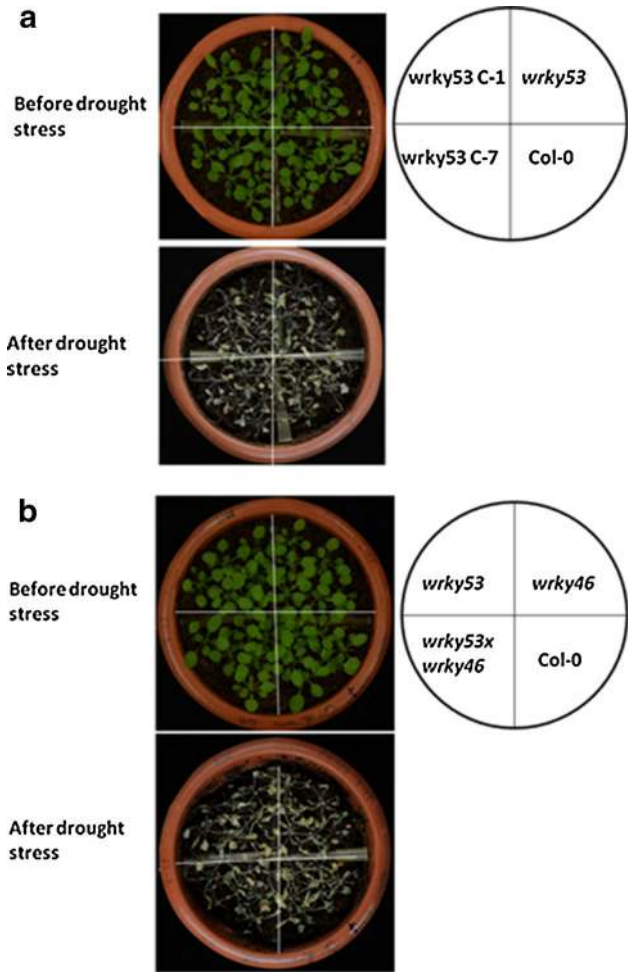

Fig. 2 T-DNA insertion mutants drought tolerance assay. **a** Drought stress was imposed on 4-week-old *wrky53* mutant and complementary lines. **b** Drought stress was imposed on 4-week-old single (*wrky53* and *wrky46*) and double (*wrky53 wrky46*) mutants

survived, but all the Col-0 plants survived. These results suggested that the 53OV plants were hypersensitive to drought stress relative to the Col-0 plants.

In response to osmotic stress, plants are able to control their water content and reduce water loss. We explored the involvement of water balance regulation in the observed drought-stress phenotype of the 53OV lines. To monitor the rate of water loss, rosettes were detached and their fresh weight changes were measured over 3.5 h. Leaves of the wild-type plants exhibited significantly lower water loss than those of the 53OV plants (Fig. 3c). These results indicated that the increased rate of water loss was attributable to increased dehydration in 53OV lines.

ABA-induced stomatal closing is impaired in 53OV lines

Stomata serve as a major gateway for transpiration-based water loss in plants. In response to drought stress, stomata often close to limit water loss. Usually, the phytohormone ABA induces stomatal closure in the dehydration state. We first studied the leaf abaxial epidermis with a microscope and found no significant difference in stomatal density or guard cell size between the 53OV lines and Col-0 plants under natural growth conditions (data not shown). We next investigated the sensitivity of the guard cells to ABA, using epidermal peels of 53OV lines and Col-0 incubated in a buffer solution under strong light conditions for 3 h to fully open the stomata. The peels were then treated with 5 μM ABA for 2 h (Pei et al. 1997). The ratio of stomatal length to width was calculated to determine the degree of stomatal closure. For Col-0 plants, this ratio was only slightly greater than for 53OV plants when exposed to light. After treatment with ABA, the stomata of the 53OV lines opened wider than those of Col-0 (Fig. 4a, b). These results suggested that the more rapid water loss observed in the overexpression lines could mainly be ascribed to impaired stomatal closure.

Drought stress can cause increased accumulation of ABA, which plays a critical role in stomatal movement. To determine whether ABA levels were affected, we quantified the ABA content in the different genetic plant lines tested. However, no significant differences were observed in the endogenous ABA level between the 53OV lines and Col-0 (Fig. 4c). These results indicated that the impaired stomatal closure of the 53OV lines was independent of ABA levels.

AtWRKY53 promotes the reduction of H_2O_2 content and starch metabolism in guard cells

Drought and osmotic stress can cause reactive oxygen species (ROS) producing in plant cells; H_2O_2 is one of the most significant of these ROS. As an important second messenger, H_2O_2 plays a key role in stomatal movement and acts downstream of ABA. To investigate the effect of H_2O_2 levels on stomatal movement, we measured H_2O_2 accumulation in guard cells following ABA treatment, using a fluorescent dye, 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). Less H_2O_2 accumulation was observed in 53OV lines compared with Col-0 plants (Fig. 5a, b), suggesting that a reduction of H_2O_2 levels in guard cells probably underlies the impaired stomatal closure in the 53OV lines.

It has been demonstrated that light-dependent starch degradation plays an important role in stomatal opening by promoting the synthesis of malate in guard cells. To investigate the effect of starch and malate content, we isolated guard cell protoplasts of the different genetic plant lines and quantified their starch and malate content. The 53OV lines contained significantly less starch than Col-0

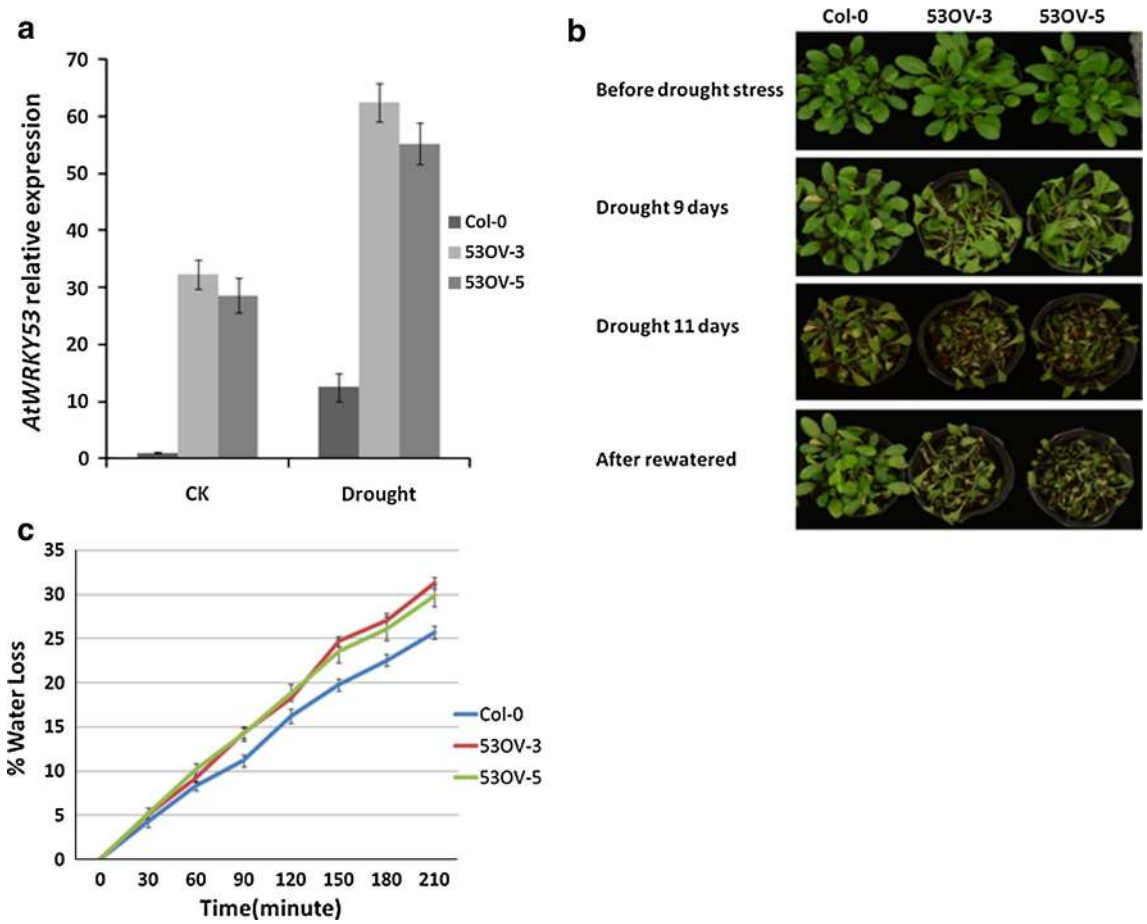

Fig. 3 *AtWRKY53* over expression lines were hypersensitive to drought. **a** Identification of the 53OV lines. *AtWRKY53* expression was detected in Col-0 and 53OV lines under control and drought treatment for 2 h. *Error bars* indicate SE ($n = 3$). **b** Drought tolerance assay of 53OV lines. Drought stress was imposed on 4-week-old seedlings for 11 days. Drought experiments were repeated four times and at least 35 plants for each individual line were used in each repeated experiment and one representative picture was shown. **c** Comparison of the rate of water loss from detached rosettes between 53OV lines and Col-0 plants. *Error bars* indicate SE ($n = 10$ plants)

(Fig. 5c). In contrast, the 53OV lines accumulated more malate in guard cells (Fig. 5d).

In summary, these results indicated AtWRKY53 reduced H_2O_2 levels and promoted starch metabolism in guard cells.

AtWRKY53 up-regulates the expression of *CAT1* and *CAT2* under drought conditions

It has previously been reported that AtWRKY53 expression can be induced by exogenous H_2O_2, which in turn aids the regulation of cellular redox and ROS homeostasis by directly promoting the expression of catalase (Miao et al. 2004). To further confirm the effect of AtWRKY53 on H_2O_2 levels in guard cells through the regulation of catalase expression, we determined the expression level of the three catalase genes, *CAT1* (AT1G20630), *CAT2* (AT1G58030) and *CAT3* (AT1G20620), under drought conditions in the Col-0 and 53OV lines (Fig. 6). The results of qRT-PCR showed all three genes were up-regulated under drought treatment. However, while *CAT1* mRNA levels showed no significant difference between the plant lines, the expression levels of *CAT2* and *CAT3* were higher in the 53OV lines compared with Col-0. These results further suggested activated AtWRKY53 positively up-regulated the expression of *CAT2* and *CAT3* under drought stress.

AtWRKY53 positively regulates the expression of *QQS*

The qua-quine starch (*QQS*, AT3G30720) gene (Li et al. 2009b), which encodes a PUF (protein with unknown functions) with no identified sequence homologs and no predicted structural motifs, has been reported to promote starch metabolism, a process regulated by AtWRKY46 in the guard cells (Ding et al. 2014). As AtWRKY53 also plays a role in stomatal movement by regulating starch

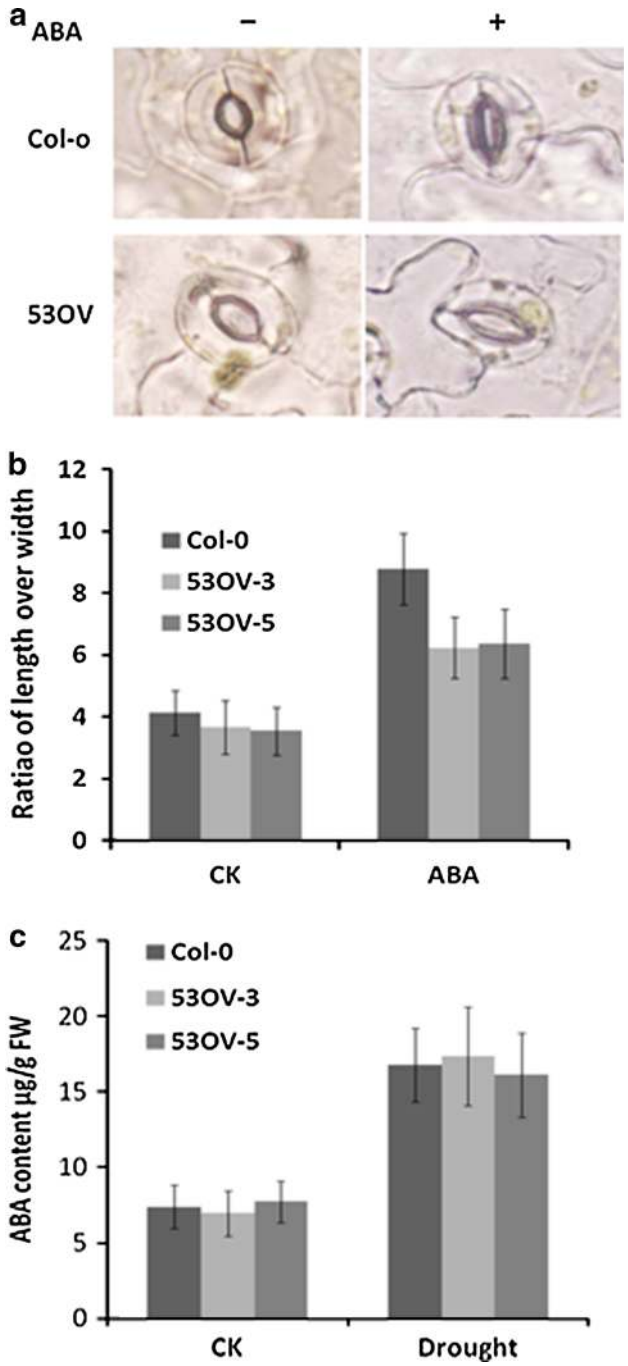

Fig. 4 ABA-induced stomatal closing is impaired in 53OV lines. **a**, **b** Stomatal closure measurement with (CK) or without ABA treatment. The data represent the mean ± SE from 30 stomata measured for each time point, from three independent experiments. Epidermal peels of indicated genotypes were treated with or without ABA for 2 h after stomatal pre-opening under light for 3 h, and the stomatal aperture was measured by microscope. **c** ABA content as determined by ELISA under normal condition (CK) and drought treatment. *Error bars* indicate SE ($n = 3$ experiments, $^*P < 0.05$). *FW* fresh weight

degradation, we analyzed the expression of *QQS* in the Col-0 and 53OV lines to reveal whether AtWRKY53 regulates the expression of *QQS*. Compared with Col-0, higher expression levels were observed in the 53OV lines (Fig. 7a).

It has been reported that AtWRKY53 can regulate the expression of target genes by binding to the typical *cis*-element W-box (TTGACC/T) or the TGAC core sequence in the promoter region of the target gene (Miao et al. 2004). To determine whether *QQS* is directly regulated by AtWRKY53, chromatin immunoprecipitation (ChIP) experiments were conducted using a transgenic Pro*WRKY53-myc-WRKY53* line. Following ChIP with an anti-*c-myc* antibody, enrichment for specific *QQS* promoter fragments in the precipitate was determined by qPCR. Mouse IgG was used as a negative control. ChIP-qPCR revealed that AtWRKY53 was enriched in the *QQS* promoter fraction (Fig. 7b, c). These results suggested that WRKY53 directly up-regulated the expression of *QQS*.

Discussion

WRKY transcription factors function in response to bio/abiotic stress. AtWRKY53 has been reported to play a central role in senescence regulation (Zentgraf et al. 2010). Other studies have indicated that AtWRKY53 also functions as a positive regulator of basal resistance (Miao and Zentgraf 2007; Murray et al. 2007). A recent study implied that the protein product of *TcWRKY53*, a homologous gene of *AtWRKY53* in *Thlaspi caerulescens*, can negatively regulate the osmotic stress tolerance of transgenic tobacco (Wei et al. 2008). According to our research, the transcripts and protein products of *AtWRKY53* accumulate rapidly under drought conditions (Fig. 1c, d), indicating that *AtWRKY53* is a drought stress response factor.

Multiple factors affect the movement of stomatal. Low CO_2 concentrations, high light intensity and high humidity can all induce stomatal opening, whereas stomatal closure is promoted by high CO_2 concentrations, darkness, drought and the plant hormone, ABA (Shimazaki et al. 2007). ABA is closely involved in abiotic stress responses. Drought can dramatically stimulate de novo ABA biosynthesis. We analyzed the mRNA levels of several genes involved in ABA biosynthesis, such as *ABA1*, *ABA2*, *ABA3* and *NCED3*. However, none of these genes showed significantly different expression levels between the *wrky53* mutant, Col-0 and 53OV lines (data not shown). We next quantified the ABA content in the different genetic plant lines tested, and again, there were no significant differences

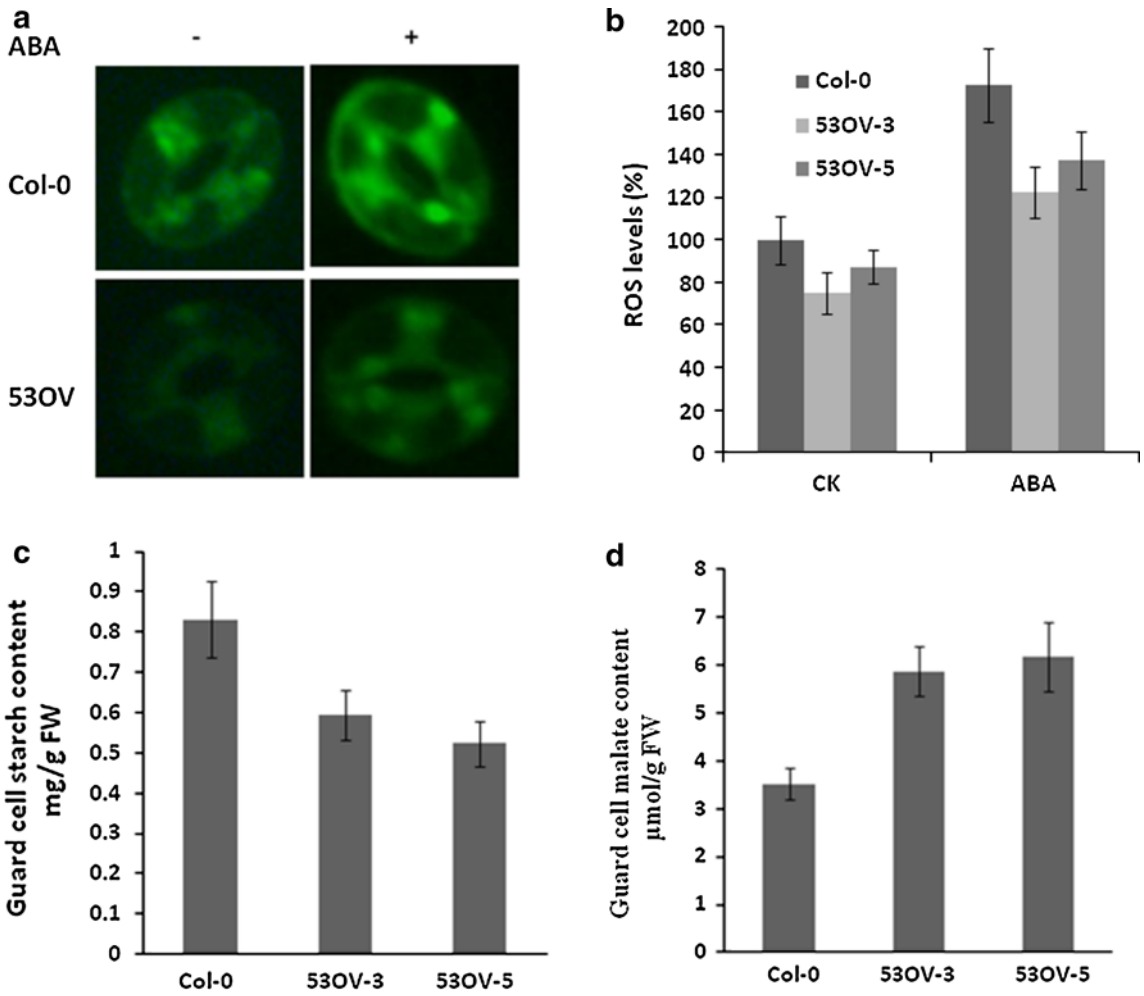

Fig. 5 AtWRKY53 promotes the reduction of H_2O_2 content and starch metabolism. **a** ROS accumulation in guard cells of Col-0 and 53OV line by using a fluorescence dye, 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA). **b** Quantification of ROS levels in guard cells of Col-0 and 53OV lines with (CK) or without ABA treatment. The fluorescent intensity of guard cells in Col-0 before ABA treatment was taken as 100 %. Three independent experiments were done with similar results. *Error bars* indicate SE ($n = 30$, $^*P < 0.01$). **c** Comparison of starch content in the guard cells of Col-0 and 53OV lines. Three independent repeats were done with similar results. *Error bars* indicate SE ($n = 3$, $^*P < 0.01$). **d** Comparison of malate content in the guard cells of Col-0 and 53OV lines. Three independent repeats were done with similar results. *Error bars* indicate SE ($n = 3$, $^*P < 0.01$)

in the endogenous ABA levels between these (Fig. 4c). These results suggested that the role of AtWRKY53 in drought stress response is independent of ABA. H_2O_2 plays a key role in stomatal movement and acts downstream of ABA. Our data showed that less H_2O_2 accumulation was observed in 53OV lines compared with Col-0 plants (Fig. 5a, b). The results of qRT-PCR indicated that AtWRKY53 positively up-regulated the expression of *CAT2* and *CAT3* under drought stress (Fig. 6). These data suggested that AtWRKY53 may play a role in the inhibition of stomatal closing by reducing the H_2O_2 content.

Light-dependent starch degradation plays an important role in stomatal opening by promoting the synthesis of sucrose and malate in guard cells (Vavasseur and Raghavendra 2005; Shimazaki et al. 2007). We also found that the 53OV lines accumulated less starch but more malate in guard cells than Col-0 plants (Fig. 5c, d). These results suggested that AtWRKY53 may affect the degradation of starch in guard cells. It has been reported that *QQS* is involved in stomatal opening by promoting the degradation of starch in guard cells (Ding et al. 2014). Indeed, qRT-PCR and CHIP-qPCR results showed that AtWRKY53 directly activated *QQS* expression (Fig. 7). Therefore, all of these results indicated that AtWRKY53 may plays a dual role in stomatal movement, on one hand through preventing stomatal closing by reducing H_2O_2 content, and on the other hand, through keeping stomata open by promoting starch degradation.

To confirm the role of AtWRKY53 in stomatal movement, we investigated whether *AtWRKY53* was expressed

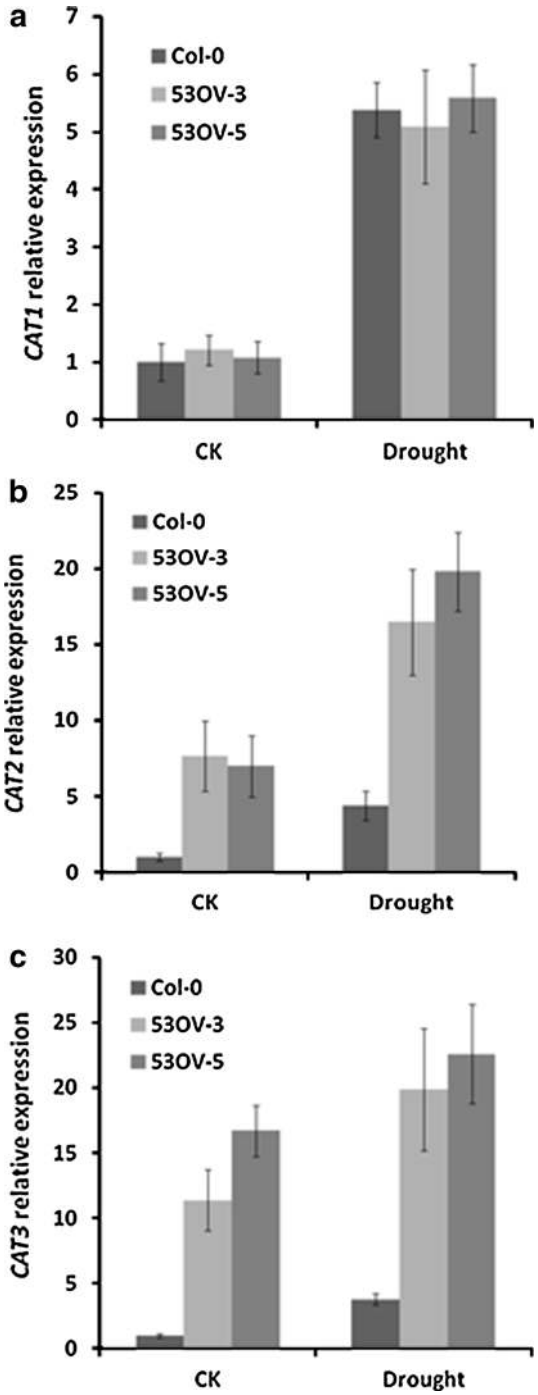

Fig. 6 Expression of *WRKY53* downstream genes under drought treatment and normal condition (CK). RNAs were extracted from the roots of 3-week-old Col-0 and 53OV plants. *ACTIN2* was used as internal reference gene. Three independent repeats were done with similar results. *Error bars* indicate SE ($n = 3$)

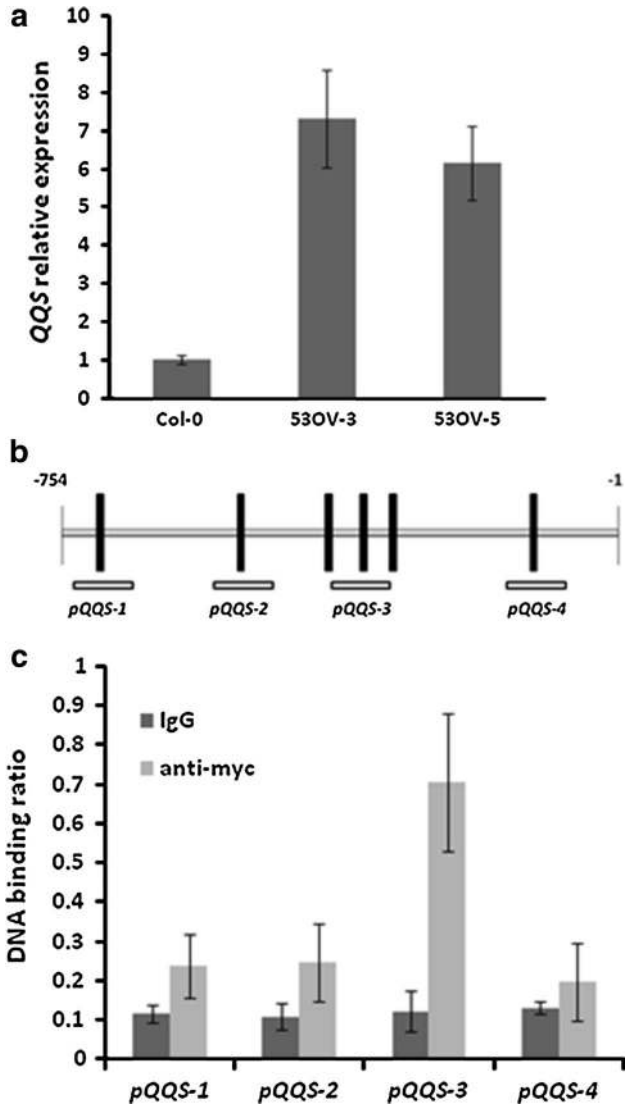

Fig. 7 AtWRKY53 involves starch metabolism in guard cells by directly regulating *QQS*. **a** Expression of *QQS* in Col-0 and 53OV lines. *ACTIN2* was used as internal reference gene. Three independent repeats were done with similar results. *Error bars* indicate SE ($n = 3$). **b** The promoter structure of *QQS*. The 754-bp length *QQS* promoter is indicated by the *gray column*; *black columns* indicate TGAC core sequences present in the promoter; *white columns* indicate the genomic DNA fragments from the *QQS* promoter that were selected for amplification during the ChIP assay. **c** ChIP analysis showing the in vivo binding of AtWRKY53 to the *QQS* promoter; 3-week-old *ProWRKY53-myc-WRKY53* seedlings were used for the ChIP analysis. IgG was used as a negative control. Three independent repeats were performed. *Error bars* indicate SDs ($n = 3$)

in the guard cells. We generated GUS reporter lines using the *AtWRKY53* native promoter and determined that this protein is indeed expressed in guard cells (Supplementary Fig. S3). However, after drought treatment, the expression of AtWRKY53 was observed not only in guard cells, but also in mesophyll cells and vascular tissue (Supplementary Fig. S4). This result suggested that AtWRKY53 may play other roles in non-guard cells under drought conditions. It is well known that over-accumulation of H_2O_2 under stress conditions can result in cell damage. We analyzed the H_2O_2 content of 3-day-old *wrky53*, Col-0 and 53OV seedlings following osmotic treatment. All seedlings

showed no obvious fluorescence signal under normal conditions, whereas under osmatic stress conditions, H_2O_2 accumulation in cotyledons was decreased (*wrky53* > Col-0 > 53OV) (Supplementary Fig. S5). Our data suggested that AtWRKY53 may play a role in controlling ROS homeostasis to reducing oxidative damage in plant cells under osmotic conditions.

To gain further insight into the functions of AtWRKY53, we generated *wrky53* complemented lines and *wrky53 wrky46* double-mutant plants. However, neither the single mutant plant (*wrky55* or *wrky46*) nor the double-mutant plants showed any differences in survival rate compared with Col-0 after drought treatment (Fig. 2). As the *Arabidopsis* group III WRKY transcription factors is currently thought to comprise 13 members, our data suggested that other factors may exist with possible functional redundancy, and AtWRKY53 may play only a minor role in drought stress response in Col-0.

The molecular mechanisms underlying plant tolerance to drought stress are still not fully understood because of the complex nature of this response. Many genes involved in stomatal opening are down-regulated by drought stress. Nonetheless, a negative regulatory mechanism may exist that promotes stomatal opening and maintains CO_2 influx under drought conditions. In summary, our data demonstrated that the activated expression of AtWRKY53 prevents stomatal closing by reducing H_2O_2 content and maintains stomatal opening by promoting starch degradation.

Author contribution statement Diqiu Yu designed research; Yiding Sun performed research, analyzed data and wrote the paper.

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Conflict of interest The authors declare that they have no conflict of interest.

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