

# Maize *ABP9* enhances tolerance to multiple stresses in transgenic *Arabidopsis* by modulating ABA signaling and cellular levels of reactive oxygen species

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**Abstract** The phytohormone abscisic acid (ABA) and reactive oxygen species (ROS) play critical roles in mediating abiotic stress responses in plants. It is well known that ABA is involved in the modulation of ROS levels by regulating ROS-producing and ROS-scavenging genes, but the molecular mechanisms underlying this regulation are poorly understood. Here we show that the expression of maize *ABP9* gene, which encodes a bZIP transcription factor capable of binding to the ABRE2 motif in the maize *Cat1* promoter, is induced by ABA, H<sub>2</sub>O<sub>2</sub>, drought and salt. Constitutive expression of *ABP9* in transgenic *Arabidopsis* leads to remarkably enhanced tolerance to multiple stresses including drought, high salt, freezing temperature and oxidative stresses. *ABP9* expressing *Arabidopsis* plants also exhibit increased sensitivity to exogenously applied ABA during seed germination, root growth and stomatal closure and improved water-conserving capacity. Moreover, constitutive expression of *ABP9* causes reduced cellular levels of ROS, alleviated oxidative damage and reduced cell death, accompanied by elevated expression of many stress/ABA

responsive genes including those for scavenging and regulating ROS. Taken together, these results suggest that *ABP9* may play a pivotal role in plant tolerance to abiotic stresses by fine tuning ABA signaling and control of ROS accumulation.

**Keywords** Transcription factor *ABP9* · ABA · Reactive oxygen species · Stress tolerance · Gene expression

## Introduction

Plants are frequently exposed to a variety of abiotic stresses such as drought, salt, and extremes of temperature, which constrain plant growth and crop productivity worldwide. Understanding how plants tolerate these stresses is a vital prerequisite for developing strategies to improving plant stress tolerance. Plants sense and adapt to dynamic stresses by altering their genome expression profiling, growth pattern and cellular metabolism and mobilizing various defense mechanisms (Bartels and Sunkar 2005). A common metabolic change provoked by stresses is the accelerated generation/accumulation of reactive oxygen species (ROS) in various cellular compartments (Mittler 2002). While excess amount of ROS can cause oxidative damage to cells during abiotic stresses, recent studies have shown that ROS at low concentrations function as signaling molecules to regulate plant protective stress responses including ABA-induced activation of Ca<sup>2+</sup> channel and stomatal closure and induction of defense gene expression (Desikan et al. 2001; Pei et al. 2000; Rizhsky et al. 2002; Zhang et al. 2001). The dual roles of ROS imply the necessity to tightly control the cellular levels of ROS. Plants possess a ROS gene network encoding components

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of both ROS-producing and ROS-scavenging pathways to secure an appropriate ROS level and signaling while minimizing their toxic effects during environmental stresses (Apel and Hirt 2004; Mittler et al. 2004). Elucidating the molecular mechanisms that regulate ROS homeostasis and signaling in response to abiotic stress could therefore provide useful insights for generating crop plants with improved tolerance to various environmental stress conditions.

The phytohormone abscisic acid (ABA) is critical for plant growth and development and plays a pivotal role in controlling stress responses through regulating a set of ABA/stress-responsive gene expression including ROS network genes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Finkelstein et al. 2002; Jiang and Zhang 2002; Yamaguchi-Shinozaki and Shinozaki 2006; Zhu and Scandalios 1994). Functional dissection of ABA-responsive gene promoters has identified several types of ABA-responsive cis-elements (ABREs) among which the G-box like sequence (C/T)ACGTGGC functions as a strong one (Busk and Pagès 1998). Several ABRE-binding transcription factors such as ABF/AREB/ABI5 have been identified and shown to play important roles in modulating ABA and stress responses (Finkelstein and Lynch 2000; Kim 2006; Uno et al. 2000). However, it is still largely unclear about how ABA signaling regulates the cellular levels of ROS in response to various abiotic stresses.

In maize, CAT genes encode three biochemically distinct isozymes including CAT1, CAT2 and CAT3, which catalyze the decomposition of H<sub>2</sub>O<sub>2</sub> and thus act as a group of major enzymatic scavengers of ROS. It has been shown that a 9-bp G-box-containing sequence (5'-CCACGTGG A-3') in the maize *Cat1* promoter, designated ABRE2, is responsible for ABA-induced *Cat1* expression (Guan and Scandalios 1998; Guan et al. 2000). In our previous work, we isolated an ABRE2-binding factor, named *ABP9* (for ABRE Binding Protein 9) by using ABRE2 of maize *Cat1* promoter as a bait in yeast one-hybrid screening of a maize cDNA library. We showed that *ABP9* encodes a transcription factor of bZIP family capable of trans-activating the expression of a downstream reporter gene by specific binding to the ABRE2 motif in yeast cells (Wang et al. 2002). Further, we showed that *ABP9* expression improves the photosynthetic capacity of transgenic *Arabidopsis* plants under stress conditions (Zhang et al. 2008). In this study, we show that *ABP9* overexpressing transgenic *Arabidopsis* plants exhibit enhanced tolerance to various abiotic stresses, including drought, salt, freeze temperature and oxidative stress. Our results suggest that *ABP9* plays a critical role in regulating ABA responses and modulation of the cellular levels of ROS.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis* plants were grown aseptically in a growth chamber (22°C, ~120 μmol photons m<sup>-2</sup> s<sup>-1</sup> under a 16-h light/8-h darkness photoperiod). Seeds were surface sterilized in 75% ethanol containing 0.05% (v/v) Tween-20 for 10 min followed by three times rinse with 95% ethanol and then plated on half strength Murashige-Skoog (1/2 MS) medium (Sigma-Adrich) (Murashige and Skoog 1962) solidified with 0.8% agar. 1 or 2-week-old seedlings were transferred from plates into pots filled with compost soil. Unless stated otherwise, the 1/2 MS medium was supplemented with 1% sucrose.

Maize (*Zea mays* L.) inbred line Qi319 was used in this work. Before sowing, the seeds were imbibed and incubated on moisture filter paper at 28°C in the dark for 48 h, then were sown in compost soil and grown in a growth chamber (28°C, 12-h light/12-h darkness photoperiod).

### Electrophoretic mobility shift assays

To prepare *ABP9* recombinant protein, the full-length cDNA of *ABP9* (Genebank accession No. GU237073) was PCR amplified using anchor primers from pPC86-*ABP9* (Wang et al. 2002). The PCR fragment with *Xba*I and *Xho*I restriction sites at 5' and 3' ends, respectively, was cloned into pGEM<sup>®</sup>-T Easy (Promega) vector resulting in pGEM<sup>®</sup>-T Easy-*ABP9*. Then the full-length cDNA of *ABP9* was released by *Xba*I digestion of pGEM<sup>®</sup>-T Easy-*ABP9* and blunt-ended with Klenow enzyme followed by *Not*I digestion, and then cloned in frame into *Sma*I/*Not*I-digested prokaryote expression vector pGEX-4T-1 (GE Healthcare) to generate the construct pGEX-4T-1-*ABP9*. pGEX-4T-1-*ABP9* was transformed into the *Escherichia coli* (*E. coli*) strain BL21 cells to produce a GST fusion protein. The GST-*ABP9* fusion protein was purified using MicroSpin<sup>®</sup> GST purification module (GE Healthcare). The 19 bp oligos containing ABRE2 (5'-GAAGTCCACGTGGAGGTGG-3') or its mutant form mABRE2 (5'-GAAGTaatcatgttcGGTG G-3') was annealed and labeled with [ $\gamma$ -<sup>32</sup>P]-ATP using DNA 5' End-Labeling system (Promega). Binding reaction containing 4 μg purified *ABP9* protein, labeled probe DNA (20,000–50,000 cpm), 25 mM HEPES-KOH (pH 8.0), 50 mM KCl, 10% glycerol, and 1 mM dithiothreitol in 20 μl volume was incubated on ice for 15 min. When performing a competition assay, unlabeled probes were added 30 min prior to the addition of the <sup>32</sup>P-labeled probes. Binding reactions were separated on 5% non-denaturing PAGE gels in 0.5× Tris-borate-EDTA buffer. After

electrophoresis, gels were dried and visualized by autoradiography.

#### Trans-activation assay in maize cells

To prepare the effector plasmid pAHC25-ABP9, the full-length cDNA of *ABP9* was isolated from pGEM<sup>®</sup>-T Easy-ABP9 by *XbaI/XhoI* double digestion, followed by blunt-ending with Klenow enzyme, then cloned into the *SmaI/Ecl136II* sites of the vector pAHC25 to replace the *GUS*-coding region. To construct the reporter plasmid pIGA, pIG46 (Ono et al. 1996), which consists of the CaMV 35S minimal promoter and *GUS* gene, digested with *XhoI* and filled-in with Klenow, was ligated with 4 copies of ABRE2. Effector plasmid pAHC25-ABP9 and reporter plasmid pIGA were co-transformed into maize Black Mexican Sweet (BMS) suspension cells by biolistic method. Briefly, the cells maintained in 50 ml liquid MS medium (Sigma–Aldrich) supplemented with 2.0 mg l<sup>-1</sup> 2,4-D and 3% sucrose were subcultured into fresh culture and treated with 3% PEG 8000 at 26–28°C in the dark on the shaker of 150 rpm for overnight before bombardment. The plasmids were introduced into osmotic-treated cells on the glass microfibre filters (Whatman, GF/A) using 1100 psi rupture pressure. After bombardment, the glass microfibre filters with the bombarded cells were transferred to MS agar medium and cultured at 26–28°C in the dark for 2 days. In situ assay of GUS activity was determined as described (Jefferson et al. 1987).

#### Generation of *Arabidopsis* transgenic plants

The full-length cDNA of *ABP9* was isolated by *XhoI*-digestion of pGEM<sup>®</sup>-T Easy-ABP9 and blunt-ended by the Klenow enzyme, followed by *XbaI* digestion. The cDNA fragment was then cloned into *XbaI* and *Ecl136II* double digested pBI121 vector to generate the pBI121-ABP9 plasmid, in which *ABP9* is under the control of the cauliflower mosaic virus 35S promoter (CaMV 35S). pBI121-ABP9 was introduced into the *Agrobacterium tumefaciens* strain GV3101 through electroporation and used to transform *Arabidopsis thaliana* (ecotype Columbia) using a floral dip method (Bechtold et al. 1993). Transgenic plants were selected by kanamycine resistance and confirmed by PCR analysis. Two representative T3 homozygous lines (5P2 and 5P3) with different expression level of *ABP9* were chosen for detailed analyses.

#### Stress tolerance assays

One-week-old young seedlings aseptically grown on 1/2 MS agar medium were transferred into pots filled with the same amount of compost soil and grown for another 1 or

2 weeks before stress treatments were applied. For drought tolerance assay, soil-grown plants were fully watered, and then withheld irrigation for 4 weeks, followed by re-watering plants. Survival rates were scored 1 week after re-watering. For salt tolerance assay, soil-grown plants were treated with progressively applied high salt stress by irrigating plants with NaCl solutions of stepwisely increasing concentrations (50, 100, and 200 mM) every 4 days and lasting at the concentration of 200 mM for 12 days when chlorophyll contents were measured according to the method (Lichtenthaler 1987). Cold treatment was performed by transferring the plants into a low temperature incubator for desired durations (–4°C, Sanyo, MIR-253). After 7 days-recovery under normal growth conditions, the survival rates were recorded. For oxidative stress treatment, leaves of similar developmental stages (7th and 8th rosette leaves) were detached from 3 or 4 week-old plants aseptically grown on 1/2 MS agar medium and floated abaxial side up in 2 μM methyl viologen (MV, Sigma–Aldrich) solution under controlled growth conditions. Chlorophyll contents of the detached leaves were measured as described (Lichtenthaler 1987).

#### ABA response and water loss assays

For germination assay, seeds were surface-sterilized and sown on MS medium (3% sucrose and 0.8% agar, pH 5.7) supplemented with or without different concentrations of ABA (Sigma–Aldrich). Seeds were stratified at 4°C for 3 days before growing at 22°C under light conditions. To score seed germination, the percentages of seeds with fully emerged radicles were determined in three independent experiments.

For root growth assay, seeds were germinated after stratification and grown for 48 h on ABA-free MS medium, seedlings were then transferred to MS medium supplemented with different concentrations of ABA and grown vertically. The primary root lengths were measured 10 days after the transfer. The root growth ratio refers to the root length on ABA medium normalized to that on ABA-free medium.

Stomatal aperture assays were performed essentially as previously described (Pei et al. 1997). Briefly, detached rosette leaves were first floated in a buffer containing 20 mM KCl, 5 mM Mes-KOH, pH 6.15, and 1 mM CaCl<sub>2</sub> at 150 μmol photos m<sup>-2</sup> s<sup>-1</sup> for 2 h followed by addition of 10 μM ABA. 2 h later, apertures were recorded on epidermal strips using a digital video camera (AxioCam HR) attached to an inverted microscope (Leica DMR) equipped with image analysis software (Axio Vision 4.0).

For water loss assay, detached rosette leaves of the same developmental stage were placed abaxial side up in open

Petri dishes and left on the lab bench. The fresh weight was monitored at the indicated time points.

#### Detection of ROS and cell death

A histochemical staining procedure was used to detect superoxide and hydrogen peroxide in situ as described (Fryer et al. 2002). Rosette leaves sampled from seedlings with or without ABA or NaCl treatment were infiltrated with nitroblue tetrazolium (NBT) (Amresco, 1 mg ml<sup>-1</sup> NBT in 10 mM sodium azide and 10 mM phosphate buffer, pH 7.8) or 3, 3'-diaminobenzidine (DAB) solutions (Sigma–Aldrich, 1 mg ml<sup>-1</sup> DAB-HCl, pH 3.8), respectively. The stained leaves were imaged after removal of chlorophylls by boiling in 96% ethanol for 10 min. Cell death was examined by Evans Blue staining as described (Kim et al. 2003) with minor modifications. Briefly, detached leaves from plants untreated or treated with 50 mM NaCl for 1 h were vacuum-infiltrated in 0.1% Evans Blue (w/v; Sigma–Aldrich) for 15 min and then kept under vacuum for 6 h, then washed three times (15 min each) with a phosphate-buffered saline containing 0.05% (v/v) Tween 20 followed by boiling in 96% ethanol for 10 min to remove chlorophylls.

ROS production in intact guard cells was detected using 2, 7-dichlorofluorescein diacetate (H<sub>2</sub>DCF-DA, Fluka) as described (Pei et al. 2000). Epidermal peels were floated in an incubation buffer (30 mM KCl, 10 mM Mes-KOH, pH 6.15) for 2 h under the light of 150 μmol photons m<sup>-2</sup> s<sup>-1</sup> at 22°C to induce stomatal opening and then loaded with 50 μM H<sub>2</sub>DCF-DA. After 10 min of dye loading, the epidermal strips were washed three times with the incubation buffer. For ABA treatment, 10 μM ABA was added to the incubation buffer and the epidermal strips were further incubated for 2 h before dye loading. Guard cells were visualized by confocal laser microscope (Leica TCS SP2) with excitation at 488 nm and emission at 525 nm and images were acquired using the Leica Confocal Software version 2.6 Build 1537. The DCF fluorescence emission of guard cells was quantified using the Leica Qwin software.

Determination of cellular levels of ROS by Electron Spin Resonance (ESR) spectroscopy was performed essentially according to the protocol as described (Capani et al. 2001) with minor modifications. Briefly, plants of the same developmental stage grown aseptically on 1/2 MS agar medium were collected, weighed and homogenized on ice in 600 μl ice-cold spin trap solution containing 100 mM α-phenyl-N-tert-butyl nitron (PBN, Sigma–Aldrich) and 2 mM diethylenetriamine-pentacetic acid (DPTA) in phosphate-buffered saline (PBS, pH 7.4). After homogenization, 250 μl ethyl acetate were added, vortexed for 30 s and centrifuged at 12,000 rpm for 15 min at 4°C for complete phase separation. Then the ethyl acetate phase

was transferred to a clean tube for ESR measurement. The Bruker 200D SRC ESR spectrometer was set as followings: center field, 3385 Gauss; scan width, 400 Gauss; modulation amplitude, 3.2 G; microwave power, 20 mW; receiver gain, 4.0 × 10<sup>5</sup>.

#### RNA analysis

Reverse transcriptase-coupled PCR (RT-PCR) analysis was performed to analyze *ABP9* expression in Maize. For dosage analysis of NaCl, H<sub>2</sub>O<sub>2</sub> and ABA effects, roots of soil-grown maize seedlings with three fully expanded leaves were submerged after the removal of soil into the NaCl, H<sub>2</sub>O<sub>2</sub> and ABA solutions of different concentrations for 3 days and 24 h, respectively. While for time course analysis, plants were put into NaCl, H<sub>2</sub>O<sub>2</sub> and ABA solutions of given concentrations for 12 h and sampled at the desired time points. Drought treatment was performed by transferring plants to pots with different soil water contents (SWC) for 3 days. Pots were covered with saran wrap and weighed daily to maintain the SWC of 13% (0.13 g H<sub>2</sub>O g<sup>-1</sup> dry soil), 10% (0.10 g H<sub>2</sub>O g<sup>-1</sup> dry soil) and 8% (0.08 g H<sub>2</sub>O g<sup>-1</sup> dry soil). For dehydration treatment, plants after the removal of soil were kept on tissue paper and left on bench for 6 h. Plants grown on soil without treatment were used as control 1 (CK1), and plants treated with distilled water were used as control 2 (CK2). Total RNAs were isolated from samples treated with different stresses using RNAgents<sup>®</sup> Total RNA Isolation System (Promega). RNA samples were reverse transcribed with SuperScript<sup>™</sup> II Reverse Transcriptase (Invitrogen). PCR was conducted at linearity phase of the exponential reactions with *ABP9*-specific primer pairs as follows: forward 5'-catgacgctggaggacttct -3' and reverse 5'-ttgacgaaacacagagc-3'. Transcripts of maize *actin1* (GenBank accession No. J01238) amplified with specific primers (forward 5'-cactctctacaacgagctccg-3' and reverse 5'-taatcaaggccaa cgtaggca-3') were used as the internal control.

RNA gel blotting was performed to analyze the expression of *ABP9* in transgenic *Arabidopsis* plants. Total RNA was isolated from 3-week-old seedlings untreated or treated with 200 mM NaCl for 1 h and 2 h, 100 μM ABA for 3 h or 30% PEG for 3 h using Trizol reagent (Sigma–Aldrich), then was subjected to electrophoresis on formaldehyde/agarose gel and transferred to Zeta-probe nylon membrane (Bio-Rad) according to standard protocols. The amount of ethidium bromide stained rRNA was used as the control for RNA loading. RNA blots were probed with the <sup>32</sup>P-labeled *ABP9* full length cDNA (Prime-a-Gene<sup>®</sup> Labeling System, Progema). Hybridization was performed according to the method of Church and Gilbert (Church and Gilbert 1984).

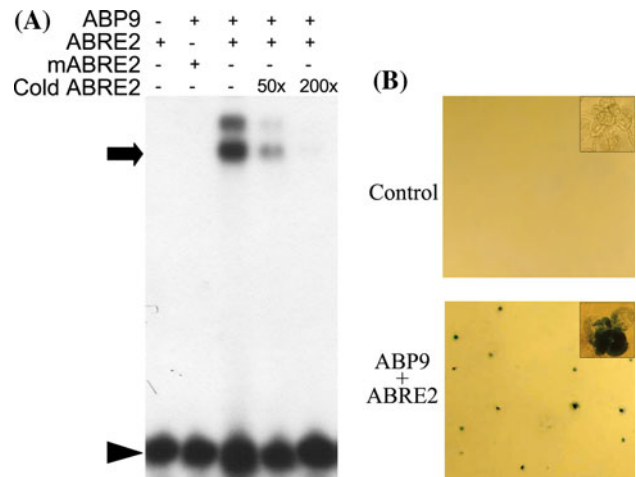
Real-time quantitative reverse transcriptase-PCR (qRT-PCR) was performed to validate the expression changes of *ABP9*-regulated genes in *ABP9* transgenic *Arabidopsis* plants. The first-strand cDNA was synthesized using 1  $\mu$ g of total RNA and ReverTra Ace- $\alpha$ <sup>®</sup> (Toyobo) kit. PCR amplification was done in a 20  $\mu$ l reaction volume containing primers, SYBR Premix Ex Tag<sup>™</sup> (TaKaRa) and diluted cDNA templates on a DNA Engine Optico 2 thermal cycler (MJ Research). Gene-specific primer pairs used here were listed in Supplementary material 6. The cycle threshold ( $C_T$ ) values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined and the relative fold differences were calculated by  $2^{-\Delta\Delta C_T}$  method using the *actin2* gene as an endogenous reference and the wild type as a calibrator (Livak and Schmittgen 2001). Samples were run in triplicate on each 96-well plate and were repeated three times for each experiment. Two experiments on independently grown plant materials were performed to confirm the reproducibility of the results.

## Results

*ABP9* encodes a transcription activator that directly binds in a sequence-specific manner to ABRE2 of maize *Cat1* promoter in vitro and trans-activates gene expression via specific interaction with ABRE2 in plant cells

We previously showed that in yeast cells *ABP9* was able to trans-activate a reporter gene expression through specific binding to the ABRE2 motif of maize *Cat1* promoter (Wang et al. 2002). To test whether *ABP9* can specifically in vitro bind to ABRE2, we conducted an electrophoretic mobility shift assay (EMSA) using purified GST-*ABP9* fusion protein and radio-labeled ABRE2 or mABRE2 as the probes. As shown in Fig. 1a, the mobility of radio-labeled wild-type ABRE2 was retarded by GST-*ABP9* (not by GST itself, data not shown) and this interaction was disrupted by competition of unlabeled ABRE2. In contrast, GST-*ABP9* failed to change the mobility of radio-labeled mABRE2. This result indicates that *ABP9* indeed physically binds to ABRE2 and the binding is sequence-specific. Interestingly, two shift bands appeared in the *ABP9*-ABRE2 interaction, suggesting that *ABP9* may function as a homo-dimer.

We next tested whether *ABP9* can activate gene expression through binding to ABRE2 in plant cells. We co-transformed an effector construct (pAHC25-*ABP9*) in which *ABP9* is driven by an ubiquitin promoter and a reporter plasmid (pIGA) where *GUS* is under the control of ABRE2-35S mini chimeric promoter, into maize BMS

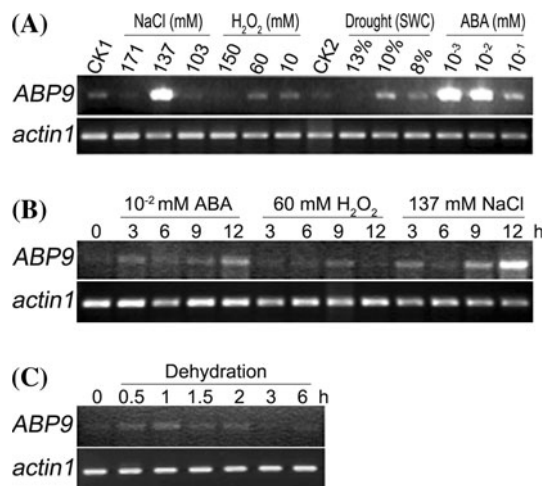


**Fig. 1** ABRE2-binding specificity and trans-activation activity of *ABP9*. **a** EMSA assay showing that *ABP9* binds specifically to ABRE2. Purified *ABP9* protein was added to reactions containing <sup>32</sup>P-labeled ABRE2 or mABRE2 probe with or without unlabeled probe (cold ABRE2). The unlabeled ABRE2 as competitors were added as 50 or 200 times of the labeled ABRE2 probe. Arrow marks the *ABP9*-ABRE2 binding complex, arrowhead indicates free probe. **b** Histochemical assay of trans-activation activity of *ABP9* in maize suspension cells. The *GUS* reporter construct containing ABRE2 in the promoter was co-transformed by bombardment into maize cells with *ABP9* effector plasmid (lower panel) or with empty vector as the background control (upper panel) and stained with X-Gluc buffer. The insets within the panels show a magnified picture of stained cells

suspension cells. Histochemical staining assay showed that the *GUS* reporter gene was activated in maize cells by *ABP9* via interaction with ABRE2 motif in the chimeric promoter (Fig. 1b). These data clearly indicate that *ABP9* also functions as an ABRE-dependent transcription activator in plant cells.

Expression of *ABP9* in maize is inducible by abiotic stresses and ABA

To determine whether *ABP9* is regulated by abiotic stresses and ABA, we performed semi-quantitative RT-PCR with maize seedlings treated with different stresses or ABA. Compared to well-watered soil-grown (CK1) or water-treated (CK2) maize seedlings, the expression of *ABP9* is significantly induced by treatments with medium levels of NaCl (137 mM) or ABA ( $10^{-3}$  or  $10^{-2}$  mM) and slightly induced by H<sub>2</sub>O<sub>2</sub> (10 and 60 mM) and drought (8 and 10% soil water content) treatments (Fig. 2a). Interestingly, higher levels of NaCl (171 mM), H<sub>2</sub>O<sub>2</sub> (150 mM) and ABA ( $10^{-1}$  mM) were not effective in inducing *ABP9* expression in maize seedlings (Fig. 2a). Next we conducted a time course experiment to examine the kinetics of *ABP9* expression by various abiotic stresses. Induction of *ABP9* by ABA ( $10^{-2}$  mM) and NaCl (137 mM) can be clearly detected 3 h after the treatments, whereas induction by



**Fig. 2** *ABP9* expression in maize is inducible by ABA and abiotic stresses. **a** RT-PCR analysis of *ABP9* expression under NaCl, H<sub>2</sub>O<sub>2</sub>, ABA and drought treatments at different dosages. CK1 and CK2 represent the untreated soil-grown and water-grown control plants, respectively. **b**, **c** Time course examination of *ABP9* expression under treatments of 10 μM ABA, 60 mM H<sub>2</sub>O<sub>2</sub>, 137 mM NaCl (**b**) and dehydration (**c**). Pre-treatments of maize seedlings with ABA and stresses were described in the “Materials and methods” section. Maize *actin1* was used as an internal control. Experiments were repeated at least 5 times with independent biological samples showing consistent results

H<sub>2</sub>O<sub>2</sub> (60 mM) became clear 9 h after the treatment (Fig. 2b). Notably, induction of *ABP9* by dehydration was most obvious during the first 2 h of treatment and became undetectable thereafter (Fig. 2c). These results suggest that *ABP9* may be involved in ABA and abiotic stress responses in plants.

#### Constitutive expression of *ABP9* in *Arabidopsis* leads to enhanced tolerance to multiple stresses

To investigate the function of *ABP9* in plant response to abiotic stress, transgenic *Arabidopsis* plants expressing the *ABP9* cDNA under the control of the CaMV 35S promoter (35S-*ABP9*) were generated and eight independent T3 homozygous lines were obtained. Northern blot analysis revealed that *ABP9* expressing transgenic plants accumulated higher levels of *ABP9* mRNA under either normal growth or stress conditions with the treatment of 200 mM of NaCl (1 or 2 h), 100 μM ABA (3 h) or 30% PEG (3 h), indicating that *ABP9* is constitutively expressed in these transgenic plants (Fig. 3a, b). Two representative lines (5P2 and 5P3), with medium and high expression levels of *ABP9* were selected for this study.

Next, we examined drought stress response of the transgenic plants compared to wild type plants. Drought stress was imposed by continuously withholding water to soil-grown plants. As shown in Fig. 3c, under normal

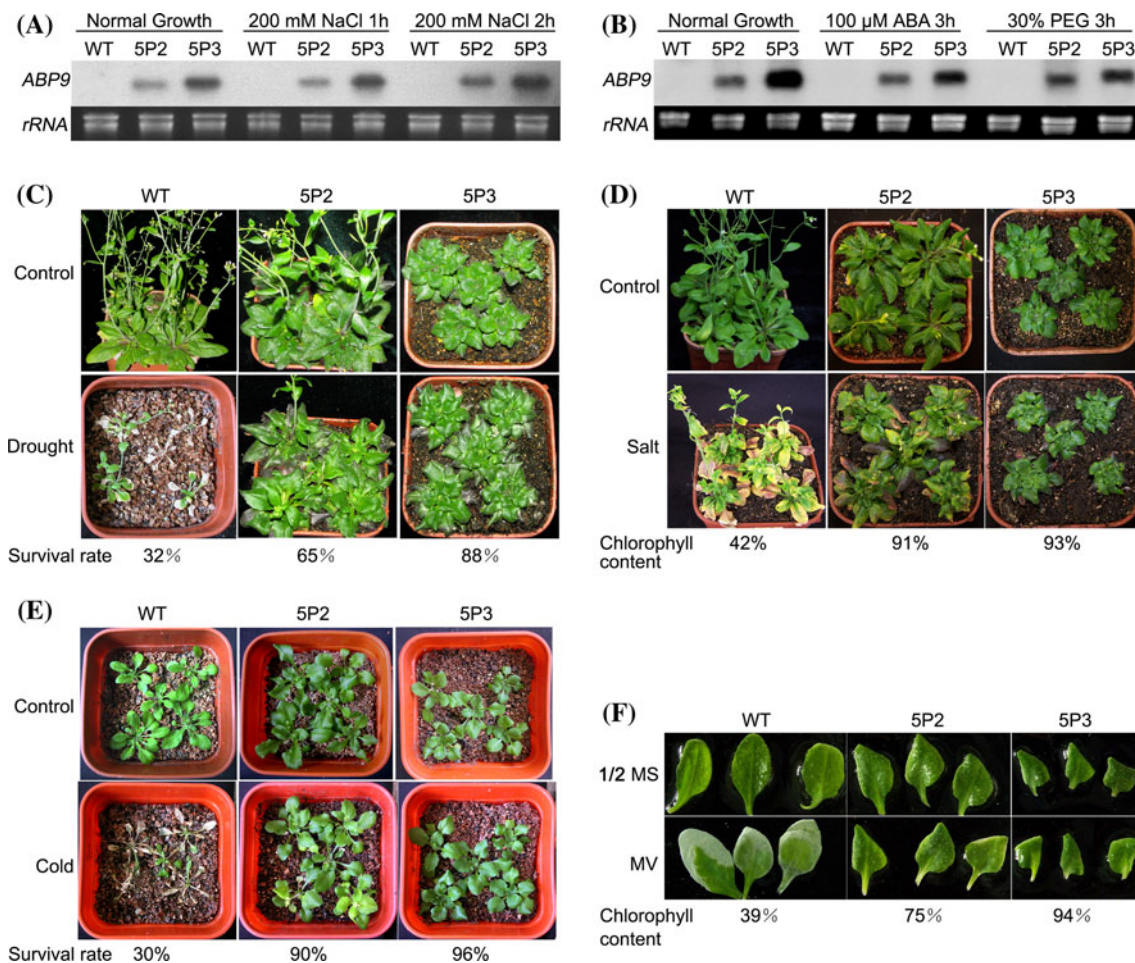
growth conditions, growth and development were somewhat delayed in the *ABP9* overexpressing transgenic line 5P2 and 5P3 and this delay is much more significant in 5P3, in which *ABP9* expression is 2–3 fold higher than in 5P2 (Fig. 3a, b). During 4-week exposure to drought, almost all the 35S-*ABP9* transgenic plants kept growing vigorously, whereas wild-type plants became withered, chlorotic and bleached. After resuming water, 65% of 5P2 and 88% of 5P3 plants survived to maturity. In contrast, more than 60% of wild-type plants were completely dehydrated and failed to recover.

For salt tolerance assay, we examined the performance of transgenic plants under progressively applied and relatively long-term high salt stress conditions. Plants were irrigated with stepwise increasing concentrations of NaCl solution and then lasting at the concentration of 200 mM for 12 days. Most leaves of 35S-*ABP9* plants kept green whereas wild-type leaves gradually became yellow and purple. Measurement of chlorophyll contents by the end of treatment revealed that the chlorophyll contents of 5P2 and 5P3 remained ~91 and 93% of untreated plants, respectively, whereas the chlorophyll content of treated wild-type plants dropped to 42% of untreated plants (Fig. 3d).

For cold tolerance assay, plants were exposed to –4°C freezing temperature for 6 h. We noticed that soon after the cold treatment, wild-type plants all collapsed with leaves showing a “boiled” phenotype in color, while most of the transgenic plants kept their original morphological structure and leaf color. After a recovery period of 7 days under normal growth conditions, at least 90% of the transgenic plants resumed their growth and development, whereas only 30% of wild-type plants survived yet grew unhealthily. The other 70% of wild-type plants failed to recover and died (Fig. 3e).

We also analyzed the oxidative stress tolerance of 35S-*ABP9* transgenic plants by assessing their response to methyl viologen (MV), a herbicide causing chlorophyll degradation and the leakage of cell membrane by generating ROS (Kurepa et al. 1998). Rosette leaves from transgenic and wild-type plants were incubated in 1/2 MS liquid medium containing 2 μM MV. After 72 h of incubation, the wild-type leaves became partially bleached. By contrast, the transgenic leaves remained greener than wild-type ones (Fig. 3f). Quantitative assay revealed that in comparison with mock-treated control leaves, wild-type leaves decreased by 61% of their chlorophyll contents after MV treatment, whereas the transgenic leaves only decreased by 25% (5P2) and 6% (5P3), respectively.

Taken together, these results indicate that constitutive expression of *ABP9* causes enhanced tolerance to drought, salt, low temperature, and oxidative stresses in transgenic plants.



**Fig. 3** Constitutive expression of *ABP9* enhances tolerance to drought, salt, cold and oxidative stresses in transgenic *Arabidopsis*. **a, b** RNA gel blot analysis of *ABP9* expression in wild type (WT) and *35S-ABP9* transgenic plants (5P2 and 5P3) under normal growth and salt stress (**a**), ABA and PEG treatment conditions (**b**). Plants grown on 1/2 MS agar plates were untreated or treated with 200 mM NaCl for 1 h and 2 h, 100 μM ABA or 30% PEG for 3 h. Equal amounts (8 μg) of total RNAs separated by electrophoresis on formaldehyde-agarose gels and rRNA was used as loading control. **c** Drought tolerance in *35S-ABP9* transgenic plants. Intact plants were drought stressed by withholding water for 4 weeks, then were rewatered for 1 week before photograph was taken. Survival rates were calculated from the results of three independent experiments ( $n \geq 30$  for each experiment). Untreated plants grown under normal conditions as control were shown in the *top panels*. **d** Capacity of *35S-ABP9* transgenic plants to tolerate salt stress. The plants were treated with progressively increased concentrations of NaCl solutions ranging from 50 to 200 mM and kept irrigated with 200 mM NaCl for

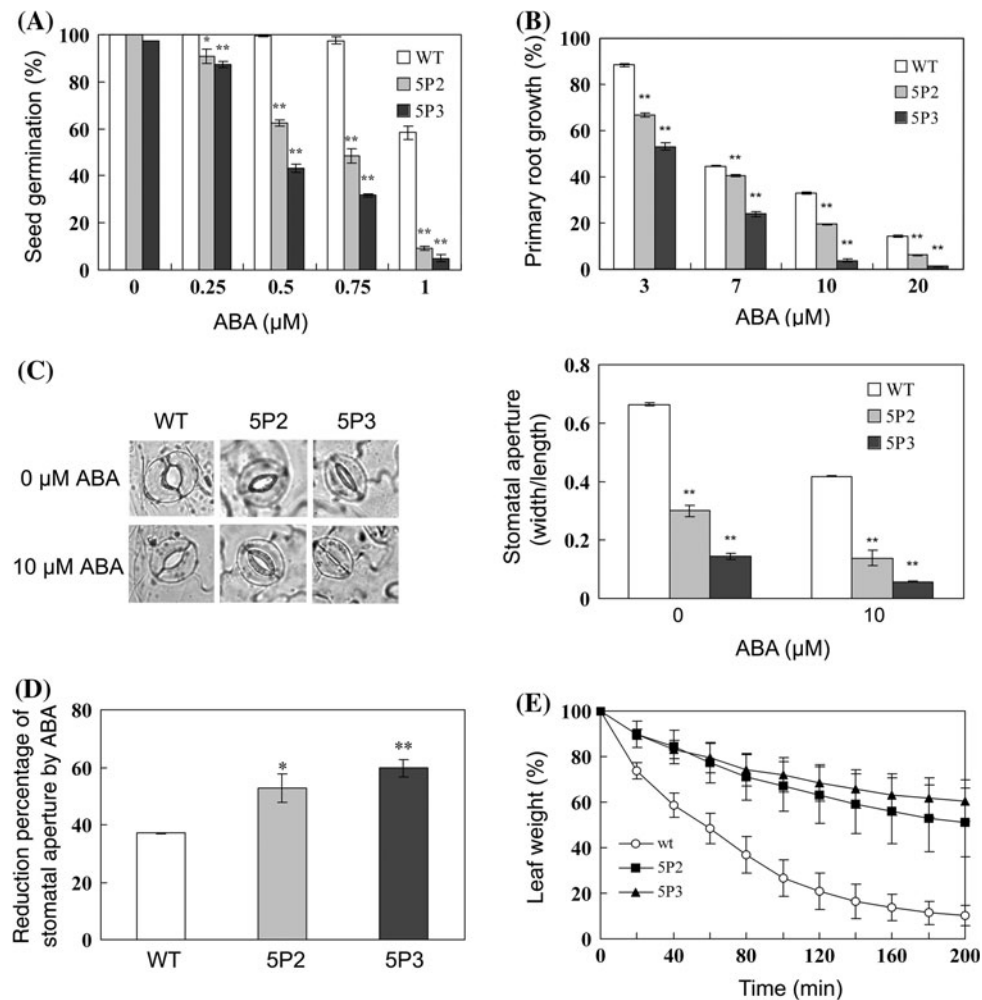
12 days, then chlorophyll contents were determined. Percentage shown are chlorophyll content of salt-stresses plants relative to that of untreated ones from the results of three independent experiments with 5 intact seedlings determined for each experiment. Untreated plants grown under normal conditions as control were shown in the *top panels*. **e** Freeze tolerance of *35S-ABP9* transgenic plants. Plants were exposed to freezing temperature  $-4^{\circ}\text{C}$  for 6 h and recovered for 1 week under normal growth conditions. Survival rates were counted from the results of three independent experiments ( $n \geq 30$  for each experiment). Untreated plants grown under normal conditions as control were shown in the *top panels*. **f** Tolerance to oxidative stress in *35S-ABP9* transgenic plants. Rosette leaves from wild-type (WT) plants and transgenic lines (5P2 and 5P3) were floated in 1/2 MS medium containing 2 μM MV for 72 h before photographs were taken. Chlorophyll contents compared to that of mocked-treated samples are shown at the *bottom*. Shown are representative photographs from 30 seedlings in triplicates

#### Constitutive expression of *ABP9* in *Arabidopsis* enhances ABA sensitivity

Given the facts that *ABP9* is an ABRE2-dependent transcription activator and its expression is induced by ABA, we next examined the effect of *ABP9* on other ABA-mediated responses including inhibition of seed

germination and root growth, promotion of stomatal closure and water conservation in *35S-ABP9* transgenic plants.

In the absence of exogenously applied ABA, the germination percentage of *35S-ABP9* seeds was similar to that of wild-type seeds when scored at 72 h on MS medium. However, compared to wild-type seeds, germination of *35S-ABP9* seed is more sensitive to exogenously applied



**Fig. 4** Constitutive expression of *ABP9* enhances ABA sensitivity and water-conserving capacity of *35S-ABP9* transgenic *Arabidopsis* plants. **a** Germination rates of wild-type (WT) and *35S-ABP9* seeds (5P2 and 5P3) exposed to 0, 0.25, 0.5, 0.75, and 1  $\mu\text{M}$  ABA at 72 h after stratification. Data represent means  $\pm$  SD of three independent experiments with 40 seeds per genotype and experiment. Asterisks indicate significant differences between the WT and 5P2 or 5P3 plants ( $*P < 0.05$ ,  $**P < 0.01$ , Students *t* test). **b** Primary root growth of WT, 5P2 and 5P3 seedlings. The germinated seeds plated in ABA-free MS medium for 48 h after stratification were transferred to plates supplemented with 0, 3, 7, 10 and 20  $\mu\text{M}$  ABA and root elongation was scored after 10 days. Each value is the mean  $\pm$  SD of at least 30 seedlings from three independent experiments. Asterisks indicate significant differences between the WT and 5P2 or 5P3 plants

( $**P < 0.01$ , Students *t* test). **c** Stomatal aperture measurements of wild-type (WT) and *35S-ABP9* transgenic lines (5P2 and 5P3) in response to 0 and 10  $\mu\text{M}$  ABA. Values are means  $\pm$  SD of 75 stomata from three independent experiments. Asterisks indicate significant differences between the WT and 5P2 or 5P3 plants ( $**P < 0.01$ , Students *t* test). **d** Percentage of ABA-induced stomatal closure in (e) of wild-type (WT) and *35S-ABP9* transgenic plants (5P2 and 5P3). Asterisks indicate significant differences between the WT and 5P2 or 5P3 plants ( $*P < 0.05$ ,  $**P < 0.01$ , Students *t* test). **e** Transpirational water loss in wild-type (WT) and *35S-ABP9* transgenic plants (5P2 and 5P3). Leaf weight is expressed as the percentage of initial fresh weight (FW). Each data point represents the mean of duplicate measurements (5 detached leaves for each measurement)

ABA (concentrations ranging from 0.25 to 1  $\mu\text{M}$ ), and the sensitivity is dependent on the level of *ABP9* expression (Fig. 4a). Measurement of root length showed that the *ABP9* transgenic plants exhibited a greater reduction in response to exogenously applied ABA, in comparison with wild-type plants (Fig. 4b).

In addition, measurement of stomatal aperture showed that *35S-ABP9* transgenic plants had much smaller stomatal aperture than wild type in the absence of

exogenously applied ABA (Fig. 4c). When exposure to the treatment of 10  $\mu\text{M}$  ABA, the stomatal aperture of 5P2 and 5P3 plants was reduced by 54 and 60%, respectively, relative to that of untreated ones, whereas the stomatal aperture of wild-type plants only reduced by 37% (Fig. 4d). These results suggest that *ABP9* expression confers enhanced sensitivity in ABA-induced stomatal closure to *35S-ABP9* transgenic plants. Consistent with this, the *ABP9* expressing transgenic plants also exhibited an



enhanced capacity to conserve water, as revealed by delayed loss of fresh-weight measured in detached leaves under dehydration conditions (Fig. 4e).

Taken together, these results demonstrate that *ABP9* plays a positive role in regulating ABA-mediated inhibition of seed germination and root growth as well as promotion of stomatal closure.

The *ABP9* expressing plants display reduced ROS levels and alleviated oxidative damage and cell death

As ABA is known to be involved in regulating ROS levels and responses in plants, and *ABP9* expressing *Arabidopsis* plants displayed enhanced tolerance to the ROS-generating agent MV (Fig. 3f), we speculated that *ABP9* may be involved in the control of ROS levels during normal growth and in response to stress and ABA. To test this, we compared ROS levels in *35S-ABP9* transgenic and wild type plants under normal growth and stress/ABA treatment conditions.

We first examined the cellular levels of  $O_2^{\cdot-}$  and  $H_2O_2$ , two prominent ROS species involved in stress signaling and oxidative injuries, under normal growth and salt stress conditions by NBT and DAB staining, respectively. As shown in Fig. 5a, the steady state levels of both  $O_2^{\cdot-}$  and  $H_2O_2$ , visualized as dark blue and deep brown products, respectively, were largely reduced in leaves of *35S-ABP9* transgenic plants compared to wild-type plants under normal growth and salt stress conditions. Similarly, the accumulation of  $O_2^{\cdot-}$  and  $H_2O_2$  in ABA-treated *35S-ABP9* transgenic plants were also clearly lower than that in wild-type plants treated with ABA (Fig. 5b).

We next measured the cellular level of  $H_2O_2$  in guard cells with or without ABA treatment using  $H_2DCF-DA$ , a fluorogenic probe reporting ROS levels within cells by detecting enzymatically formed  $H_2O_2$ . As shown in Fig. 5c and 5d, although ABA treatment significantly increased  $H_2O_2$  levels in both wild type and *35S-ABP9* transgenic plants,  $H_2O_2$  levels in guard cells of *35S-ABP9* transgenic plants was much lower than that in wild-type plants either in the presence or absence of 10  $\mu$ M ABA. Interestingly, quantitative analysis revealed that the level of  $H_2O_2$  in ABA-treated guard cells of 5P2 and 5P3 transgenic plants increased by 33 and 37%, respectively, relative to the untreated control. In contrast,  $H_2O_2$  level in ABA-treated guard cells of wild type plants only increased by 14% relative to the untreated control (Fig. 5d). These results suggest that *ABP9* expression renders plant cells an enhanced capacity to constrain the absolute amount of  $H_2O_2$  to a lower level in both the rest and ABA-stimulated cells, but allows a higher relative increase of  $H_2O_2$  in response to ABA stimulation.

Since highly reactive free radicals such as  $OH^{\cdot}$ , resulting from reactions between  $O_2^{\cdot-}$  and  $H_2O_2$ , can cause lipid

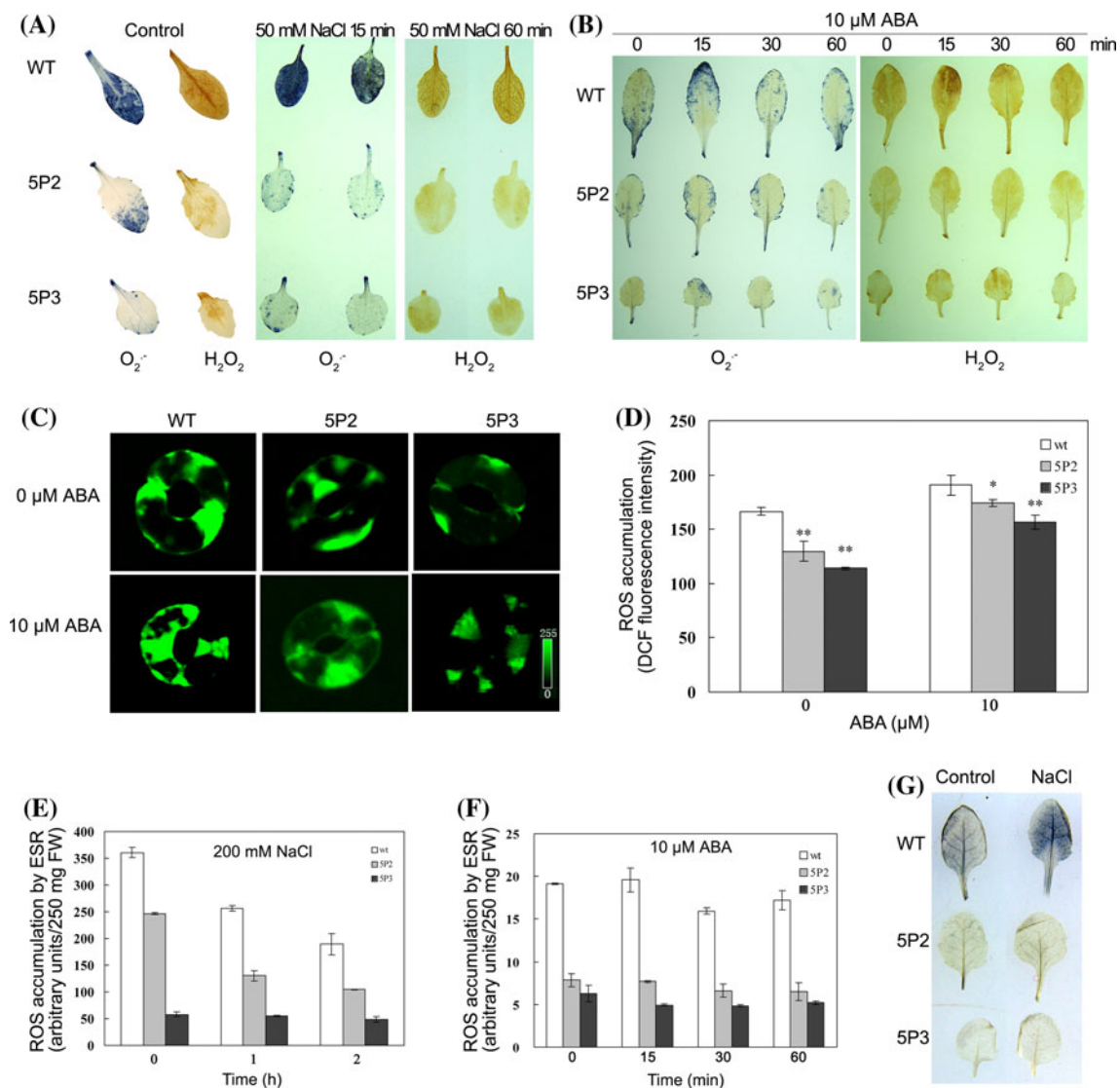
peroxidation by generating carbon-centered lipid free radicals (Halliwell 2006), our observations that *ABP9* down-regulates the total cellular levels of both  $O_2^{\cdot-}$  and  $H_2O_2$  lead us to speculate that *ABP9* may alleviate cellular oxidative damages caused by ROS. To test this, the steady state level of carbon-centered lipid free radicals was detected by ESR as spin adducts of PBN, a lipophilic spin-trap that is commonly used to detect lipid-derived carbon-centered radicals (Garlick et al. 1987). Quantification of ESR signals revealed that the cellular levels of lipid radicals in transgenic lines 5P2 and 5P3 were much reduced in comparison with wild-type plants under both normal growth conditions or treated with salt stress and ABA. Further, the radical levels in 5P2 and 5P3 reversely correlate with the expression levels of *ABP9* in these two independent transgenic lines (Fig. 5e, f). These observations suggest that the cellular oxidative damage resulting from secondary reactions of initially-formed reactive oxygen species is alleviated by *ABP9*. Consistent with this notion, Evans Blue staining assay showed the decreased cell death in *35S-ABP9* transgenic plants under either normal growth or salt stress conditions, compared with wild type plants (Fig. 5g).

Taken together, these results demonstrate that *ABP9* plays a critical role in controlling the homeostasis of cellular levels of ROS, thus preventing cells from oxidative damages and decreasing cell death.

Constitutive expression of *ABP9* enhances the expression of stress/ABA-responsive genes including ROS-scavenging/signaling genes

To investigate the molecular mechanism by which *ABP9* regulates ABA signaling, ROS accumulation and thus stress tolerance, we performed microarray analysis to measure global changes of gene expression in 5P2 and 5P3 transgenic plants in comparison with wild-type plants using the *Arabidopsis* Affymetrix GeneChip<sup>®</sup> ATH1 array containing probe sets representing approximately 24000 genes. The chip assay showed that under normal growth conditions, a total of 365 and 607 genes, respectively in 5P2 and 5P3 transgenic plants, were significantly up-regulated (twofold cutoff) relative to wild-type plants (Supplementary material 2, 3). When treated with 200 mM NaCl for 1 h and 2 h, 357 and 373 genes with levels greater than two fold change over wild-type plants were identified in the 5P2 transgenic line, respectively (Supplementary material 4, 5).

To verify the microarray results, we selected 19 well-documented stress-inducible genes for qRT-PCR analysis. Among which, 14 were shown to be both stress- and ABA-inducible (Supplementary material 7). As shown in Fig. 6a, under normal growth conditions, 12 genes displayed elevated expression in both 5P2 and 5P3 transgenic plants,

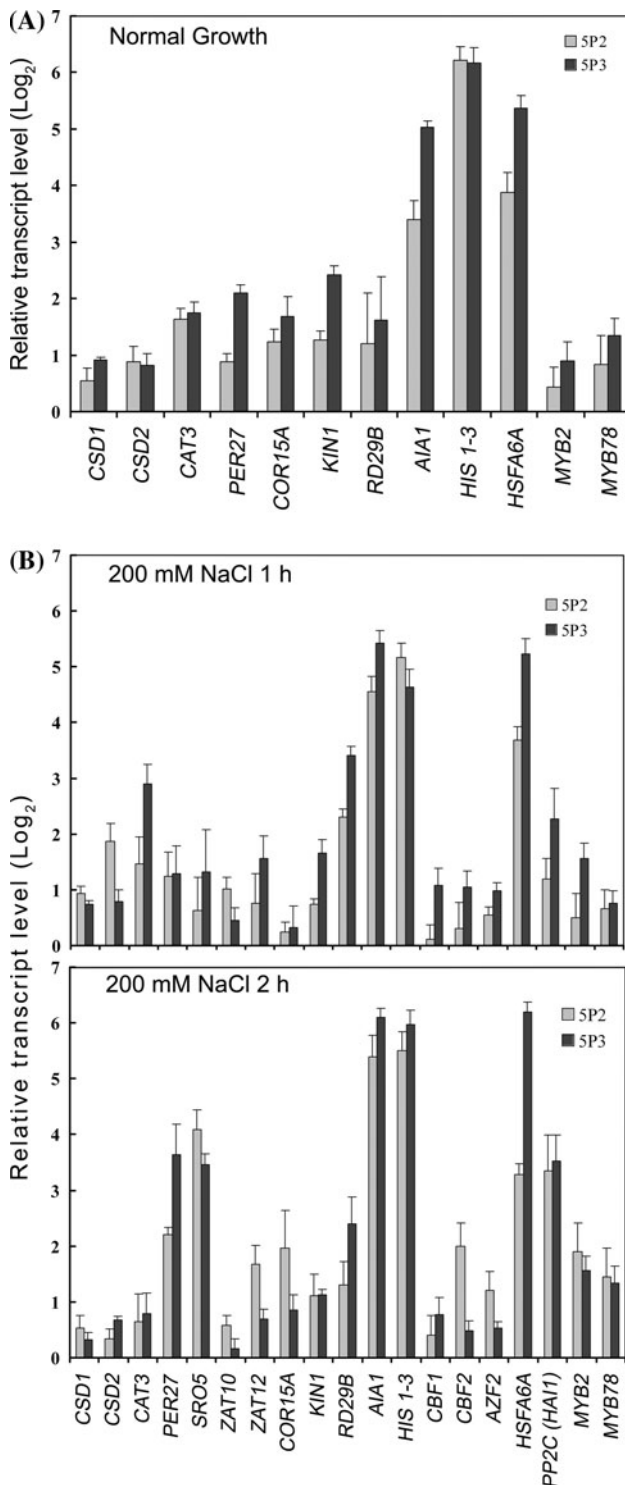


**Fig. 5** Constitutive expression of *ABP9* decreases ROS levels and cell death in *35S-ABP9* transgenic *Arabidopsis* plants under normal growth, ABA and salt stress conditions. **a, b** Cellular levels of  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$  are reduced in leaves of *35S-ABP9* transgenic plants under normal growth (Control), salt (**a**) and ABA (**b**) conditions. Detached leaves from wild-type (WT) and transgenic plants (5P2 and 5P3) pretreated with 50 mM NaCl or 10  $\mu\text{M}$  ABA were stained with DAB and NBT to visualize  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$ , respectively. All experiments were repeated at least 3 times, and about 15 leaves collected from multiple seedlings (4–5-week-old) were inspected in each experiment. **c** Representative microscopy images of ROS production in intact guard cells indicated by fluorescent dye DCF. Epidermal peels were loaded with  $\text{H}_2\text{DCF-DA}$  for 10 min after the incubation with 10  $\mu\text{M}$  ABA for 2 h. The pseudo-color key is shown at the bottom of lower panel and applied to pixel intensity (0–255) for all fluorescence images. **d** Quantification of ROS production in guard cells in (**c**) of wild-type and *35S-ABP9* plants following ABA treatment. Data are from measurements of pixel intensity in whole cells determined that are treated with or without ABA in three independent experiments

relative to wild-type plants. These genes are (i) ROS-scavenging enzymes *CSD1*, *CSD2*, *CAT3* and *PER27* (Mittler et al. 2004); (2) stress- and ABA-inducible

(means  $\pm$  SD). For the wild type (WT),  $n = 93$  cells without ABA treatment,  $n = 100$  cells with ABA treatment; for *35S-ABP9* transgenic line 5P2,  $n = 125$  cells without ABA treatment,  $n = 220$  cells with ABA treatment; and for 5P3,  $n = 120$  cells without ABA treatment,  $n = 140$  cells with ABA treatment. Asterisks indicate significant differences between the WT and 5P2 or 5P3 plants ( $*P < 0.05$ ,  $**P < 0.01$ , by Student's *t* test). **e, f** ESR spin trapping detection of carbon-centered free radicals by PBN in wild type and *35S-ABP9* transgenic plants in response to salt (**e**) and ABA (**f**). Intensity of the ESR spectra signal in plants untreated and treated with 200 mM NaCl or 10  $\mu\text{M}$  ABA was measured and expressed in arbitrary units. FW, fresh weight. Experiments were performed three times. Shown are means  $\pm$  SD of three replicates from one experiment. All differences between the WT and 5P2 or 5P3 plants are significant at  $P < 0.05$  using Student's *t* test. **g** Cell death staining with Evans blue before (Control) and 60 min after the treatment of 50 mM NaCl. The experiment was repeated 3 times, and 10 leaves collected from multiple seedlings (4–5-week-old) were inspected in each experiment

protective or regulatory genes *COR15A*, *KINI*, *RD29B*, *AIA1*, *HIS1-3*, *MYB2*, *MYB78* (Abe et al. 1997; Fujita et al. 2005; Thomashow 1999; Xin et al. 2005); (3)



**Fig. 6** Constitutive expression of *ABP9* enhances the expression of stress- and ABA- responsive genes. Seedlings grown on agar plates were untreated or treated with 200 mM NaCl for 1 h and 2 h. Transcript level of each gene before (a) and after the treatment (b) detected by qRT-PCR, normalized to the endogenous control (*actin2*) relative to the calibrator (wild type), is given by  $2^{-\Delta\Delta C_T}$ . The Figure was plotted by Log<sub>2</sub> values of relative transcript levels vs. genes. For each gene, the expression level in the WT plants was defined as 0. Data represent means  $\pm$  SD of three replicates

heat-inducible transcription factor *HSFA6A* (von Koskull-Döing et al. 2007). It is noteworthy that *HIS1-3*, *AIA1* and *HSFA6A* show highest induction among these genes. Further, among the 19 selected genes, 7 of them show enhanced expression only under salt treatments (Fig. 6b). These genes include *SRO5*, *Zat10* and *Zat12* which are involved in ROS signaling to regulate ROS-scavenging gene expression (Borsani et al. 2005; Mittler et al. 2006; Rizhsky et al. 2004), three stress- and ABA-responsive transcription factors *CBF1*, *CBF2*, *AZF2* (Sakamoto et al. 2004; Stockinger et al. 1997), and the protein phosphatase *PP2C* (*HAI1*)/*At5g59220* (Fujita et al. 2009; Xin et al. 2005).

Since *ABP9* binds to a G-box type ABRE in a sequence-specific manner, we next searched for candidate ABREs in the promoters (2,000-bp region upstream of the start codon) of the 19 *ABP9*-regulated genes described above using the ACGT core-containing ABRE motifs available in the plantCARE database (<http://sphinx.rug.ac.be:8080/PlantCare/>) (Lescot et al. 2002). This analysis identified 13 genes (*CSD1*, *CAT3*, *SRO5*, *ZAT10*, *COR15A*, *RD29B*, *KIN1*, *AIA1*, *HIS1-3*, *CBF1*, *CBF2*, *AZF2*, and *PP2C(HAI1)/At5g59220*) that contain at least one G-box type ABREs with the sequence (C/T)ACGTG, among which 10 genes (*ZAT10*, *COR15A*, *RD29B*, *KIN1*, *AIA1*, *HIS1-3*, *CBF1*, *CBF2*, *AZF2* and *PP2C(HAI1)/At5g59220*) have been reported to be both stress- and ABA-inducible (Supplementary material 7). Our results suggest that *ABP9* may mediate enhanced stress tolerance through regulating the expression of these downstream target genes either directly or indirectly.

## Discussion

Modulation of ROS to appropriate levels is critical for tolerance to abiotic stress given the facts that ROS act as both signaling molecules at low concentrations (Pei et al. 2000; Prasad et al. 1994) and damaging agents when accumulated in excess in plants (Desikan et al. 1998; Levine et al. 1994). Previous studies have suggested that the cellular ROS levels are modulated by ABA via regulation of ROS-producing and ROS-scavenging genes (Fryer et al. 2003; Kwak et al. 2003; Zhu and Scandalios 1994). However, the transcription factors and mechanisms involved in the regulation of ROS genes and hence ROS levels by ABA remain unclear. In this report, we demonstrated that (1) *ABP9*, a bZIP protein of maize, in vitro directly binds in a sequence-specific manner to a G-box type ABRE in the promoter of a maize antioxidant gene *Cat1*, and trans-activates ABRE-dependent expression of downstream reporter gene in plant cells (Fig. 1); (2) the expression of *ABP9* in maize is ABA- and stress-inducible (Fig. 2); (3) Constitutive expression of *ABP9* in transgenic *Arabidopsis* leads to enhanced tolerance to multiple stresses, and to alleviate

oxidative damage (Fig. 3); (4) Constitutive expression of *ABP9* also confers hyper-sensitivity to ABA during germination, root growth, stomatal closure and increased capability to conserve water (Fig. 4); (5) Constitutive expression of *ABP9* down-regulates cellular ROS levels induced by ABA and stress, and decreases cell death (Fig. 5); (6) Constitutive expression of *ABP9* results in elevated expression of many ABA- and stress-inducible genes and those for scavenging and regulating ROS (Fig. 6). These results clearly indicate that *ABP9* is an ABRE-binding transcription activator that acts as a positive regulator of ABA signaling and is involved in modulation of ROS accumulation in plants to confer an enhanced tolerance to various abiotic stresses by reducing the oxidative damages of ROS and cell death.

H<sub>2</sub>O<sub>2</sub> functions as essential signaling molecule in ABA-induced stomatal closure (Pei et al. 2000). Interestingly, we found that although ABA treatment significantly increased H<sub>2</sub>O<sub>2</sub> levels in both *35S-ABP9* transgenic and wild type plants, the net H<sub>2</sub>O<sub>2</sub> increase by ABA as compared to untreated control in guard cells of *35S-ABP9* transgenic plants was much higher than that in wild-type plants (Fig. 5d) accompanied by enhanced ABA-induced stomatal closure in transgenic plants (Fig. 4c, d), and meanwhile the total H<sub>2</sub>O<sub>2</sub> level in guard cells of *35S-ABP9* transgenic plants was much lower than that in wild-type plants either in the absence or presence of exogenously applied ABA (Fig. 5c, d). These results suggest that *ABP9*, on one hand, induces a higher ROS increase to induce stomatal closure, while on the other hand, constrains the total ROS levels by decreasing total ROS production/accumulation, thus alleviating oxidative damage and cell death under stress conditions. Thus, *ABP9* may play a dual role in the harmonious regulation of ROS production and signaling by ABA and abiotic stresses.

Our genome-wide gene expression and qRT-PCR analyses provided further evidence supporting the above notion. We identified 19 genes that were up-regulated in transgenic *Arabidopsis* lines constitutively expressing *ABP9* under normal growth and/or salt stress conditions (Fig. 6). Among them, *CSD1*, *CSD2*, *CAT3*, and *PER27* encode major antioxidant enzymes for removing superoxide and hydroperoxide in various cellular compartments. *ZAT10*, *ZAT12* and *SRO5* are all regulatory components in ROS signaling, and all can be induced by multiple abiotic stresses including oxidative stress and are found capable of enhancing stress tolerance of plants via elevating the expression of reactive oxygen-defense transcripts (Borsani et al. 2005; Davletova et al. 2005; Mittler et al. 2006; Rizhsky et al. 2004; Vogel et al. 2005). The observation that these genes were up-regulated in *ABP9* overexpressing transgenic plants under normal growth and/or salt stress conditions suggests that *ABP9* enables the plants to detoxify ROS efficiently and to tightly control ROS accumulation and thus enhances stress tolerance via mobilizing an

array of ROS-scavenging enzymes and activating signaling molecules regulating ROS-scavenging genes.

Our transcriptome analysis also revealed that expression of many stress/ABA-responsive protective and regulatory genes, is drastically up-regulated in *ABP9* overexpression plants, including *COR15A*, *KIN1*, *RD29B*, *AIA1*, *HIS1-3*, *ATMYB2*, *ATMYB78*, *CBF1*, *CBF2*, *AZF2*, *PP2C (HAI1)/At5g59220*, and *HSFA6A*. All these 12 members were evidenced as both stress- and ABA-inducible (Supplementary material 7), and some of these genes have been clearly identified as positive regulators of ABA signaling such as *MYB2*, *PP2C (HAI1)* (Abe et al. 2003; Fujita et al. 2009) or as downstream targets of ABA-signaling pathways such as *COR15A*, *KIN1*, *RD29B*, *AIA1*, *HIS1-3* (Fujita et al. 2005). Therefore, their up-regulation in *ABP9* overexpressing transgenic plants under normal growth and/or salt stress conditions (Fig. 6) provides further molecular validation that *ABP9* may act as an upstream positive regulator in ABA and stress signaling. Previous studies have reported that enhanced expression of stress-responsive genes such as *COR15A*, *KIN1*, *RD29B*, *AIA1*, *HIS1-3* or overexpression of transcription factor genes such as *MYB2*, *CBF1*, *CBF2*, *AZF2* imparts tolerance to low temperature and water stress in plants (Abe et al. 2003; Gilmour et al. 2004; Sakamoto et al. 2000). In addition, a role of heat shock transcription factor genes, such as *HSFA6A* and *HSFA4A*, in stress tolerance and ROS signaling has also been reported (Chao et al. 2007; Miller and Mittler 2006). Thus, the up-regulation of these stress tolerance marker genes and signaling molecules or transcription factors may collectively set the molecular basis for *ABP9*-mediated enhancement of abiotic stress tolerance. Future studies will be aimed to unravel the direct target genes of *ABP9* in maize and to explore its utilization in engineering transgenic crops with enhanced tolerance to multiple abiotic stresses.

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