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The analysis of an *Arabidopsis* triple knock-down mutant reveals functions for MBF1 genes under oxidative stress conditions

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ABSTRACT

Transcriptional co-activators of the multiprotein bridging factor 1 (MBF1) type belong to a small multigenic family that controls gene expression by connecting transcription factors and the basal transcription machinery. In this report, a triple knock-down mutant (*abc*−) for the *Arabidopsis thaliana MBF1* genes *AtMBF1*a, *AtMBF1*b and *AtMBF1*c was generated. The phenotypic characterization using oxidative agents such as hydrogen peroxide and methyl viologen revealed that the *abc*− mutant was more sensitive to oxidative stress. The triple knock-down mutant, *abc*− was also sensitive to osmotic stress mediated by high concentrations of sorbitol. Furthermore, the *abc*− phenotype was partially or completely rescued by *AtMBF1c* cDNA over-expression (*abc*− +c) depending on physiological and developmental conditions. *AtMBF*1s regulate the expression of *ABR1*, which is a member of the ethylene-response factor family and acts as ABA repressor. Thus, we conclude that *AtMBF*1 gene family may function as a regulatory component of the cross-talk node between ethylene, ABA and stress signal pathways. Furthermore, higher levels of a HSP70 mRNA and an immunoreactive HSP70 protein were detected in the *abc*− mutant. The participation of *MBF1*c as a possible negative regulator of *HSP* genes was discussed.

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Introduction

Transcriptional regulation plays a central role in the exertion of genomic information during complex biological processes in all organisms. Binding of transcription factors to cis-acting elements must be carried out by the RNA polymerase complex in order to initiate and maintain transcription. A category of eukaryotic proteins named co-activators, which enhance transcription by interacting with both general and gene-specific transcription factors has been identified and isolated from humans, animals, fungi and plants (Takemaru et al., 1997; Zhu et al., 2000; Tsuda et al., 2004). Multiprotein bridging factor 1 (*MBF1*) is a highly conserved transcriptional co-activator gene family involved in the regulation of diverse physiological processes (Liu et al., 2003; Jindra et al., 2004).

The first *MBF1* gene family member in plants was reported by Zegzouti et al. (1999) and was named ER24 by ethylene-responsive transcriptional co-activator. In *Arabidopsis thaliana* there are three different genes (*AtMBF1*a, *AtMBF1*b and *AtMBF1*c) encoding MBF1 proteins (Tsuda and Yamazaki, 2004). The extent of amino acid sequence identity indicates that *AtMBF1*a and *AtMBF1*b belong to group I, and *AtMBF1*c to group II. The expression of *MBF1*c increases in response to pathogen infection, salinity, drought, heat, methyl viologen (MV), hydrogen peroxide (H_2O_2), abscisic acid (ABA) and salicylic acid (SA) (Tsuda et al., 2004; Tsuda and Yamazaki, 2004; Suzuki et al., 2005). *MBF1*c over-expression enhances the tolerance to heat and osmotic stresses in *A. thaliana* transgenic plants by partially activating the ethylene-response signal transduction pathway (Suzuki et al., 2005). Constitutive expression of *MBF1*a in *A. thaliana* led to elevated salt tolerance, insensitivity to glucose and resistance to fungal disease (Kim et al., 2007). Suzuki et al. (2008) reported that *MBF1*c is a key regulator of basal thermotolerance and provided evidence for the existence of a coordinated heat stress-response

Abbreviations: *abc*−, triple mutant line for AtMBF1 genes; *abc*− +c, triple mutant complemented with AtMBF1c cDNA; H_2O_2, hydrogen peroxide; MBF1, multiprotein bridging factor 1; MV, methyl viologen; NBT, nitro blue tetrazolium; $O_2^{\cdot-}$, superoxide

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network involving SA-, trehalose- and ethylene-signaling pathways under the control of *MBF1*c. However, the functional contribution of *MBF1*c to the defense response is still obscure.

The aim of this work was to analyze the function of *MBF1* genes in young *A. thaliana* seedlings. We characterized the triple knock-down mutant, *abc*− under oxidative and osmotic stress conditions.

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh. Col-0 (*A. thaliana*) plants were grown at 22–24 °C under fluorescent light l20 μmol photons $m^{-2}\,s^{-1}$ with a 16-h photoperiod. For soil growth, seeds were sown on organic substrate placed for 2 d at 4 °C in the dark to break residual dormancy and then transferred to normal growth conditions. Plants were watered twice a week. For stress treatments, seeds were sterilized with 30% sodium hypochlorite and 0.02% Triton X-100 for 15 min with vigorous shaking, then washed three times in sterile water and plated on ATS agar medium or half-strength Murashige and Skoog (MS) medium. Media were supplemented with 1% sacarose and solidified with 0.8% agar. For oxidative and osmotic stresses, methyl viologen (MV) and sorbitol, respectively, were used. After the plates were placed at 4 °C for 2 d in the dark to break residual dormancy, seedlings were grown in vertical position during different periods of time.

Identification and genotyping of knock-down mutants

T-DNA insertion mutants for *AtMBF1*a (*At2g42680*), *AtMBF1*b (*At3g58680*) and *AtMBF1*c (*At3g24500*) genes (GARLIC_427_G03=427, GARLIC_33_C08=33, GARLIC_141_D08=141, respectively) were identified from the Syngenta Collection in the Columbia background (Sessions et al., 2002). Confirmation of the T-DNA insertion was done by performing PCR using a combination of a T-DNA border primer (5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′) and a gene-specific primer: (Line 427_G03) 5′-GTTCTGCAACCGCCTTTTGACA-3′; (Line 33_C08), 5′-GCTTCTCTTGTGATTTTCTTTC-3′; (Line 141_D08), 5′-TCGAATTCTCCAGAAACCAATC-3′, for *AtMBF1*a, *AtMBF1*b and *AtMBF1*c, respectively. To remove additional insertions or other background mutations two backcrosses to wild-type (WT) plants were performed for each line. Homozygous lines were identified by BASTA selection on MS agar plates and PCR genotyping. Double and *abc*− triple mutants were generated from genetic crosses of single mutants and identified through BASTA selection and PCR genotyping. For genetic complementation, *AtMBF1*c cDNA was cloned into the pBI121 vector (Chen et al., 2003) and transformed into the *abc*− mutant plants using *Agrobacterium tumefaciens* strain GV3101pMP90RK. Then, T1 transformants of *abc*− +c plants were identified based on their resistance to kanamycin. *AtMBF1*c expression levels were measured by real-time-RT-PCR (Mastercycler® ep *realplex*, Eppendorf).

Stress assays

For germination assays, *abc*−, *abc*− +c and WT seeds harvested at the same time were surface sterilized and incubated at 4 °C for 2 d in the dark. The seeds were sown on filter paper wetted with sterile H_2O or 100 μM MV and incubated for 2 d in the growth chamber. Then the percentage of germination was scored according to Boyes et al. (2001). Osmotic stress assays were performed on agar plates containing 0.5 × MS plus 0, 200 or 400 mM sorbitol and incubated for 2 d in the growth chamber. Seeds with fully emerged radicles were counted. For growth stage, seeds were surface sterilized and placed in rows on agar plates containing 0.5 × MS plus H_2O_2 or 5 μM MV. Percentage of seedlings at growth stage 0.7 was scored after 5 d (Boyes et al., 2001). All experiments were performed at least in triplicates. WT seeds were included in each plate as internal control.

For transcript analysis, *abc*−, *abc*− +c and WT seeds harvested at the same time were surface sterilized and placed in rows on agar plates containing 0.5 × MS plus water or 10 nM MV. Then, plates were incubated at 4 °C for 48 h and placed vertically in a growth chamber for 7 d.

Cell death determination

Five-day-old seedlings grown on ATS agar were transferred to liquid ATS medium with or without 15 mM H_2O_2. Cell death was monitored in roots and cotyledons after 1.5 h or 24 h, respectively. To assess cell viability, seedlings were stained with 1% Evan's blue solution (Sigma) and observed under light microscope (Nikon Eclipse E200). For quantification, stained roots from 20 seedlings, per line and per treatment were cut 1 cm above the apical tip and incubated in 100 μL 100% DMSO for 20 min. The absorbance was measured in a spectrophotometer (Ultrospec™ 1100) at 565 nm.

Detection of superoxide ($O_2^{\cdot-}$)

A. thaliana seedlings grown in soil were treated with 50 μM MV for 90 min under light. Then, leaves were incubated with 0.2% nitro blue tetrazolium (NBT) in 50 mM sodium phosphate pH 7.5. Leaves were destained overnight with ethanol 96% and analyzed under lupe (Nikon). $O_2^{\cdot-}$ is the major oxidant species responsible for reducing NBT which is observed as a dark spot. Observations were carried out in three independent experiments and representative leaves from *abc*−, *abc*− +c and WT were shown.

Measurement of electrolyte leakage

Leaflets from 2-week-old seedlings were transferred to 1 mL of H_2O, 50 μM MV or 15 mM H_2O_2 and incubated for 4 h with continuous light in the growth chamber. The conductivity of the suspending solution was measured with a conductance meter (Twin Compact Meter-Horiba, Norhampton, UK) before and after autoclaving at 120 °C for 30 min to release the total electrolytes. The conductivity was scored at least for 4 plants per line and per treatment. Electrolyte leakage was expressed as a percentage of total electrolytes.

RNA preparation and RNA gel blot analysis and hybridization

Total RNA was isolated using TRIzol® reagent as described by the manufacturer (Invitrogen). For RNA gel blot analysis, total RNA was separated on a 1.5% agarose gel containing 1.2 M glyoxal and transferred to N+ Hybond membranes (Amersham Biosciences, USA). The RNA on membranes was hybridized with ^{32}P-labeled DNA probes in 0.5 M Na_2HPO_4, 7% SDS, and 10 mM EDTA pH 7.2 at 65 °C for 16 h. Each ^{32}P-labeled-specific DNA probe was produced from each corresponding PCR product using the *Prime-a-Gene*® labeling system (Promega). Sequences of primers used for preparation of specific probes were the following sets: 5′-ACTGATGTAGCA-3′ and 5′-CAACTATGTGATGAAAAGACCCAAG-3′ for *AtMBF1*a probe, 5′-AAGTGTAGAACAAAGCTCTTAAAGG-3′ and 5′-ATAATGACAAAAGGTTCCAAACAGC-3′ for *AtMBF1*b probe,

5′-TGTTCCTTTCTCTCAATTCATCGAC-3′ and 5′-CATTTATCAAACA-AAACAACAAGAC-3′ for *AtMBF1*c probe.

cDNA probes corresponding to *ABR1* (*At5g64750*), *APX1* (*At1g07890*), *GST1* (*At1g02930*) genes were used for RNA gel blots. Membranes were washed twice in 2× saline-sodium citrate buffer (SSC) containing 0.1% SDS at 42 °C for 15 min and then washed in 1 × SSC containing 0.1% SDS at 42 °C for 30 min. Finally, membranes were incubated at −80 °C and the signal was detected by autoradiography. RNA gel blot assays were repeated three times with independently isolated RNA. Band intensities were quantified using the Gel-Pro Analyzer 4.0 software.

Real-time-RT-PCR measurements

RNA for real-time-RT-PCR was prepared with TRIzol® reagent according to the manufacturer's instructions. RNA (2 μg) was used for reverse transcription reactions using M-MLV reverse transcriptase (Promega). Quantitative PCRs were carried out using a Mastercycler Realplex apparatus (Eppendorf) in a 20 μL final volume containing 0.8 μL 10 × SYBR® Green Master Mix reagent (Applied Biosystems), 0.02 pmol of each primer, 3 mM $MgCl_2$, 0.2 μL of the reverse transcription and 0.5 units Platinum®*Taq* DNA polymerase (Invitrogen). Fluorescence was measured at 72 °C during 40 cycles. Specific primers were designed using publicly available sequences (http://www.arabidopsis.org). The sets of primer sequences were: 5′-TCCGGTGATTAACACGAAGA-3′ and 5′-CACAGCCTGATTAGGAACAGC-3′; 5′-GGCTGAGGCAGATGAGTTCGA-GGA-3′ and 5′-GGCCAGCACCGCCGCTACCA-3′ for *AtMBF1*c and *HSP70* (*At3g12580*), respectively.

Protein gel blot analysis

For protein gel blot assays total proteins from *A. thaliana* leaflets (50 mg fresh weight) were extracted in sample buffer (Laemmli, 1970). Total proteins were boiled for 5 min and analyzed by electrophoresis on 12% SDS-PAGE. Then, proteins were transferred to a nitrocellulose membrane. The polyclonal antibody raised against a chloroplast HSP70 from *Pisum sativum*, CSS1 at a dilution of 1: 3000 was used (Rial et al., 2003). The blot was allowed to react with anti-rabbit IgG conjugated with alkaline phosphatase and revealed with 5-bromo-4-chloro-3-indolylphosphate/NBT substrate according to the procedure recommended by the manufacturer (Sigma). Band intensities were quantified using the Gel-Pro analyzer 4.0 software.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and post hoc comparisons were done with Tukey's multiple range test to determine statistical significance among genotypes and treatments at $P < 0.05$ level. The statistical software program used was SigmaStat 3.1.

Results

Generation of AtMBF1 knock-down mutant (abc−)

There would be functional redundancies among *AtMBF1*s since amino acid sequences are highly similar and the gene expression suggest some overlaps in their patterns (Tsuda and Yamazaki, 2004). To evaluate functions of the entire *AtMBF*1 gene family, the triple knock-down mutant, *abc*− was characterized. We searched the GARLIC sequence-indexed mutant collections for T-DNA insertions in *MBF1* genes. Three different mutant lines that carry single T-DNA insertions in each *MBF1*a, *MBF1*b and *MBF1*c were obtained. In the GARLIC_427_G03 and GARLIC_33_C08 mutants, the T-DNA was inserted in the 5′-untranslated region, 60 and 98 nucleotides upstream of the *At2g42680* and *At3g58680* start codons, respectively. In the GARLIC_141_D08 mutant, the T-DNA insertion site was located within the *At3g24500* open-reading frame, 29 nucleotides upstream of the stop codon (Fig. 1A).

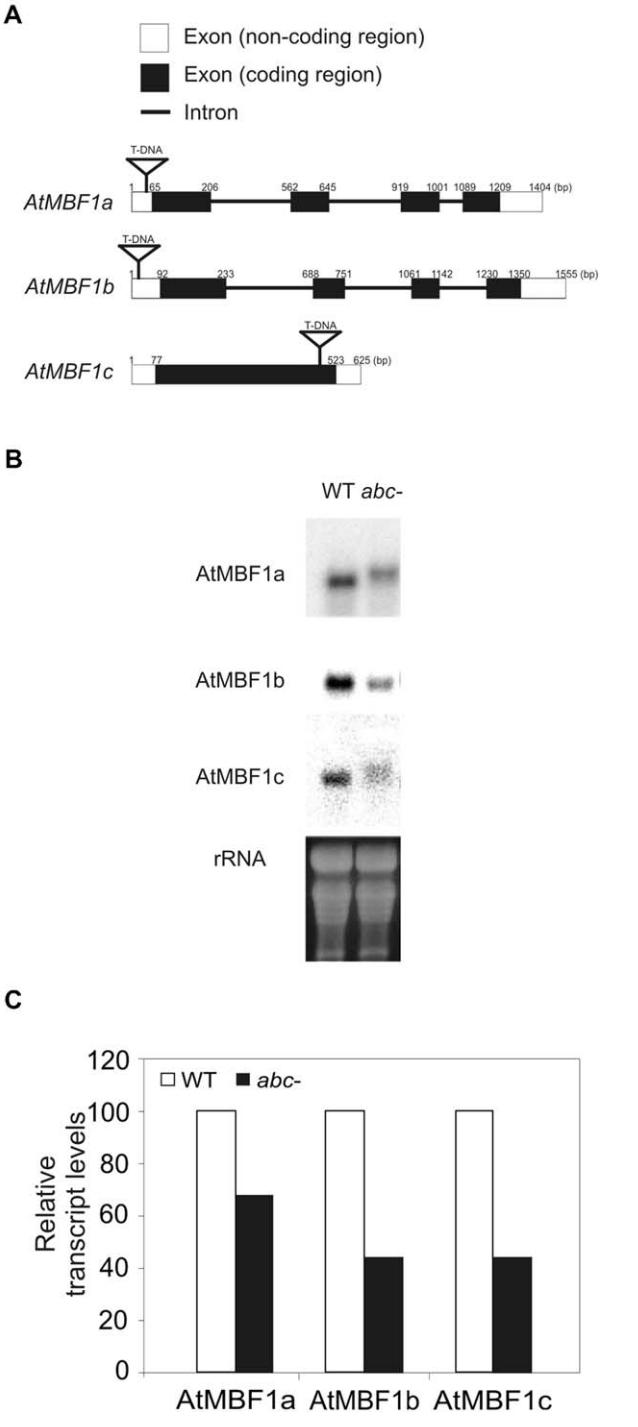

Fig. 1. Expression analysis of *AtMBF1* genes in the *abc*− mutant. (A) Scheme of the *AtMBF1*a, *AtMBF1*b, *AtMBF1*c genes. The position of the T-DNA insertion within *MBF1*s in the GARLIC lines is shown. (B) RNA gel blot analysis of *MBF1*s in *abc*− and WT seedlings. Total RNA isolated from *A. thaliana* seedlings was blotted and hybridized with [^{32}P]-labeled probes. rRNA corresponds to ribosomal RNAs. (C) Quantification of (B) by Gel-Pro Analyzer 4.0. The expression level of each transcript is expressed as percentage of WT level.

The single mutants showed no apparent defective phenotypes, at least under the growth conditions used (data not shown).

The triple mutant *abc*− was generated through crossings and screened by PCR genotyping. From the segregating populations derived from the genetic crossings homozygous *abc*− appeared at rates similar to the expected ratios. RNA gel blot assays were performed in order to evaluate AtMBF1 transcript levels. The transcript sizes were the expected ones for each *MBF1*, 675, 726 and 625 bp for *AtMBF1*a *AtMBF1*b and *AtMBF1*c, respectively (Fig. 1B). A possible explanation for the mRNA size shifts in the T-DNA lines of *MBF1*a and *MBF1*c is that by virtue of weak terminator sequences, transcript read-through from T-DNAs might be produced, resulting in producing aberrant transcripts together with native genes (Ülker et al., 2008). As shown in Figs. 1B and C, *AtMBF1*a, *AtMBF1*b and *AtMBF1*c expression decreased in *abc*− mutant. In this study, we focused on functions of the entire *AtMBF*1 family. In addition, the *abc*− mutant line complemented with *AtMBF1c* cDNA driven by the CaMV 35S promoter (*abc*− +c) was generated to evaluate if the observed phenotypes are due to the loss-of-function mutations of *AtMBF1*s. We assume that *AtMBF1*c driven by the constitutive promoter can compensate functions of the entire *AtMBF*1 family since amino acid sequences are highly conserved among *AtMBF*1s. Real-time-RT-PCR conducted with AtMBF1c probe showed higher transcript levels in *abc*− +c than in *abc*− and WT lines (Supplemental Fig. 1). Global observations of seeds and plant morphology during the life cycle did not show visual differences between *abc*−, *abc*− +c and WT (data not shown).

Analysis of the abc− mutant under different oxidative stress conditions

Firstly, the sensitivity of WT seedlings to the minimum H_2O_2 concentration was determined in a dose-response assay (data not shown). Cell death in roots and cotyledons was detected at a minimum of 15 mM H_2O_2 after 1.5 and 24 h of treatment, respectively (Supplemental Fig. 2A). To quantify cell death, the dye of stained roots was eluted and the absorbance of Evan's blue solution was measured. The highest value of absorbance revealing cell death symptoms was detected in the *abc*− mutant. The *abc*− sensitivity to oxidative stress was partially reverted by *AtMBF1*c constitutive expression in the *abc*− +c line (Fig. 2A). In addition, cotyledons of the *abc*− mutant were more sensitive to H_2O_2 treatments than those of WT (Supplemental Fig. 2B). The effect of MV as a different oxidative agent was also assayed. MV has largely been used as an inductor of oxidative injury through $O_2^{\cdot-}$ production in plants (Scarpeci et al., 2008). The reduction of NBT by $O_2^{\cdot-}$ to insoluble formazan spots was analyzed in non-treated and MV-treated leaves (Fig. 2B, upper and lower panels, respectively). The *abc*− mutant evidenced a high level of $O_2^{\cdot-}$ in MV-treated leaves and its phenotype was partially rescued by *AtMBF1*c over-expression. To evaluate the extent of cell damage caused by 15 mM H_2O_2 and 50 μM MV in *abc*− seedlings, electrolyte leakage was measured. The *abc*− mutant presented 1.5–3 folds higher electrolyte leakage than WT, which suggests that the membrane is likely to be impaired in these seedlings under such conditions (Fig. 2C).

Seedlings tolerance to MV was also analyzed during the germination process. In seeds, MV has a toxic effect by generating $O_2^{\cdot-}$ in the mitochondria (Mees, 1960). First, the minimum concentration affecting germination in WT seeds was tested. Approximately 42% inhibition was observed in WT seeds after 100 μM MV-treatment (Fig. 3A). The *abc*− mutant was strongly sensitive to 100 μM MV during germination (Fig. 3A).The *abc*− +*c* and WT seeds showed similar percentages of germination,

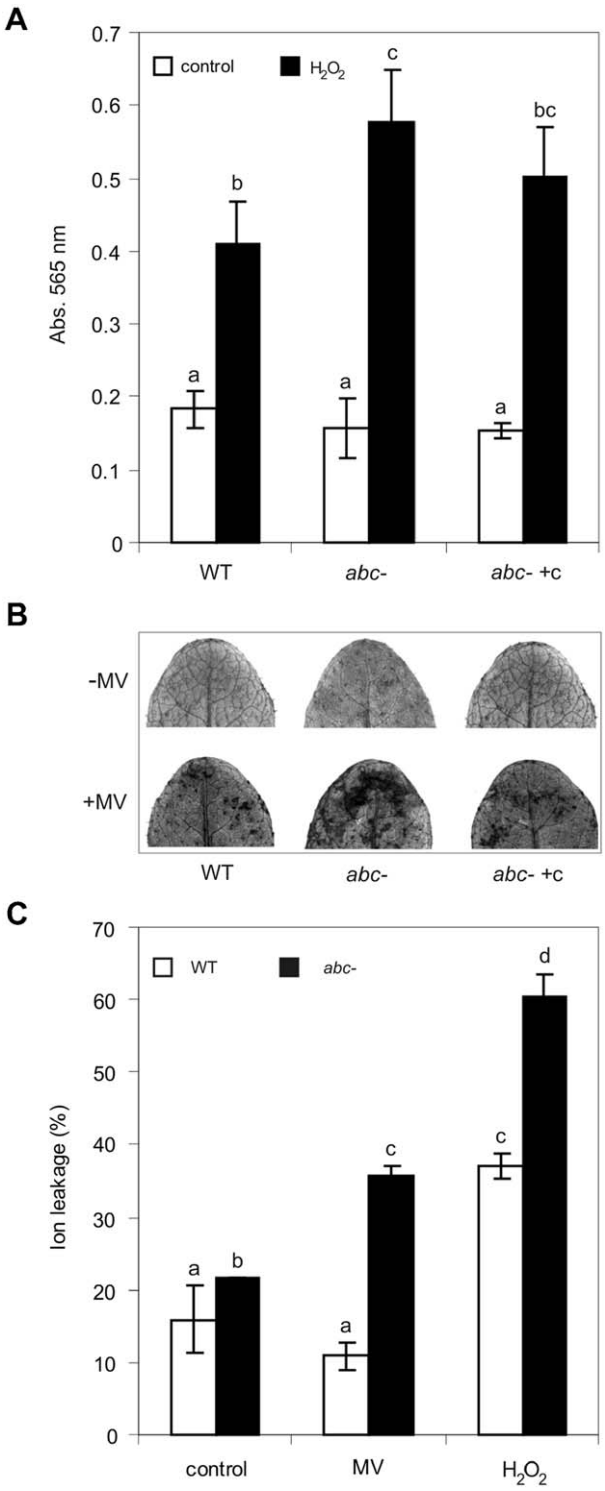

Fig. 2. The triple mutant *abc*− shows susceptibility to oxidative agents. (A) Quantification of cell death in primary roots of 5-d-old *A. thaliana* seedlings. WT, *abc*− and *abc*− +c seedlings were treated with 15 mM H_2O_2 or distilled H_2O as control for 1.5 h. Cell death was measured by Evan's blue staining and quantified spectrophotometrically. (B) Photographs show $O_2^{\cdot-}$ accumulation in leaflets from 2-week-old seedlings in the absence (upper panel) or in the presence of 50 μM MV (lower panel). (C) Two-week-old-soil-grown WT and *abc*− seedlings were treated with distilled H_2O, 50 μM MV or 15 mM H_2O_2. Electrolyte leakage was expressed as a percentage of total electrolytes. Data are mean values (±SD) of at least three independent experiments. Different letters indicate significantly different values between treatments or genotypes ($P < 0.05$).

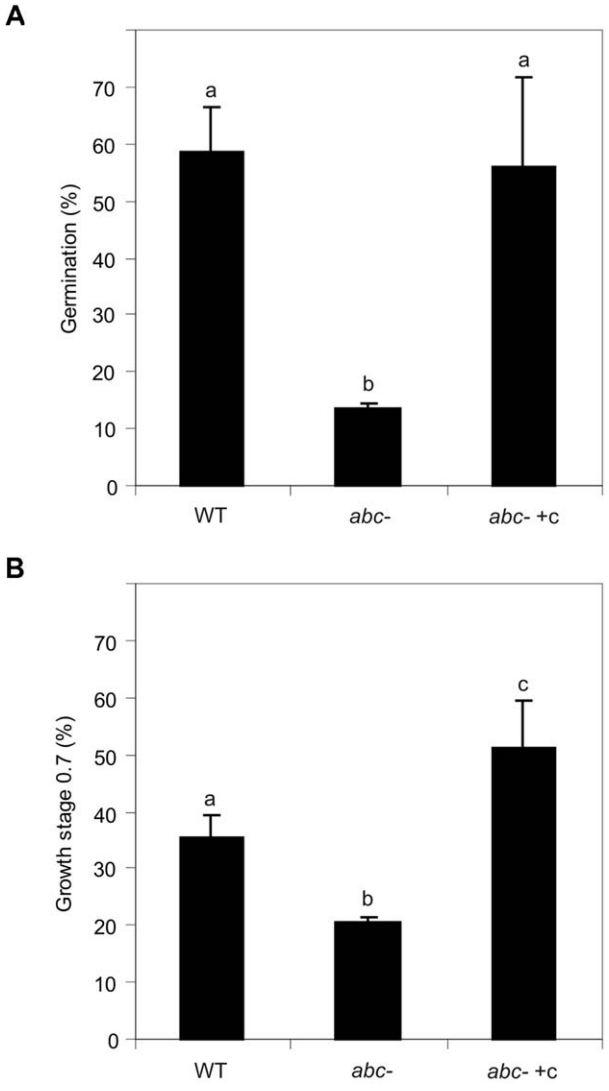

Fig. 3. Sensitivity of *abc*− seeds and seedlings to MV. (A) Germination was scored in WT, *abc*− and *abc*− +c after incubation for 48 h with 100 μM MV (B) Growth stage 0.7 was scored in 5-d-old seedlings grown in the presence of 1 μM MV. Values represent the percentage of total seeds per line. Data are mean values (±SD) of at least three independent experiments. Different letters indicate significantly different values between genotypes ($P < 0.05$).

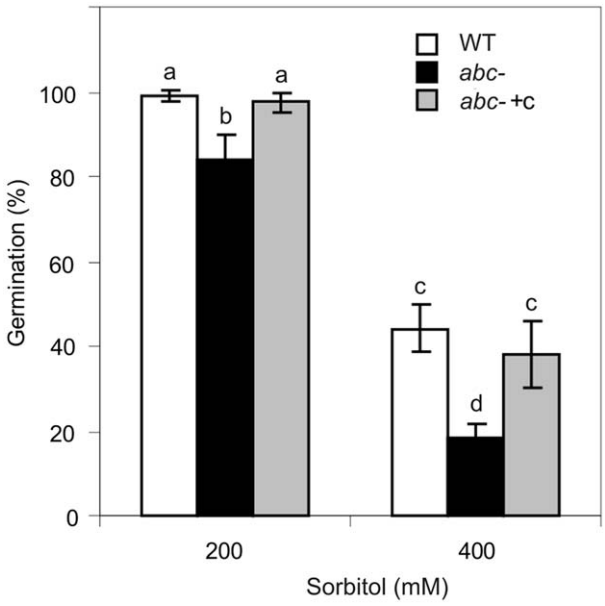

Fig. 4. Sensitivity of *abc*− seeds to osmotic stress mediated by sorbitol. WT, *abc*− and *abc*− +c seeds were germinated on media containing 200 or 400 mM sorbitol. Seeds with fully emerged radicle were counted as germinated. Data are mean values (±SD) of at least three independent experiments. Different letters indicate significantly different values between treatments or genotypes ($P < 0.05$).

indicating that *AtMBF1c* over-expression was able to fully rescue the mutant phenotype (Fig. 3A). Since very few MV-treated seedlings reached the developmental stage 0.7, we assume that the treatment imposed caused a severe oxidative stress (Boyes et al., 2001). Seedling ability to growth under oxidative conditions was also tested. WT, *abc*− and *abc*− +c seeds were germinated in ATS agar medium containing 5 μM MV. Although germination was not affected, growth retardation of *abc*− seedlings was detected (Fig. 3B). The triple knock-down mutant, *abc*− presented a lower percentage (20%) of seedlings at stage 0.7 than WT (35%). However, in the complemented line, *abc*− +c 51% of seedlings reached the stage 0.7. In this particular assay, the enhanced tolerance of *abc*− +c seedlings may be explained by the over-expression of *MBF1c* as described by Suzuki et al. (2005). The data indicated that the *abc*− mutant was more sensitive to different oxidative stress conditions. The knock-down phenotypes were partially or completely rescued by complementation with *AtMBF1c* over-expression depending on the developmental stage and stress condition.

Analysis of the abc− mutant under osmotic stress

Seed germination is a key developmental process in the plant life cycle. Salt sensitivity is most evident at the germination and early growth stages, and ROS produced under osmotic stress cause secondary oxidative stress. To characterize the physiological response of the *abc*− mutant under osmotic stress, germination was tested under different sorbitol concentrations (Fig. 4). Compared with WT, the *abc*− mutant presented a significant diminution of germination in a dose dependent manner. At 400 mM sorbitol, the *abc*− mutant germinated approximately 60% less than WT. The *abc*− phenotype was completely rescued by constitutive expression of *AtMBF1c* in *abc*− +c seeds. In the absence of sorbitol nearly 100% germination rate was observed in all genotypes (data not shown). The results suggest that *AtMBF1s* regulate the tolerance to osmotic stress conditions during germination.

Altered gene expression in abc− and abc− +c during oxidative stress

To examine the expression of different stress-responsive genes, the transcript levels of an AP2/ERF (*ABR1*), a cytosolic ascorbate peroxidase 1 (*APX1*) and glutathione S-transferase 1 (*GST1*) were tested in 7-d-old seedlings grown in the absence or presence of 10 nM MV (Fig. 5A). The densitometric analysis indicated that the ABR1 transcript level is reduced in *abc*− compared with WT in the absence of MV. Additionally, ABR1 expression decreased upon the MV-treatment in WT and *abc*− +c while it did not decrease in *abc*−, meaning that mechanism leading to reduction of ABR1 expression is compromised in *abc*−. APX1 and GST1 transcript levels were very similar in all lines with or without MV-treatment (Fig. 5B). In conclusion, susceptibility of *A. thaliana* seedlings to oxidative stress includes altered *ABR1*gene expression.

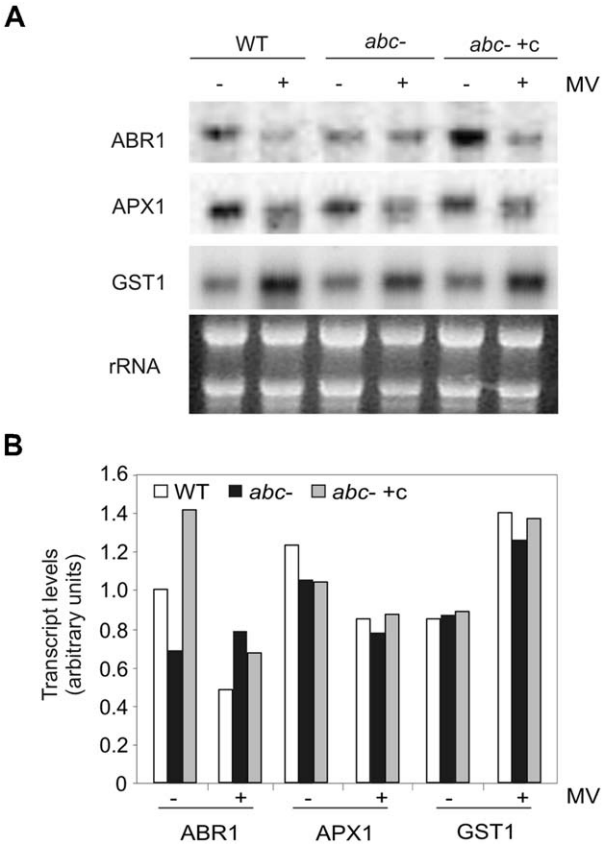

Fig. 5. RNA blot analysis of stress-responsive genes (*ABR1, APX1* and *GST1)* in 7-d-old seedlings. (A) Total RNA from untreated or 10 nM MV-treated seedlings was blotted and hybridized with [^{32}P]-labeled probes. (B) Densitometric analysis from RNA gel blot as indicated in (A). The expression level of each transcript is expressed as percentage of WT levels.

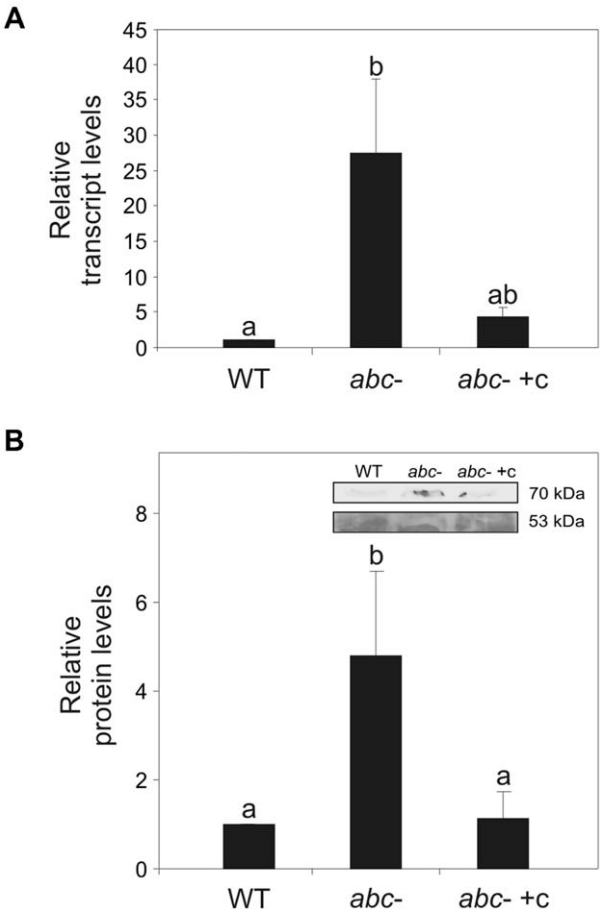

Fig. 6. *HSP* expression in *A. thaliana* seedlings. (A) Expression of HSP70 mRNA by Real-time-RT-PCR. Total RNA was extracted from 2-week-old seedlings. Tubulin was used as internal control. Data are mean values (± SD) of three independent experiments. Different letters indicate significantly different values between genotypes ($P < 0.05$). (B) Quantification of an immunoreactive HSP (70 kDa) in 7-d-old seedlings. Relative intensities of the HSP70 bands were normalized to Rubisco large subunit (52.7 kDa) by densitometry. Data are mean values (± SD) of three independent experiments. Different letters indicate significantly different values between genotypes ($P < 0.1$). Inset shows a representative protein gel blot.

Constitutive expression of stress-responsive HSP genes is augmented in the abc− mutant

To evaluate if *AtMBF*1 expression could be correlated with alterations of other stress-responsive genes, a heat-shock gene, *HSP70* was tested in all lines by Real-time-RT-PCR (Scarpeci et al., 2008). *HSP70* was up-regulated in *abc*− seedlings (Fig. 6A). Upon complementation with *AtMBF1*c, HSP70 transcript level was similar to WT. Furthermore, a similar tendency was measured at protein level by using anti-CCS1 antibodies. The anti-CCS1 antibodies revealed an immnuoreactive HSP of an expected size (70 kDa) (Fig. 6, inset). Semiquantitative examination of the protein gel blot indicated that the relative amount of immunoreactive HSP70 protein was somewhat increased in the *abc*− mutant when compared with WT and complemented lines (Fig. 6). Such increase was much more prominent in heat-shocked WT seedlings used as control (data not shown).

Discussion

We should be aware of functional redundancies when we analyze a gene family. In this report, the characterization of a triple knock-down mutant of *MBF1*s (*abc*−) allowed us to reveal new functions for *AtMBF1* genes. Studies using knock-down mutants have been used to functionally characterize some transcription factors and co-activators in *Arabidopsis* (Xu et al., 2006). Considering that over-expression of specific genes may not fully reveal their *in vivo* functions, loss-of-function mutants are valuable to confirm or further dissect gene function. Indeed, the combination of results obtained from the analysis of loss of function and over-expressing mutants would ensure a better comprehension of *AtMBF1* functions.

The knock-down *abc*− mutant showed high susceptibility to the stress imposed during germination and early growth stages. The *abc*− phenotype was partially or fully rescued by *AtMBF1*c gene in the *abc*− +c line depending on the developmental stage and the stress condition. This finding may be explained by similar evidences reported by Suzuki et al. (2005) who suggest that a constitutive expression of *MBF1*c in *A. thaliana* transgenic plants alters the accumulation of specific transcripts under normal growth conditions and during stress. Since different stresses may disrupt the cellular homeostasis by specific manners, the plant cell might counteract the damage by different mechanisms. It could be also possible that each stress opens out specific defense mechanisms in young seedlings and the participation of *AtMBF1*s might be different depending on the stress condition imposed. During germination, *AtMBF*1s participate in the tolerance to osmotic and oxidative stress as was shown for *TAF10,* a TATA-box binding protein associated factor (TAF) (Gao et al., 2006). In general, seed tolerance to stress involves activation of intricate networks of signaling pathways. Our results indicated that *AtMBF1s* regulate the expression of the *ABR1* gene during normal

growth and stress conditions. *ABR1* is a member of the AP2/ERF transcription factor superfamily which encodes an APETALA2(AP2)-Domain transcription factor and functions as an ABA repressor. Its disruption leads to hypersensitivity to osmotic stress conditions (Pandey et al., 2005). According to our findings, Suzuki et al. (2005) described that among the transcription factors that are up-regulated in transgenic *Arabidopsis* plants over-expressing *MBF1c*, APETALA2 (AP2)-Domain transcription factor (*At5g52020*) revealed the highest level of induction. In addition, the authors suggested that *AtMBF1*c expression enhances the tolerance of transgenic plants to abiotic stress by perturbing the ethylene-response signal transduction pathway. Nevertheless, Tsuda et al. (2004) had previously reported that the up-regulation of *AtMBF1*c expression by ABA treatment might be a result of combinational effects of ABA and abiotic stress. In addition, a very close connection between ethylene and ABA signaling pathways has been revealed (Wang et al., 2007). ABA is a phytohormone that regulates seed dormancy and plays important roles in adaptation of both early seedling development and vegetative growth to abiotic stress. Thus, we conclude that the hypersensitivity of the *abc*− mutant to osmotic and oxidative stresses may be due to a perturbed regulation of ABA signaling pathway. *AtMBF1*s may function as regulatory components of the cross-talk between ABA, ethylene and stress signaling pathways during germination and early developmental stages of *A. thaliana* seedlings. A possible link with other hormones that control the dormancy and plant development might also exist.

We agree that *AtMBF1s* may act as negative regulators of HSP in *A. thaliana* seedlings. Suzuki et al. (2005) described that transcripts encoding classical HSPs did not accumulate in transgenic plants over-expressing *MBF1*c. They suggested that the enhanced tolerance of these plants to osmotic and heat-shock stress was associated with the expression of other stress-responsive genes rather than with the constitutive expression of HSPs. In plants, there is genetic evidence for a positive and negative transcriptional regulation of heat-shock genes through two classes of heat-shock factors (HSFs): activators and repressors, respectively. Thus, *AtMBF1s* may also be modulators of specific HSFs counteracting transcriptional activation either through DNA binding or through protein-protein interaction. Furthermore, HSFs and MBF1 proteins are highly conserved among eukaryotes. The potato transcriptional co-activator StMBF1 is up-regulated in response to oxidative and heat-shock stress (Arce et al., 2006). Considering these facts, we agree with Miller et al. (2008) who proposed wide roles of *MBF1*c in the plant responses to different stresses.

Finally, our data together with previous evidences support a role for *AtMBF1* gene family in the cross-talk between ethylene, ABA and stress signaling pathways. Further studies will be necessary to reveal specific functions for each gene and how they are functionally related. However, these evidences might also be of impact to plant genetic manipulation and agriculture.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jplph.2009.09.003.

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