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Arabidopsis DREB2C modulates ABA biosynthesis during germination

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ABSTRACT

Plant dehydration-responsive element binding factors (DREBs) are transcriptional regulators of the APETELA2/Ethylene Responsive element-binding Factor (AP2/ERF) family that control expression of abiotic stress-related genes. We show here that under conditions of mild heat stress, constitutive overexpression seeds of transgenic *DREB2C* overexpression *Arabidopsis* exhibit delayed germination and increased abscisic acid (ABA) content compared to untransformed wild-type (WT). Treatment with fluridone, an inhibitor of the ABA biosynthesis abrogated these effects. Expression of an ABA biosynthesis-related gene, 9-*cis*-epoxycarotenoid dioxygenase 9 (*NCED9*) was up-regulated in the *DREB2C* overexpression lines compared to WT. DREB2C was able to trans-activate expression of *NCED9* in *Arabidopsis* leaf protoplasts *in vitro*. Direct and specific binding of DREB2C to a complete *DRE* on the *NCED9* promoter was observed in electrophoretic mobility shift assays. Exogenous ABA treatment induced *DREB2C* expression in germinating seeds of WT. Vegetative growth of transgenic *DREB2C* overexpression lines was more strongly inhibited by exogenous ABA compared to WT. These results suggest that *DREB2C* is a stress- and ABA-inducible gene that acts as a positive regulator of ABA biosynthesis in germinating seeds through activating *NCED9* expression.

1. Introduction

The plant growth and development are precisely orchestrated by endogenous signals and external cues. The phytohormone ABA is a key internal factor in regulating many aspects of plant growth and development, including seed and bud dormancy, seed germination and vegetative growth, inhibition of flowering, regulation of stress-responsive genes, stomatal closure, and alteration of susceptibility to pathogen infection [1]. Uncovering molecules that control tissue ABA content is therefore important for manipulating many of these plant processes.

Expression and regulation of ABA biosynthetic genes at the molecular level is not fully understood [2]. Through a combination of forward genetic studies and biochemical analyses, several components of the ABA biosynthetic pathway have been elucidated in *Arabidopsis* [2–4]. The first step of the ABA biosynthetic pathway that leads to ABA deficiency when inactivated by mutation is the conversion of zeaxanthin to *trans*-violaxanthin by zeaxanthin epoxidase, the enzyme encoded by *ABA1*. *trans*-Violaxanthin is converted to *trans*-neoxanthin, by a reaction dependent on the product of the *ABA4* locus. *trans*-Neoxanthin, is isomerized to 9-*cis*-neoxanthin by an as-yet unidentified enzyme. The first and rate limiting committed step in ABA biosynthesis is the cleavage of 9-*cis*-neoxanthin by the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED) to yield xanthoxin. Nine putative *NCED* genes are encoded in the *Arabidopsis* genome, providing functional redundancy. All of the above reactions take place in the chloroplast. Xanthoxin, however, moves from plastids to the cytoplasm where it is converted via a number of steps to ABA-aldehyde. The last step in the generation of ABA-aldehyde is catalyzed by a short chain dehydrogenase/reductase-like enzyme encoded by *ABA2*. ABA-aldehyde is then converted into ABA by abscisic aldehyde oxidase 3 (AAO3) that requires a sulfurated molybdenum cofactor. The sulfurase that produces the functional cofactor is encoded by *ABA3*. Most ABA biosynthetic genes such as *ABA1-4*, *NCED*s and *AAO3* are induced by endogenous developmental signals and up-regulated by abiotic stresses to various extents [5–8]. Thus, unraveling the transcriptional regulation of ABA biosynthetic genes is crucial for understanding ABA biosynthesis and regulation.

The plant-specific dehydration-responsive element (*DRE*; TACCGACAT) is one of the major *cis*-acting elements that regulate gene transcription during abiotic stress. It functions as a component of both ABA-dependent and ABA-independent pathways of abiotic stress response [9]. DRE binding factors (DREBs) have been reported to function in drought, high salinity, oxidative stress, and heat responses in *Arabidopsis* [10–13]. Among DREBs, the DREB2s

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have been reported to directly regulate expression of *heat shock transcription factor A3* (*HsfA3*) under conditions of heat stress [13,14].

Here we provide genetic evidence that transgenic overexpression of *DREB2C* in *Arabidopsis* leads to delayed seed germination under mild HS or in response to exogenous ABA and elevation of tissue ABA content. Furthermore, we provide molecular and biochemical evidence that DREB2C can up-regulate the ABA biosynthetic gene *NCED9* in germinating *Arabidopsis* seeds. Our results suggest that DREB2C serves as a positive regulator for ABA biosynthesis in germinating seeds under heat stress conditions.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana L. Heynh. plants used in this study were in the Columbia (Col-0) background. The transgenic *35S:DREB2C* lines and *Promoter*$_{DREB2C}$*:GUS Arabidopsis* line used in this study have been described by Lim et al.[11] and Chen et al. [15], respectively. Homozygous T_3 lines were used.

Plants were either grown aseptically or on soil in a growth chamber at 22 °C under the long-day conditions (16 h light/8 h dark, 100 μE m^{-2} s^{-1}). For aseptic growth, seeds were sterilized and plated on half-strength MS medium (1/2MS) containing vitamins, 0.25% phytagel, and 0.05% MES (pH 5.7) but lacking phytohormones. The plates were incubated at 4 °C for 3 d in the dark to break residual dormancy and then transferred to the growth chamber.

2.2. Fluridone treatment and seed germination

Seeds were collected from fully matured siliques of dehydrated plants of the same age for seed germination assays and were stored in the dark at room temperature until use. Stratified seeds (>80 seeds for each replicate) were germinated on 1/2MS medium without or with 10 μM fluridone (Duchefa) supplement. The plates were transferred to growth chambers set to 22 °C or 33 °C and germination was scored daily through 3 d after transfer to the growth chamber.

2.3. ABA assay

ABA extraction was performed by a slight modification of the procedure described by Lin et al. [2]. Plant tissues were homogenized in liquid nitrogen, and extracted by overnight agitation in extraction buffer (80% methanol and 2% glacial acetic acid) at 4 °C in the dark. Insoluble material was removed by centrifugation at 4000 rpm for 15 min and the clear supernatant was evaporated to dryness at 4 °C in a speedvac. The powdery residue was dissolved in 100% methanol plus 0.2 M $NH_4H_2PO_4$ (pH 6.8). To reduce background signals in the immunoassay for ABA, each extract was first passed through a polyvinylpolypyrrolidone micro-spin column (Sigma–Aldrich) to remove pigments and then through a Sep-Pak C18 cartridge (Waters) to remove non-polar compounds. The Sep-Pak elute was dried in a speedvac and suspended in TBS buffer for immunoassay [16]. ABA concentration was determined by ELISA using the Phytodetek ABA kit (Agdia). Absorbance was measured at 405 nm using a Multiskan® FC microplate photometer (ThermoFisher Scientific).

2.4. Analysis of gene expression

Comparative analysis of gene expression levels was performed by RT-PCR as described previously [11]. Several aliquots of each cDNA sample were subjected to 30 cycles of PCR with primers for *Arabidopsis Actin2* and the volume of each cDNA required to give an arbitrary fixed exponential-phase PCR signal strength was determined. This volume of each cDNA sample was then used as template for PCR amplification of the genes targeted for expression analysis. PCR was performed using the gene-specific primers listed in Supplementary Table S1.

2.5. Promoter transactivation assay

The effector construct (*35SΩ:DREB2C*) contained *DREB2C* cDNA fused in-frame at its N-terminus to a FLAG-tag sequence and has been described [13]. Constitutive overexpression of *DREB2C* in this construct was directed by a chimeric promoter consisting of the *CaMV35S* promoter and the TMV translation enhancing omega element (*Ω*). A 1.0 kb fragment of the *NCED9* upstream regulatory region that contains one core *DRE* (*cDRE*) and one partial *DRE* (*pDRE*) element, was inserted upstream of the β-glucuronidase (*GUS*) reporter gene in a *pUC19*-derived plasmid vector [17] to yield the reporter construct *pNCED9:GUS*. The *NCED9* upstream regulatory region was subjected to mutation by the megaprimer-PCR method [18] using sequence-specific primers to generate the reporter plasmids *pNCED9(mc):GUS*, *pNCED9(mp):GUS* and *pNCED9(mcmp):GUS* that harbor mutations in the *cDRE*, *pDRE* and both these elements, respectively. Protoplast isolation and polyethylene glycol (PEG)-mediated DNA transfection was performed as described [19].

2.6. Electrophoretic mobility shift assay (EMSA)

The recombinant glutathione-S-transferase (GST)-DREB2C$^{(145–528)}$ fusion protein was expressed in *Escherichia coli* BL21 (DE3) and purified by Protino® Glutathione Agarose 4B chelation affinity chromatography. EMSA was performed as described previously [13], using the [α-^{32}P]dATP end-labeled synthetic ds-DNA probes.

2.7. Histochemical GUS staining and seed germination assay

Histochemical localization assays were performed as described by Jefferson [20]. Transgenic *Promoter*$_{DREB2C}$*:GUS Arabidopsis* plants were imbibed and allowed to germinate in 1/2MS plates without or containing 10 μM ABA.

To compare the ABA effect on seed germination, seeds were primed for 3 days at 4 °C on 1/2MS medium without or with 0.5 or 1.0 μM ABA, respectively. After stratification, the plates were placed vertically in a growth chamber set to long-day conditions at 22 °C. Shoot weight, length of the primary root and ABA content were determined on day 10 after transfer to the growth chamber.

3. Results and discussion

3.1. ABA synthesis in imbibed DREB2C seed is responsible for thermoinhibition of germination

Seeds of two independent transgenic *DREB2C* overexpression lines (*35S:DREB2C*-a and -c) and untransformed WT were imbibed and stratified for 3 d in the dark at 4 °C, and then allowed to germinate under long-day conditions at 22 °C and 33 °C in order to test the effect of temperature on germination rate. Under normal growth conditions (22 °C) there was very little difference between the three lines (Fig. 1A). Slightly lower germination rates were observed in the *35S:DREB2C* transgenic lines on day1 after imbibition (1 DAI) but the germination rates of all three lines were 100% by 2 DAI. Germination rate of WT was unaffected when growth temperature was raised to 33 °C (compare Fig. 1A and B). However,

germination rate was greatly reduced in the *35S:DREB2C* transgenic lines at 33 °C, with only 30–60% of the seeds having germinated by 3 DAI compared to 100% in WT (Fig. 1B). Heat stress increases ABA concentration in plant cells [8]. Therefore, inhibition of germination in *35S:DREB2C* transgenic seeds under conditions of mild heat stress could be due to either the heightened sensitivity to ABA or elevated level of ABA. ABA measurements revealed that ABA content of WT was slightly lower than that of the *35S:DREB2C* transgenic lines in imbibed seeds at day 0 (Fig. 1C and D). ABA content decreased over time in both WT and *35S:DREB2C* transgenic lines and there was no significant difference in ABA content between the WT and *35S:DREB2C* transgenic lines by 3 DAI at 22 °C (Fig. 1C), whereas in seeds incubated at 33 °C, ABA content decreased much more slowly in the *35S:DREB2C* transgenic lines compared to WT. ABA content of WT remained approximately 20% lower than that of the two *35S:DREB2C* transgenic lines from 1 through 3 DAI (Fig. 1D). Thus altered ABA content contributes to the thermo-inhibition of germination in seeds of *35S:DREB2C* transgenic plants.

Fluridone is an inhibitor of phytoene desaturase and fluridone treatment reduces ABA biosynthesis in plants [21]. We hypothesized that if delayed germination of the *35S:DREB2C* transgenic

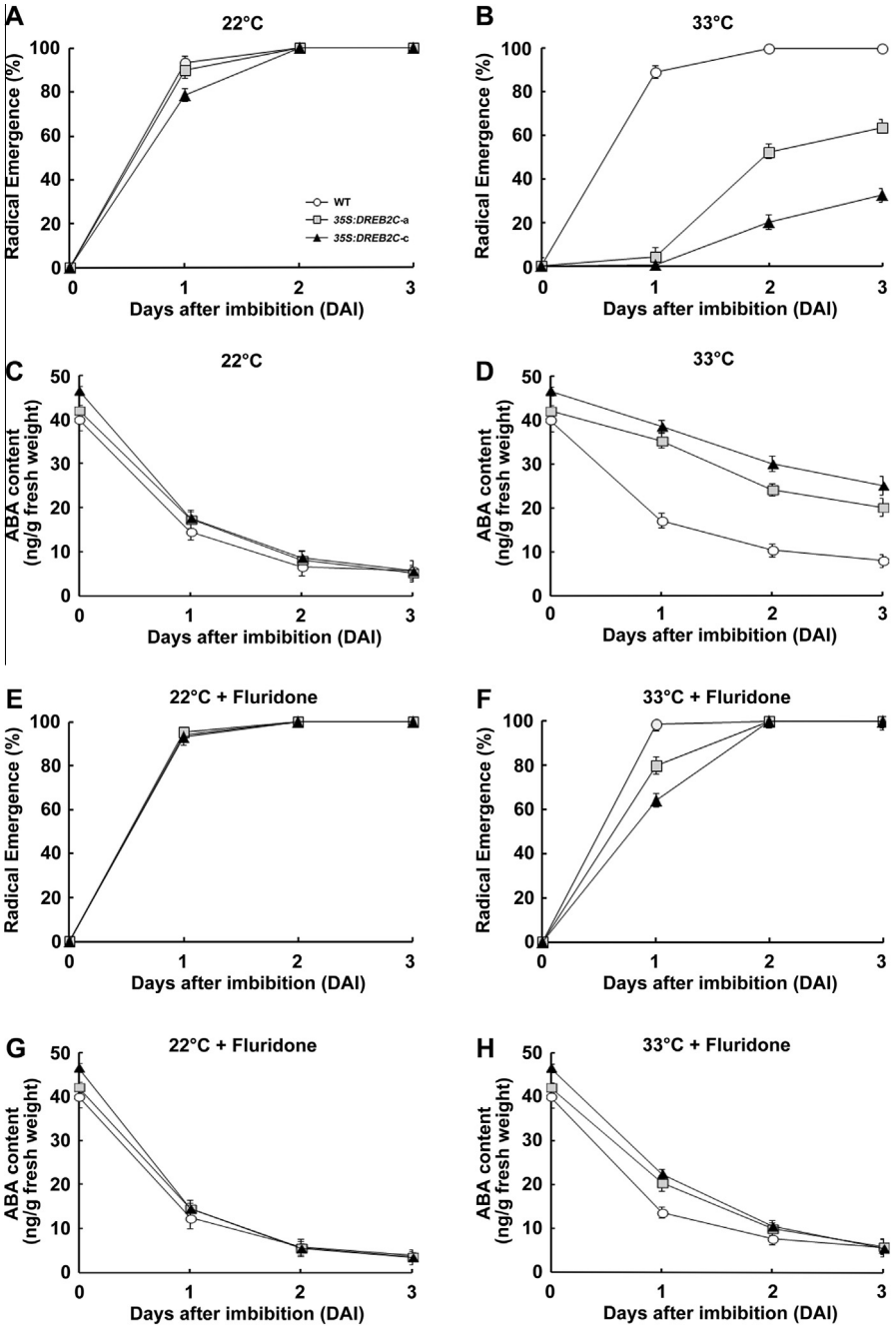

Fig. 1. Thermo inhibition of germination of *DREB2C* overexpression plants requires *de novo* ABA biosynthesis. Seeds of WT and two independent *35S:DREB2C* lines (*35S:DREB2C*-a, *35S:DREB2C*-c) were surface-sterilized, plated on 1/2MS (A–D) or 1/2MS containing 10 μM fluridone (E–H) and stratified in the dark at 4 °C for 3 d before shifting the plates to a growth chamber set to the indicated temperatures. Percent radical emergence and ABA content was measured at 24 h intervals after shifting to a growth chamber set at 22 °C (A, C, E, G) or 33 °C (B, D, F, H). Values represent mean ± *SD* ($n = 3$).

lines is due to increased ABA biosynthesis, then fluridone treatment should abrogate this phenotype. To test this hypothesis, surface-sterilized seeds of WT and *35S:DREB2C* transgenic lines were plated on medium supplemented with 10 μM fluridone, imbibed, stratified and allowed to germinate as described above, and then percent germination as well as ABA content was compared over time. Under normal growth conditions, the germination rate of WT was not affected by fluridone treatment, but slight difference in germination rates of WT and *35S:DREB2C* transgenic lines that was evident at 1 DAI on normal growth medium was abolished on the fluridone-containing medium (compare Fig. 1A and E), suggesting that germination rates of the *35S:DREB2C* transgenic lines were improved on fluridone-containing medium. This conclusion was strengthened by the observation of germination rates at 33 °C. At 33 °C, the germination rate of WT was slightly affected by fluridone treatment, but germination rates of the *35S:DREB2C* transgenic lines were much improved on the fluridone-containing medium compared to normal growth medium by 1 DAI and by 2 DAI germination rates of were the same as that of WT (compare Fig. 1B and F). At 22 °C, ABA content of imbibed seeds of WT and *35S:DREB2C* transgenic lines on medium supplemented with fluridone (Fig. 1G) were ≈3 ng lower than their ABA contents on medium without fluridone (Fig. 1C) at 1, 2, and 3 DAI. However at 33 °C, ABA content in germinating seeds of the *35S:DREB2C* transgenic lines was much lower compared to germination on fluridone-containing medium than on normal medium, reaching WT level by 3 DAI (compare Fig. 1D and H). Collectively, these results showed

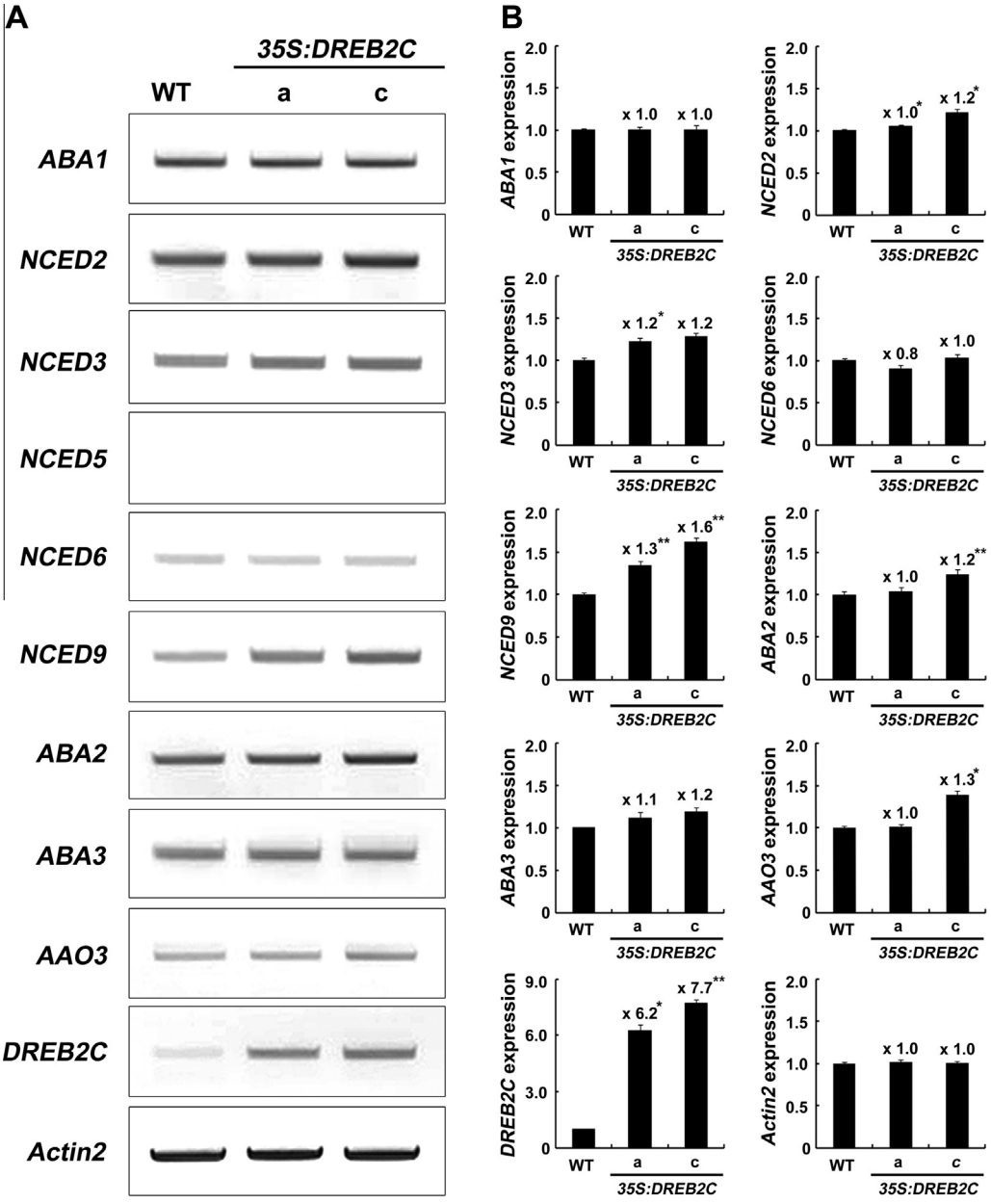

Fig. 2. *NCED9* transcript level is elevated in *DREB2C* overexpression lines. RNA was extracted from seedlings of 2 d old untransformed WT and *DREB2C* overexpressors (*35S:DREB2C*-a and -c) grown at 22 °C. (A) Shown are the results of RT-PCR analysis that was performed on total RNA. *Actin2* was amplified as loading control. Each PCR reaction was conducted in triplicate with the same results. (B) Signal intensity in the bands shown was measured using ImageJ software and expressed relative to the signal intensity of WT. Bars represent mean ± *SD* ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t* test).

that overexpression of *DREB2C* increased ABA concentration in germinating seeds and that this increase was mitigated by inclusion of an ABA biosynthesis inhibitor in the growth medium. These effects of *DREB2C* overexpression on germination inhibition were more readily evident at 33 °C. Therefore, inhibition of germination in *35S:DREB2C* transgenic seeds under conditions of mild heat stress could be due to increased ABA biosynthesis under this stress. These results suggest that DREB2C positively regulates ABA biosynthesis during seed germination.

3.2. Overexpression of DREB2C affects expression of genes encoding components of the ABA biosynthetic pathway

Next we performed quantitative RT-PCR to compare the expression of major ABA biosynthesis genes at 2 DAI by in WT and *35S:DREB2C* seedlings that were allowed to germinate at 22 °C. The genes that were examined were *ABA1*, *NCED2, 3, 5, 6 and 9*, *ABA2*, *ABA3* and *AAO3*. As shown Fig. 2, only expression of *NCED9* was slightly induced in *35S:DREB2C* seedlings under non-stressed

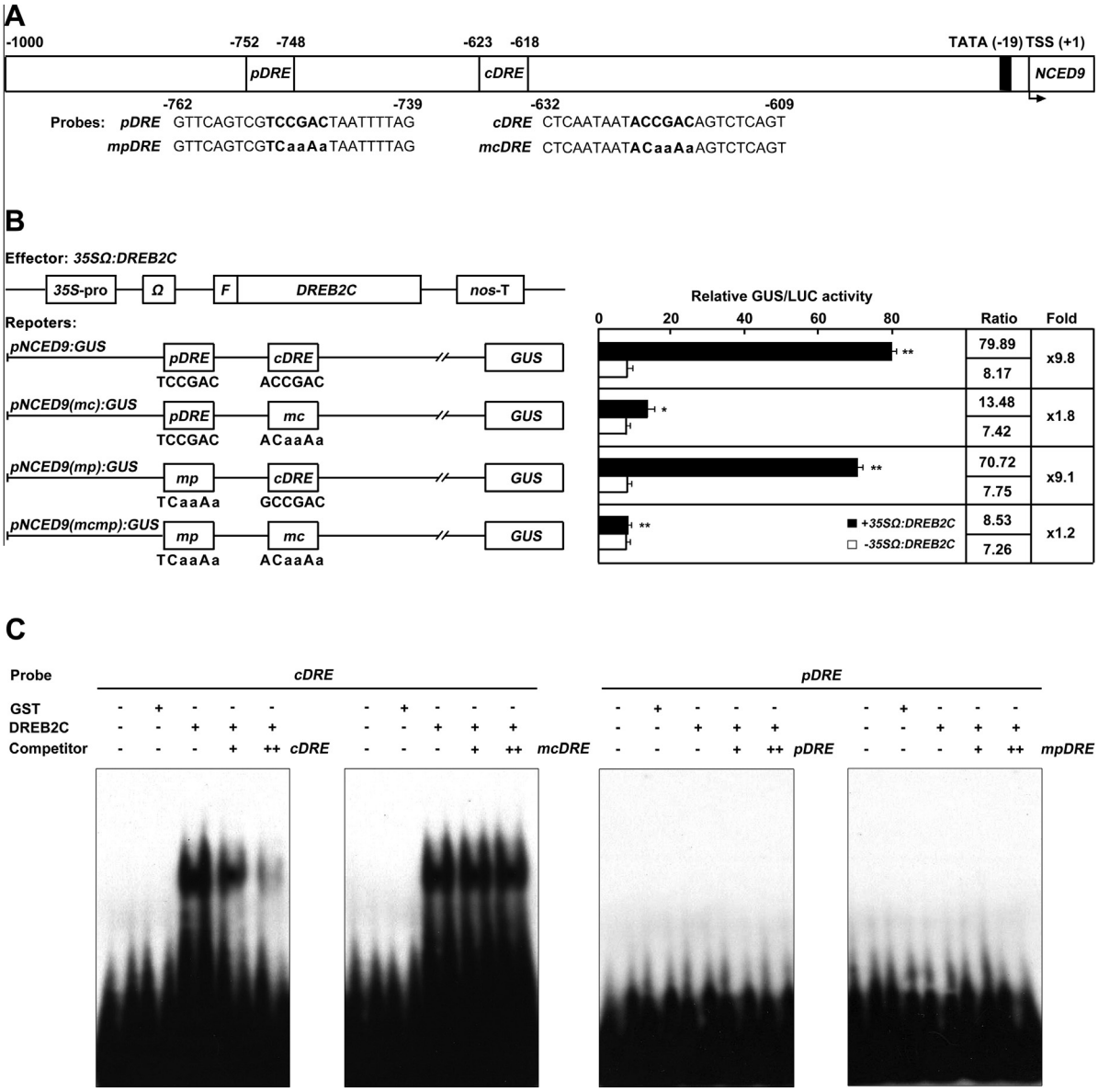

Fig. 3. DREB2C binds to *DRE* motif in the *NCED9* promoter. (A) Schematic diagram of the *NCED9* promoter indicating the location of core *DRE* (*cDRE*), partial *DRE* (*pDRE*), putative TATA-box, the predicted transcription start site (TSS) and the *NCED9* transcript (arrow). Numbers indicate nucleotide positions relative to TSS (+1). Sequences of the oligonucleotides used in EMSA (Probes) are shown. The *DREs* in the probes (in bold) are *cDRE* (at position −623 to −618 bp), *pDRE* (at −752 to −748 bp), mutant *cDRE* (*mcDRE*) and *pDRE* (*mpDRE*). Mutations indicated in lower case letters. (B) *trans*-Activation of *NCED9* by DREB2C in *Arabidopsis* leaf protoplasts. Transient assays were performed by co-transformation of the effector construct (*35SΩ:DREB2C*) with reporter constructs containing indicated *NCED9* promoter variants fused to *GUS* and a construct containing LUC (not shown) for normalization for transformation efficiency. The effector construct for constitutive overexpression of *DREB2C* contained N-terminal Flag-tagged *DREB2C* under control of the *CaMV35S* promoter (*35S*-pro) and the TMV *Ω* enhancer (*Ω*). The reporter construct *pNCED9:GUS* contained the native *NCED9* promoter fragment indicated in (A). *pNCED9(mc):GUS*, *pNCED9(mp):GUS*, and *pNCED9(mcmp):GUS* contained the same promoter fragment with indicated base substitution mutations at *cDRE* and *pDRE* in (A). The histogram denotes GUS/LUC activity in transformants receiving the corresponding reporter construct along with or without the effector construct as indicated. The mean value depicted by each bar is indicated under Ratio. Fold represents the fold-increase in GUS/LUC activity in transformants receiving both reporter and effector compared to the GUS/LUC activity in transformants receiving reporter only. Bars represent the mean ± *SD* ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test). (C) EMSA of *DRE*-binding activity of DREB2C. Shown are the autoradiograms of gels used to analyze binding reactions of the indicated composition, where minus (−) indicates omission, plus (+) indicates addition and double plus (++) indicates double amount compared to (+) for the same component. The sequence of the ^{32}P-labeled oligonucleotides used as probes and the unlabeled oligonucleotides used as competitors is shown in (A). Purified recombinant GST and GST-DREB2C$^{(148–528)}$ were used as test proteins.

conditions (22 °C). Previous reports showed that *DREB2C* and *NCED9* expression was up-regulated by high temperature [8,11] and NCED9 plays a major roles in high temperature-induced ABA synthesis and germination inhibition [8], we suggests that DREB2C

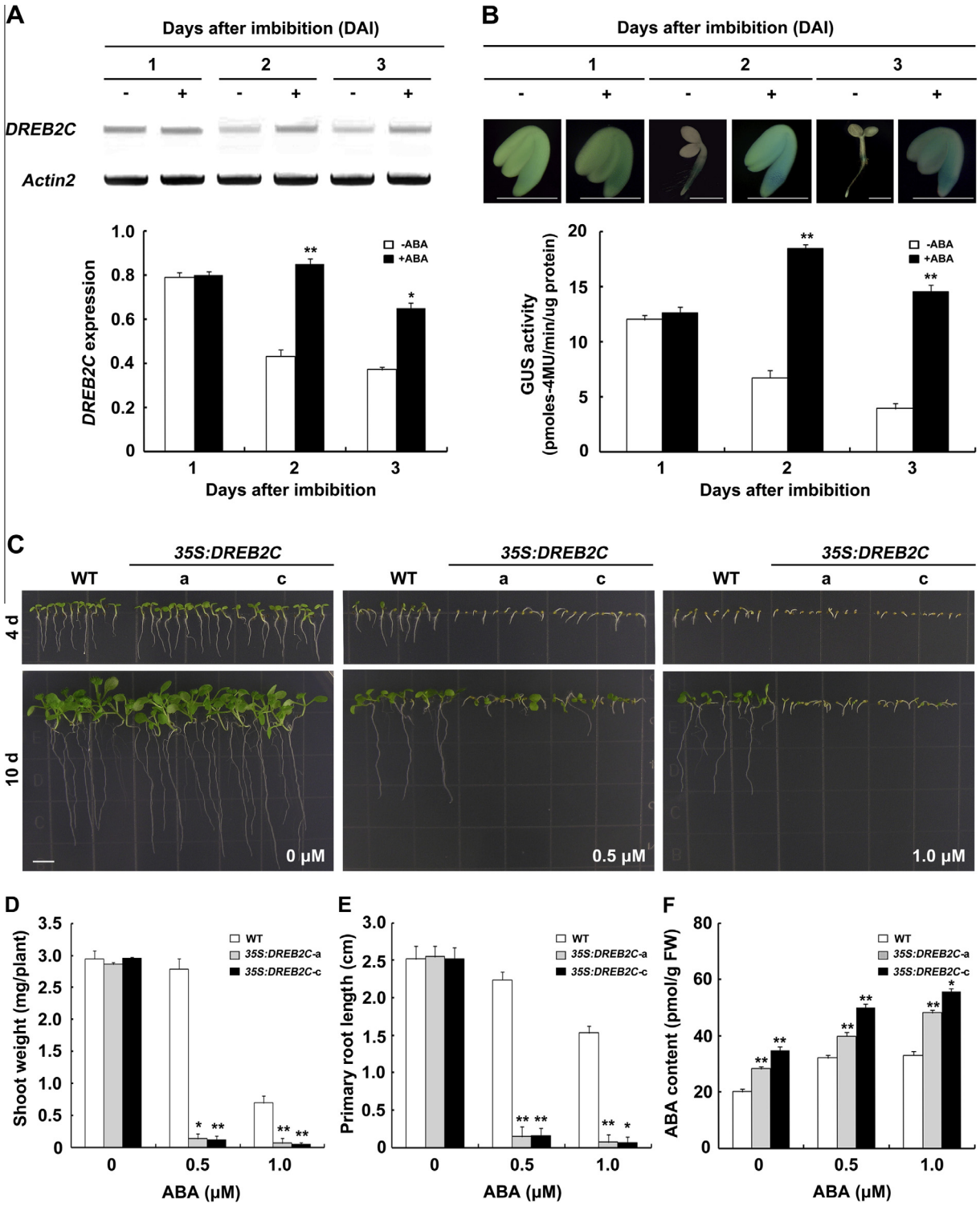

Fig. 4. Overexpression of *DREB2C* results in ABA hypersensitivity. (A) ABA treatment prevents lowering of *DREB2C* transcript level during germination. Shown are the results of a RT-PCR analysis of *DREB2C* transcript abundance in seeds of WT that were plated on 1/2MS without (−) or with (+) 10 μM ABA, stratified, and then allowed to germinate at 22 °C under a 16 h light/8 h dark cycle for the indicated periods. *Actin2* transcript abundance is shown for normalization. The histogram depicts the signal strength measured using ImageJ software. *Y*-axis values represent the fold-increase in expression of *DREB2C* compared to the *Actin2* (normalized as 1). Bars represent mean ± *SD* ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t* test). (B) *DREB2C* promoter activity was determined by histochemical GUS staining of germinated seeds of transgenic $Promoter_{DREB2C}$:*GUS* plants shown in (A). Scale bars are 1 mm. GUS activity in the germinating seeds was quantified and is shown in the histogram. Bars represent mean ± *SD* ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t* test). (C) Germination of *35S:DREB2C* seeds are hypersensitive to ABA. Seeds of WT and transgenic *35S:DREB2C* lines (*35S:DREB2C*-a and -c) were plated on 1/2MS phytoagar medium containing 0, 0.5, and 1.0 μM ABA. After stratification at 4 °C for 3 d, plates were incubated vertically at 22 °C for germination. Plates were photographed on days 4 and 10 after transfer to 22 °C. Shoot weight (D), primary root length (E) and ABA content (F) were measured on day10 after transfer to 22 °C. Three independent experiments were performed with similar results. Bars represent mean ± *SD* ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test).

may activate the transcription of *NCED9* gene during seed germination.

3.3. DREB2C trans-activates NCED9 expression

It has been reported that DREB2C can directly and specifically bind to *DRE* and *CRT* motifs on plant promoters [11]. Acting via *DRE* motifs, DREB2C functions as a robust transcriptional activator of *HsfA3* [13]. The 1-kb region upstream of the putative transcriptional start site of *NCED9* contains a core *DRE* (*cDRE*; 5′-ACCGAC-3′) and a partial *DRE* (*pDRE*; 5′-TCCGAC-3′) (Fig. 3A). The ability of DREB2C to regulate *NCED9* transcription via these motifs was evaluated by transient promoter activation assays in *Arabidopsis* leaf protoplasts using reporter constructs that contained intact or mutated *DRE* elements. As shown in Fig. 3B, the GUS/LUC activity of the *NCED9* construct that carries both intact *cDRE* and *pDRE* (*pNCED9:GUS*) was increased 9.8-fold in transformation reactions including the *35SΩ:Flag-DREB2C* effector (*35SΩ:DREB2C*) compared to reactions lacking the effector construct, indicating that DREB2C trans-activates gene expression from the *NCED9* promoter. Substitution mutations in *pDRE* had only a small effect on this fold increase in GUS/LUC activity (9.1- vs. 9.8-fold), whereas mutation of *cDRE* and both *cDRE*/*pDRE* significantly reduced this value (1.8-fold and 1.2-fold, respectively) indicating that the *cDRE* of *NCED9* had a greater role in DREB2C-directed transcriptional activation of the reporter gene than the *pDRE*.

To directly assess the ability of DREB2C to bind to the promoter of *NCED9*, we purified bacterially expressed GST-DREB2C$^{(145–528)}$ fusion protein [11] and characterized the DNA-binding ability of the recombinant protein to the *cDRE* and *pDRE* motifs in *NCED9* promoter using EMSAs. As shown in Fig. 3C, a retarded radio labeled band was observed with ^{32}P-*cDRE* as probe with GST-DREB2C$^{(145–528)}$ but not GST, indicating specific binding of DREB2C to the probe. The signal intensity of the retarded band was reduced in a concentration-dependent manner by addition of unlabeled *cDRE*, but not unlabeled mutated *cDRE* (*mcDRE*), to the binding reaction indicating that the mutated bases are required for interaction with DREB2C. There was no retarded band observed in reactions with ^{32}P-*pDRE* as probe. Together, these results show that DREB2C directly and specifically interacts with the *cis*-element on the *NCED9* promoter that is also required for activation of *NCED9* promoter-dependent transcription *Arabidopsis* mesophyll cells, namely *cDRE*.

3.4. DREB2C overexpression is associated with increased sensitivity to ABA inhibition of germination

Since imbibed *35S:DREB2C* seeds exhibited higher ABA content and delayed germination compared to WT under conditions of mild heat stress (Fig. 1) and DREB2C can activate transcription of the ABA biosynthetic gene *NCED9* (Figs. 2 and 3), we hypothesized that the ABA induced by mild heat stress maybe inducing *DREB2C* and this may lead to increased ABA content through activation of *NCED9* expression. To test this hypothesis, we compared *DREB2C* transcript abundance in WT seeds imbibed and grown in the presence and absence of ABA by quantitative RT-PCR. As shown Fig. 4A, *DREB2C* transcript levels in WT seedlings decreased gradually over 3 days without ABA in the medium (Fig. 4A), whereas *DREB2C* transcript levels remained high in seedlings receiving ABA supplement, indicating that *DREB2C* is an ABA-induced gene. The RT-PCR results concur with findings by GUS activity assays in imbibed transgenic *Promoter$_{DREB2C}$:GUS* seeds (Fig. 4B). GUS specific activity reduced over 3 d in seeds imbibed and maintained in medium lacking ABA. However in medium supplemented with a concentration of ABA that inhibits germination, GUS specific activity did not reduce over the same time period indicating activation of *DREB2C* promoter (Fig. 4B). These results led us to hypothesize that seed germination in *DREB2C* overexpression plants should be hypersensitive to exogenous ABA. Germination and seedling growth was inhibited by ABA in a concentration-dependent manner and to a greater extent in *DREB2C* transgenic lines than in WT (Fig. 4C–E). Measurements of ABA content in 10 d-old seedlings showed that tissue ABA content was proportional to the ABA content of the medium in all lines, but was always 10–25% higher in the *35S:DREB2C* transgenic lines than in WT (Fig. 4F). According to these analyses, exogenous ABA induces *DREB2C* expression while inhibiting germination and seedling growth.

Heat stress, like drought stress, cold stress and ABA application, increases tissue ABA content [2,8]. Feed-forward regulation of ABA biosynthesis by ABA has been reported to regulate plant development and stress responses, which means that the initial induction of ABA biosynthesis under stress conditions further stimulates ABA biosynthetic genes and ABA production [22]. Our results provide new evidence to support this scenario. We suggest that constitutive *DREB2C* overexpression primes this feed-forward loop so that a small increase in tissue ABA content due to application of exogenous ABA (Fig. 4) or heat stress (Fig. 1) is magnified to a much greater extent in the *DREB2C* overexpression lines compared to WT due the greater transcriptional activation of *NCED9* expression in *DREB2C* overexpressors compared to WT and is manifested as a greater delay of seed germination in *DREB2C* overexpressors compared to WT in response to exogenous ABA or mild heat stress.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.052.

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