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MBF1s regulate ABA-dependent germination of Arabidopsis seeds

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Abbreviations: ABA, abscisic acid; *abc*-, *Arabidopsis thaliana* triple knock-down mutant for *MBF1* genes; *abc*- +*c*, *abc*- mutant complemented with *AtMBF1c* gene over-expression; ABR1, absisic acid repressor; ACC, 1-aminocyclopropane-1-carboxylic acid; ERF, ethelyne responsive factor family; H_2O_2, hydrogen peroxide; MBF1, multiprotein bridging factor 1; MV, methyl viologen

Transcriptional co-activators of the multiprotein bridging factor 1 (MBF1) control gene expression by connecting transcription factors and the basal transcription machinery. In *Arabidopsis thaliana* functions of *MBF1* genes have been related to stress tolerance and developmental alterations. Endogenous ABA plays a major role in the regulation of Arabidopsis seed dormancy and germination. Seed dormancy and ABA sensitivity are enhanced in ethylene insensitive mutants suggesting that ethylene signal transduction pathway is necessary to fully develop ABA-dependent germination. In this report we showed that a triple knock-down mutant for Arabidopsis *MBF1* genes (*abc*-) has enhanced seed dormancy and displays hypersensitivity to exogenous ABA. In addition, higher ABA contents were detected in *abc*- seeds after imbibition. These evidences suggest a negative role of *MBF1s* genes in ABA-dependent inhibition of germination. The participation of MBF1s in ethylene signal transduction pathway is also discussed.

Introduction

The multiprotein bridging factor 1 (MBF1) type controls gene expression by connecting transcription factors and the basal transcription machinery.[1,2] In *Arabidopsis thaliana* there are three genes: *AtMBF1a* (*At*2g42680), *AtMBF1b* (*At*3g58680) and *AtMBF1c* (*At*3g24500), encoding MBF1 proteins.[3] Several evidences relate *AtMBF1* functions with tolerance to stress conditions.[4-7] Analysis of *AtMBF1c* overexpressing plants and null mutant unraveled the role of *MBF1c* during osmotic stress and thermotolerance.[5-7] Constitutive overexpression of *AtMBF1a* leads to elevated salt tolerance, insensitivity to glucose and resistance to fungal disease.[4] Other reports link *MBF1* functions with developmental alterations, such as plant size, leaf cell expansion and ploidy levels.[8,9] In addition, a loss-of-function *AtMBF1c* mutant, showed a reduction on seed germination.[8]

Initially, *MBF1* genes were related to ethylene signal transduction pathway. The first *MBF1* gene was identified in tomato fruits and it was named *ER24* by ethylene-responsive transcriptional co-activator.[10] *AtMBF1c* overexpressing plants accumulate transcripts associated with ethylene signaling and exhibit a stronger triple-response phenotype.[5] These results suggest that *MBF1* genes are positive regulators of ethylene signaling.

We reported the analysis of an *Arabidopsis thaliana* triple knock-down mutant for *MBF1* genes (*abc*-) under oxidative and osmotic stress conditions.[11] We showed that *abc*- mutant seedlings were more sensitive than wild type (WT) to hydrogen peroxide (H_2O_2) and methyl viologen (MV). Inhibition of seed germination by oxidative treatments and osmotic stress was enhanced by the *abc*- mutation. In addition, we showed that *AtMBF1s* regulate the expression of the Abscisic Acid Repressor (*ABR1*) gene. *ABR1* transcript levels were strongly reduced in the *abc*- mutant under normal conditions. WT seedlings treated with MV showed a reduction in *ABR1* transcript levels meanwhile, *abc*- seedlings were unable to regulate *ABR1* expression. ABR1 is a transcription factor of the Ethelyne Responsive Factor family (ERF) with an APETALA2 domain and it was described as an ABA response repressor during germination and root growth.[12] In Arabidopsis, disruption of *ABR1* gene leads to hypersensitivity to osmotic stress and to ABA application during seed germination and root growth assays.[12] In our previous report we suggested that the reduced tolerance to oxidative stress in the *abc*- may be due to a perturbed

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regulation of the ABA signaling pathway.[11] In addition, there are evidences connecting ABA and ethylene signaling cascades. The insensitive ethylene mutants *etr1* and *ein2* show phenotypes with enhanced dormancy, ABA hypersensitivity during germination and augmented levels of ABA, resembling ABA-signaling mutants.[13,14] *Ein 2* null mutant is also supersensitive to both salt and osmotic stress conditions.[15] All these data suggest that a functional ethylene signal transduction pathway is necessary to fully develop several ABA responses. As we commented before there are evidences that *MBF1* genes positively regulate ethylene signaling pathway. Thus, in this report we explored the impact of *AtMBF1* genes on inhibition of germination, a typical ABA-dependent response.

Results

Seed dormancy is enhanced in ABA-hypersensitive mutants, such as *era 1* as well as in the ethylene insensitive mutants (*ctr1, ein2 ein3*).[13-17] We analyzed the effect of *AtMBF1* mutations on seed dormancy of *abc-* seeds and compared with *ein3–1* mutant and *abc-* complemented with *AtMBF1c* gene overexpression (*abc- +c*).[11,16] Since stratification of Arabidopsis seeds breaks dormancy,[18] we determine seed germination of WT or mutant seeds previously incubated or not for 4 d at 4°C (**Fig. 1A**). Germination rate did not differ among the lines upon 24 h stratification. However, the non-stratified *abc-* seeds showed a lower germination rate (10%) compared with WT (20%). Germination of *ein3–1* was similar to *abc-*. However, germination of *abc- +c* was similar to WT indicating that *AtMBF1c* overexpression rescued the mutant phenotype. When developmental stages were analyzed at 48 h, according to Boyes et al.,[19] only 40% of non-stratified *abc-* seeds reached stage 0.7 compared with 90% of the WT (**Fig. 1B**). Eventually all the non-stratified seeds germinated and reached stage 0.7 (data not shown).

In Arabidopsis, mutants with enhanced seed dormancy also showed increased sensitivity to exogenous ABA during germination.[13,14,17] Therefore, to clarify ABA sensitivity of the *abc-* mutant, ABA dose-response experiments were performed during germination of stratified WT and mutant seeds (**Fig. 2A**). Germination rates of *abc-* and *ein3–1* seeds were 20% lower than WT for the tested concentrations. *abc- +c* showed germination rates similar to WT.

It is well known that 1-aminocyclopropane-1-carboxylic acid (ACC) is an intermediate in the conversion of methionine to ethylene and that ACC synthesis, mediated by the enzyme ACC synthase, determines the rate of ethylene production. Exogenous ACC induces ACC oxidase leading to ethylene biosynthesis.[20] Moreover, ABA regulates ACC synthase and ACC oxidase genes in mung bean and during tomato fruit ripening.[21,22] Ghassemian et al.[14] described that ABA-dependent inhibition of WT germination is partially rescued in a dose dependent manner by ACC, while ACC alone does not enhance germination in Arabidopsis. Effects of ACC on enhancement of germination in *abc-* were weaker than those in WT and the complemented line (**Fig. 2B**). Taken together these results suggest that MBF1 negatively regulates ABA-dependent inhibition of germination by positively regulating ethylene signaling.

Next, we analyzed ABA content in non-stratified mature seeds of WT, *abc-* and *abc- +c* lines imbibed for different times (**Fig. 3**). At 8 h after imbibition all seeds showed a similar increase in ABA content. A similar increase on ABA levels has been reported for non dormant Arabidopsis seeds during the first hours after imbibition.[23] At 16 h, ABA content decreased at a slower rate in *abc-* compared with *abc- +c* and WT seeds.

Discussion

The enhanced seed dormancy of *abc-* mutant seeds and their hypersensitivity to exogenous ABA (**Figs. 1 and 2A**) suggest a negative role of *MBF1s* genes in ABA-dependent inhibition of

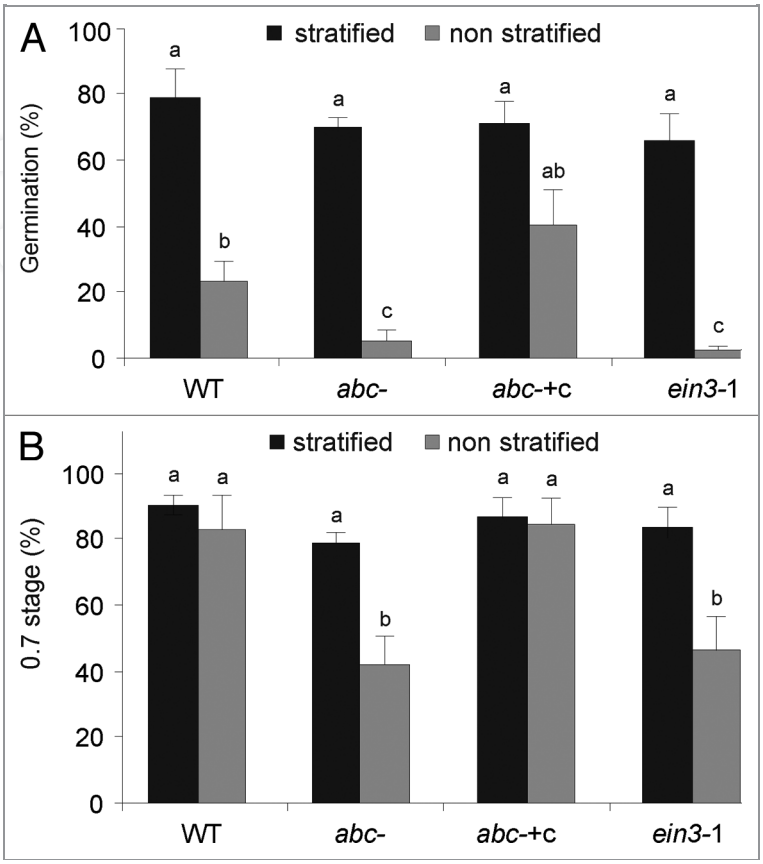

Figure 1. Mutations in *AtMBF1* genes enhance seed dormancy. Seeds from WT, *abc-*, and *abc-* +c plants were surface-sterilized and stratified or not at 4°C for 2 d in the dark to break dormancy. Seeds were plated on ATS medium with 0.8% agar and placed on a growth chamber at 23°C with a 16 h-light photoperiod. (A) The percentage of germination was scored after 24 h. (B) The percentage of 0.7 stage according to Boyes et al.[19] was evaluated after 48 h. Approximately, 100 seeds were processed per line in each experiment. Data are mean values (± SE) of five independent experiments. Different letters indicate a significant difference at $p < 0.05$ (Tukey's test).

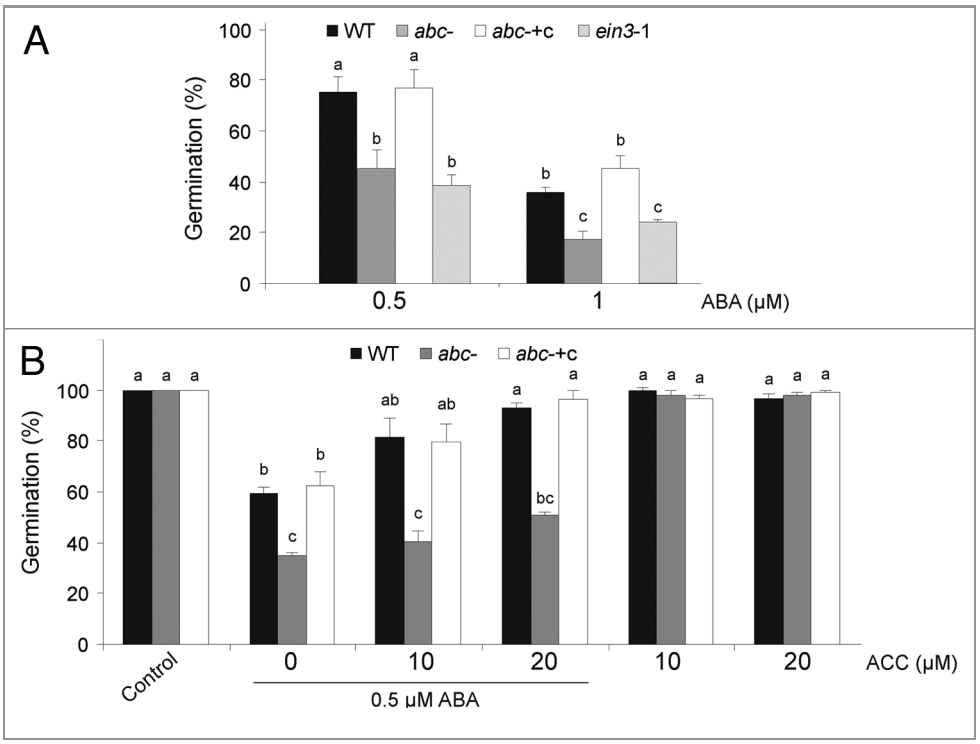

Figure 2. *abc-* mutant is hypersensitive to ABA during germination. (A) Sterilized and stratified WT, *abc-*, *abc-* +c and *ein3*-1 seeds were plated on ATS agar medium supplemented with the indicated concentrations of ABA and incubated in the growth chamber. (B) Sterilized and stratified WT, *abc-* and *abc-* +c seeds were plated on ATS agar supplemented with ABA, ACC or the combination of both as indicated. In (A) and (B) the percentage of germination was scored after 24 h. Approximately, 100 seeds were processed per line in each experiment. Data are mean values (± SE) of five independent experiments. Different letters indicate a significant difference at $p < 0.05$ (Tukey's test).

germination. Unlike ethylene insensitive mutants (*ein2*, *ein3* or *etr1*)[14,15] *abc-* root growth was not altered in the presence of exogenous ABA (data not shown), suggesting that MBF1s may modulate specific ABA-dependent responses.

Our results unravel new evidence that connects *AtMBF1* genes to the ethylene signal transduction pathway. First, *abc-* mutant resembled *ein3* responses in all the assays. Second, ABA inhibition of germination could not be fully rescue by exogenous ACC (**Fig. 1B**), suggesting that *abc-* ability to sense ACC is compromised.

An *Oryza sativa* MBF1 was reported to interact "in vitro" with ERF2 and ERF4 transcription factors.[24] Furthermore, *Arabidopsis thaliana* plants overexpressing *ERF4* have less sensitivity to ABA and are hypersensitive to osmotic stress.[25] Thus, we speculate that AtMBF1s might be interacting with specific ERFs such as

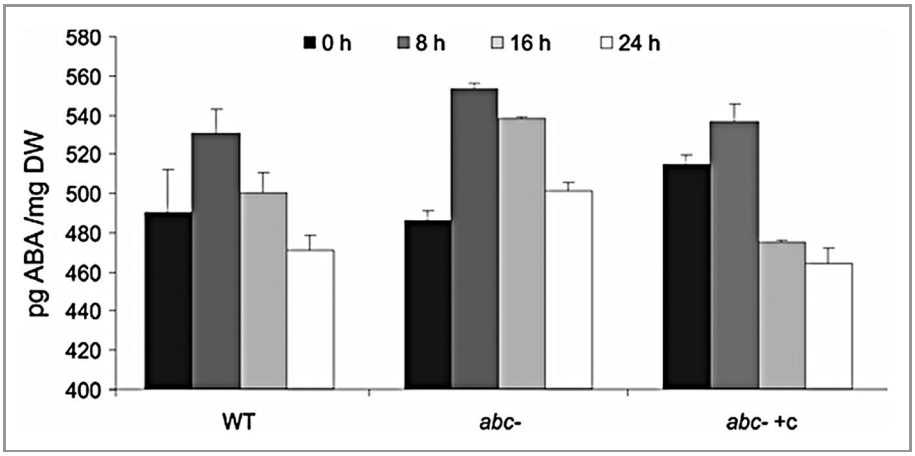

Figure 3. *abc-* mutant accumulates ABA in seeds. Seeds from WT, *abc-*, and *abc-* +c plants were imbibed in sterile water for the designated times and assayed for ABA content by radioimmunoassay. Each sample was assessed twice. The results presented are the mean value of two biological replicates ± SE.

ABR1 and ERF4 to modulate ABA-dependent germination in Arabidopsis seeds.

Moreover, higher ABA contents were detected in *abc-* seeds at 16 h after imbibition, suggesting that *AtMBF1* genes positively regulate ABA degradation during early hours after imbibition (**Fig. 3**). Supporting our data, Arabidopsis mutants insensitive to ethylene also show increased endogenous ABA concentrations. The ethylene insensitive mutant *ein2* accumulates ABA in green tissue and this accumulation is related to an increased of ABA biosynthesis.[14] ABA levels in mature dry seeds of the *etr1–2* mutant were 10-fold higher than in WT seeds.[26]

The expression pattern of some key genes regulated by ABA or ethylene in the mutants could provide additional evidences on MBF1-mediated interaction between these two hormones. Since it has been suggested that ABA and ethylene may control the hormonal biosynthesis, catabolism, or signaling of each other to enhance their antagonistic effects upon seed germination,[27] it would be interesting to further explore the influence of *MBF1* genes on these hormone cross-talks.

Materials and Methods

Plant material and growth conditions. *Arabidopsis thaliana* (L.) Heynh. wild type, *abc-* and *ein3–1* mutants used in this study were of ecotype Columbia. The *abc-* mutant line is a T-DNA insertion mutant for AtMBF1a (At2g42680), AtMBF1b (At3g58680) and AtMBF1c (At3g24500) genes. Their genetic and phenotypic characteristics have been described by Arce et al.[11] The *ein3–1* is an ethylene insensitive mutant with a loss-of-function mutation for *EIN3* gene (At3g20770).[17] Plants were grown at 22–24°C under fluorescent light 120 μmol photons m^{-2} s^{-1} with a16-h-photoperiod. 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 until senescence.

Germination Assays. To quantify dormancy, seeds 1 mo-old after harvest were surface-sterilized in 30% commercial bleach and 0.02% Triton X-100 for 15 min, rinsed four times with sterile water and stratified or not at 4°C for 2 d in the dark to break dormancy. Then, seeds were plated on ATS medium with 0.8% agar and placed on a growth chamber at 23°C with a 16 h-light photoperiod. The percentage of germination (fully emerged radicle tip) was evaluated after 24 h of incubation. The percentage of 0.7 stage according to Boyes et al.[19] was determined after 48 h of incubation. Measurements of ABA sensitivity were conducted with 1 to 3 mo-old seeds. Seeds were surface-sterilized, stratified at 4°C for 2 d in the dark. Seeds were plated on ATS medium with 0.8% agar containing various concentrations of ABA in combination or not with various concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid and placed on a growth chamber. The percentage of germination was scored after 24 h.

ABA determination. One-mo-old seeds were imbibed in sterile water for different times at 22°C, lyophilized, powdered, weighed and stored at –20°C. ABA content was determined by radio-immunoassay as described in Steinbach et al.[28] This method uses the monoclonal antibody AFRC MAC 252[29] and tritiated-ABA (Amersham-Pharmacia). Each sample was assessed twice.

Statistical analysis. The values shown in each figure are mean values ± SE. The data were subjected to analysis of variance (one-way ANOVA) and post hoc comparisons were done with Tukey's multiple range test at $p < 0.05$ level. The statistical software program used was SigmaStat 3.1.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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