Research article

Title: Abscisic acid binding transcription factors mediates proline biosynthesis and drought stress adaptation in *Arabidopsis thaliana*

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Abstract

Proline is a compatible solute and accumulates under osmotic stress, regulated by enzyme coding gene pyrroline-5-carboxylate synthetase 1 (*P5cs1*). Although stress-inducible proline accumulation is a well-sought topic, the transcriptional regulators mediating *P5cs1* under the ABA-dependent pathway is poorly understood. In the present work, we evaluated proline biosynthesis in the quadruple mutant (*abf1 abf2 abf3 abf4*) of four ABA-responsive elements (ABREs) binding factor (AREBs/ABFs) in Col-0 background. We transferred two-weeks-old plants to MS medium containing 50 µM ABA and measured shoot proline at 24, 48, 72, and 96 h of ABA application. After ABA treatment, wild type accumulated more proline in the shoots compared to *abf1 abf2 abf3 abf4*. We also observed a significant upregulation of the *P5cs1* gene in ABA treated plants compared to untreated plants. *P5cs1* expression was about four to six folds higher in wild type compared to *abf1 abf2 abf3 abf4*. However, no difference in shoot proline levels was observed between wild type and *abf1 abf2 abf3 abf4* under control condition (transferred to MS medium without ABA). Likewise, the *P5cs1* expression and shoot proline concentration at 2 h and 3 h following dehydration were significantly higher in wild type compared to *abf1 abf2 abf3 abf4*. Proline measurement upon terminal drought revealed earlier response to proline accumulation in wild type compared to the mutant. Compared to wild type, a terminal drought for one week significantly reduced fresh weight and dry weight. Tissue hydration was also significantly lower in *abf1 abf2 abf3 abf4* under drought conditions. Besides, *abf1 abf2 abf3 abf4* showed considerable membrane damage and lipid peroxidation in response to drought. Also, wild type maintained higher growth rate and biomass production under moderate water stress. The study provides compelling evidence on the role of AREBs/ABFs in ABA-mediated proline biosynthesis and drought adaptation.

Keywords: proline, ABA, ABA-responsive element binding factors, drought, adaptation

Introduction

Higher plants have evolved several physiological mechanisms to cope with environmental stress, including water limiting conditions (Shinozaki & Yamaguchi-Shinozaki 2006). Osmotic adjustment to maintain cell turgidity is one of the critical adaptive measures to accomplish

drought tolerance (Szabados & Savouré 2010; Blum 2017; Zarattini & Forlani 2017). To achieve this, the accumulation of compatible solutes like proline, soluble sugars, glycine betaine, and low molecular weight organic acids are essential protecting to maintain membrane stability and protect macromolecule structure and activity during osmotic stress (Szabados & Savouré 2010; Forlani, Trovato, Funck & Signorelli 2019). Among those, the accumulation of proline is one of the most apparent responses of plants against drought stress. The primary role of proline is to maintain membrane stability and protect macromolecule structure and activity during osmotic stress (Szabados & Savouré 2010; Forlani *et al.* 2019). Proline also acts as reactive oxygen species scavenger or activates the antioxidant like superoxide dismutase, catalases, and peroxidase (Alia et al., 2001; Signorelli et al., 2015, 2014). Therefore, understanding the genetic mechanisms modulating the proline accumulation is highly essential for its utility in establishing drought stress adaptation in plants.

Proline biosynthesis occurs through two pathways known as glutamate and ornithine. The glutamate pathway accounts for the significant proline biosynthesis catalyzed by pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) enzymes (Szabados and Savouré, 2010). The first step of proline biosynthesis is the conversion of glutamate to glutamate semialdehyde (GSA) by P5CS. GSA is converted to pyrolline-5-carboxylate (P5C), which is subsequently reduced to proline by P5CR (Forlani et al., 2019; Szabados and Savouré, 2010). There are two copies of P5cs genes in higher plants, *P5cs1* and *P5cs2*. In Arabidopsis, *P5cs2* regulates the housekeeping proline biosynthesis, while *P5cs1* is a stress-inducible homolog active in the chloroplast (Székely et al., 2008). The proline biosynthesis pathway is upregulated, and the degradation pathway is downregulated, leading to cytosolic proline accumulation under stress conditions (Szabados and Savouré).

P5cs1 transcription is considered as a hallmark for cytosolic proline deposition under drought stress conditions. In the past, many studies were made on the genetic regulation of *P5cs1* transcription activity under drought stress. Yoshiba et al. (1999) made transient expression assays using *P5cs1* promoter variants and suggested that MYB binding motif were essential for Arabidopsis *P5cs1* transcription under dehydration. Later, Jae et al. (2005) validated the role of MYB-like transcription factor for the upregulation of *P5cs1* expression via Ca^{2+} signaling under salinity in Arabidopsis. In addition, Fu et al., (2018) reported epigenetic control of *P5cs1* where decreased methylation of chromatin in the ANAC055 promoter increased the *P5cs1* expression under drought stress. Apart from osmotic stress, a recent study showed that an MYB-like transcription factor regulates the transcription of *P5cs1* in Arabidopsis seedling exposed to

phosphorus deficiency (Aleksza et al., 2017). These data suggest a complex genetic regulation of *P5cs1* transcription and its role in proline accumulation under different drought stress scenario.

Drought stress response is generally divided in ABA-dependent and ABA-independent signals (Szabados and Savouré, 2010). ABA-driven signal for proline accumulation is controlled by ABA insensitive 1 (ABI1) regulatory pathway in Arabidopsis (Savouré et al., 1997; Uno et al., 2000; Verslues and Bray, 2006). Arabidopsis *abi1* knock-out mutant showed a reduced response to external ABA treatment for proline accumulation (Verslues and Bray, 2006; Savouré *et al.*, 1997). ABI1 belongs to protein phosphatase 2C and controls the ABA responsiveness in reproductive and vegetative tissues (Cell, 1997; Rodriguez, 1998; Yoshida et al., 2006). ABA-responsive elements (ABREs) are one of the critical regulatory elements found in the promoter of osmotic-stress-responsive genes and are the direct target of ABRE binding factors (AREBs/ABFs). AREBs/ABFs belong to the basic-domain leucine zipper (bZIP) transcription family and transactivates ABA-responsive genes (Fujita et al., 2005; Yoshida et al., 2015, 2010). Four ABFs (ABF1, AREB1/ ABF2, ABF3, and AREB2/ ABF4) were identified through yeast one-hybrid screening and electrophoretic mobility shift assay, and their transactivation property was verified by heterologous expression of *lacZ* in yeast (Choi et al., 2000). Although, ABFs transcription factors are a fundamental component of ABA-driven transcription cascade, their role in modulating proline accumulation and proline mediated drought stress adaptation remained enigmatic in Arabidopsis.

Recently, we isolated a wild allele of *P5cs1* that modulated drought-inducible proline accumulation in cultivated barley, Scarlett. Polymorphisms across ABA-responsive element (ABRE) motifs in *P5cs1* promoter between wild barley and Scarlett explained the variation in drought-inducible shoot proline content. Heterologous expression of barley *P5cs1* promoter:reporter constructs in Arabidopsis protoplast (Col-0) revealed higher activation of wild barley promoter upon ABA treatment compared to Scarlett. However, the promoter activity of wild barley significantly reduced in the protoplast of quadruple mutants of ABF1, AREB1/ABF2, ABF3, and AREB2/ABF4 (*abf1 abf2 abf3 abf4*) upon ABA treatment (Muzammil et al., 2018). Therefore, the present study aims to dissect the critical role of AREBs/ABFs in proline biosynthesis under ABA signaling. The availability of quadruple mutant of four AREBs/ABFs, *abf1 abf2 abf3 abf4* was capitalized for phenotypic, biochemical, and molecular screening under external ABA application, acute dehydration, and drought conditions.

Materials and Methods

Plant materials and growth conditions

We used Arabidopsis thaliana ecotype Col-0 and quadruple mutant of four ABF transcription factors (*abf1 abf2 abf3 abf4*) in the study. *abf1 abf2 abf3 abf4* was developed in the lab of Prof. Dr. Yamaguchi through genetic crosses between T-DNA insertion lines SALK_043079 (abf1), SALK_002984 (areb1/abf2), SALK_096965 (abf3), SALK_069523 (areb2/abf4). Prof. Dr. Yamaguchi kindly provided seeds of *abf1 abf2 abf3 abf4*. We analyzed the presence/ absence of gene expression by semi-quantitative PCR (Supplementary Fig 1).

ABA treatment

Seeds were plated in half-strength MS media agar plates (2mM MES and pH 5.7). Plates were stratified at 4 ^{o}C and transferred to a growth chamber at 22/20 ^{o}C day/ night temperature, 10/ 14 light and dark period, and 100-150 µmol m^{-2} s^{-1} light intensity. For ABA treatment, two weeks old seedlings were transferred to the semi-solid MS media (0.2% Agar) supplemented with 50 µM ABA (Sigma Aldrich, A1049). For the control condition, seedlings were transferred to semi-solid MS media agar plates without ABA (Supporting information Fig. 2A). We collected fresh samples at 24, 48, 72, and 96 h of ABA treatment. Seedlings were snap-frozen in liquid nitrogen and stored at -80 ^{o}C before proline measurement and mRNA extraction. The experiment was performed in six biological replicates.

Dehydration treatment

Seeds were sown in a plastic pot (6cm*6cm*7cm) filled with a peat-based potting mixture, ED73 classic produced and marketed by Einheiterde, Germany. The pots were stratified at 4 ^{o}C for three days and transferred to the greenhouse. After germination, the seedlings were thinned, maintaining 8 to 10 seedling per pot. The water level was maintained at 1.5 times the dry weight of the soil. For dehydration, 15 days old seedlings were removed from the pots and soil adhered to the roots were washed off with water. Then, the seedlings were placed above the parafilm. Seedlings were placed above wet filter paper as control samples (Supporting information Fig. 2B). The samples were collected at 1, 2, and 3 h of dehydration, snap-frozen in liquid nitrogen and stored at -80 ^{o}C before proline determination and RNA extraction. The experiment was in six biological replicates.

Drought treatment

The growing condition was identical to dehydration treatment. We applied terminal drought to 21 days old seedlings for one week and evaluated morphological and physiological traits. Shoot fresh weight was recorded and dried at 70 ^{o}C for 24 h before taking the dry weight. Leaf water status was estimated through relative water content (RWC). For RWC measurement, rosettes were detached, and the fresh weight was recorded (FW). Then, rosettes were dipped in a falcon tube filled with 10ml deionized water for 24 h at room temperature. The rosette was removed from the falcon tube, and excess water was wiped with a paper towel before taking the turgor weight (TW). Dry weight was recorded after oven drying at 70 ^{o}C for 24 h. RWC was estimated as (FW-DW)/(TW-DW)*100. Cell wall integrity was determined by evaluating electrolyte leakage (EL) based on Bajji et al. 2001 with some modifications. Falcon tubes were filled with 10ml deionized water, and initial electrical conductivity was recorded (ECi). Rosettes were detached and placed in a falcon tube with 10ml double distilled water and stored in the dark at room temperature. Then, electrical conductivity was measured 24 h of rehydration period (ECf). After the final reading, the samples were boiled at 100 ^{o}C for 30 minutes, cooled to 25 ^{o}C, and total electrical conductivity (ECt) was measured. EL was expressed as (ECf-ECi)/(ECt-ECi)*100. Fresh samples were collected, snap-frozen in liquid nitrogen, and stored at -80 ^{o}C before malondialdehyde (MDA) and proline analysis. The experiment was performed in six biological replicates.

Evaluation of shoot growth and rosette morphology under moderate drought

Above-ground morphology of wild type and *abf1 abf2 abf3 abf4* was phenotyped in the GROWSCREEN-FLURO system, according to Barboza-Barquero et al (2015). Stratified seeds were pre-germinated and 7 d old seedlings were transferred to a pot (6cm*6cm*7cm). Plants were grown in controlled condition at 22/20 ^{o}C day/ night temperature, 10/14 h light/ dark period and 150 µmol m^{-2} s^{-1} light intensity. Two sets of wild type and *abf1 abf2 abf3 abf4* were grown in 20 biological replicates in a completely randomized design for control and moderate drought condition. During the establishment phase of one week after transplanting plants were grown under well-watered condition maintaining 60% volumetic moisture content (VMC). At 24 d when VMC reached 30%, the control pots were rewatered while the irrigation was stopped in drought set. Moderate drought was applied (maintaing 10% VMC). Drought stress was induced by withholding irrigation at 24 d until 36 d. First measurement was done 16 d after seeding. Three additional measurements were done between 20 d and 23 d. After 26 d the measurements were done everyday until 30 d and between 33 d and 36 d. Plants from drought treatment were rewatered at 36 d when shoot growth was ceased in mutant. Data were recorded at 40 d and 41

d to evaluate the recovery process. Plants were harvested to record shoot fresh and dry weight at 41 d after seeding. Projected leaf area, rosette morphology and growth rate were estimated according to Barboza-Barquero et al (2015).

Proline determination

Proline was measured from shoot samples based on Ábrahám *et al.* (2010). In short, Arabidopsis shoots were homogenized in liquid nitrogen, and proline was extracted using 0.5ml 3% sulphosalicylic acid followed by centrifuging at 12000 *g* for 5 minutes at 4 ^{o}C. The sample extract was incubated for 1 hour at 96 ^{o}C with 2.5% ninhydrin and acetic acid at a 1:1:1 ratio. The reaction was stopped on ice, and proline- ninhydrin reaction product was extracted with 1ml toluene. The absorbance of chromatophore containing toluene was measured at 520 nm using a microplate reader (TECAN Infinite 200 Pro, TECAN Group Limited, Switzerland). Shoot proline level was determined using a standard curve method and expressed as micrograms per gram fresh weight.

MDA determination

Oxidative damage of lipid membrane during drought was estimated by determining MDA based concentration using thiobarbituric acid (TBA) method (Hodges et al., 1999) adapted to a microplate-based protocol (Dziwornu et al., 2018) with some modification. Shoot samples were homogenized in liquid nitrogen, and MDA was extracted using 1.5ml of 0.1% trichloroacetic acid (TCA) followed by centrifuging at 14000 *g* for 15 minutes at 4 ^{o}C. Then, 500 µl supernatant was mixed with reaction solution I (0.01% 2,6-di-tert-butyl-4-methyl phenol (BHT) in 20% TCA) and reaction solution II (0.65% TBA, 0.01% BHT in 20% TCA) in a 1:1 ratio. Reaction and sample mix was incubated at 95 ^{o}C for 30 minutes. The reaction was stopped on ice for five minutes, and the reaction mix was centrifuged at 8000 *g* for 10 minutes at 4 ^{o}C. The absorbance was measured at 440 nm, 532 nm, and 600 nm using a microplate reader (TECAN Infinite 200 Pro, TECAN Group Limited, Switzerland). MDA concentration was expressed as nanomoles per gram fresh weight.

Statistical analysis

Data were analyzed in open access statistical computing software R. Two way ANOVA was performed to observe genotype, treatment, and interaction effects. The student's *t*-test was used for the mean comparison between genotypes for a given treatment condition. Multiple

mean comparison analysis was done using a Tukey post hoc test. Graphics were prepared using statistical platform R and Prism8.

P5cs1 mRNA expression analysis using quantitative RT-PCR

RNA was extracted from ABA and dehydration experiments from control and treatment samples for all time points. Arabidopsis shoots were homogenized in liquid nitrogen, and RNA was extracted using Monarch RNA miniprep kit (New England Biolabs, USA) following the manufacturer's instruction. The RNA concentration and quality were determined by running on 1% Agarose gel and nanodrop (NanoDrop 2000c, Thermo Fischer Scientific, USA) before cDNA synthesis.

cDNA was synthesized using LunaScript super RT mix (New England Biolabs, USA) following the manufacturer's instruction. Quantitative real-time PCR (qPCR) was performed in 96-well plates using a 7500 fast real-time PCR system (Applied Biosystems, USA). An SYBR green-based Luna Universal qPCR master mix was used in the assay with three technical replicates per sample. The qPCR run was set to initial denaturation at 95 ^{o}C for 3 minutes followed by 40 cycles (95 ^{o}C for 15 seconds, 60 ^{o}C for 1 minute). Specific amplification was analyzed using a melt curve (95 ^{o}C for 15 seconds, 60 ^{o}C for 1 minute, 95 ^{o}C for 15 seconds). Relative mRNA expression of *P5cs1* was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). qPCR experiment was performed in three biological replicates. Primers used the study is provided in supplementary table 1.

Results

The present study evaluated the role of AREB/ABF in proline biosynthesis and drought adaptation in Arabidopsis. There are four AREB/ABFs characterized in Arabidopsis that possess conserved N terminus and C terminus (basic leucine zipper) domains (Supporting information Fig. S3). A quadruple mutant of ABF1, AREB1/ABF2, ABF3, and AREB2/ABF4 was used to perform a series of experiments such as external ABA application, acute dehydration, and terminal drought stress conditions.

The promoter region of *P5cs1* harbors DNA binding motifs associated with stress-inducible transcription factors

We performed *in silico* analysis of *P5cs1* promoter across plant species, including Arabidopsis. Regulatory motifs that are targets of AREB/ABF, MYC, MYB, and WRKY transcription factors

were present in the *P5cs1* promoter of rice, wheat, barley, maize, and Arabidopsis (Supporting information (Supporting information Fig. S4). In Arabidopsis, three ABRE were present withing 500 bp upstream of the start codon (Fig. 1).

ABFs regulate proline accumulation upon ABA treatment

External ABA application induces *P5cs1* activation and proline biosynthesis in Arabidopsis (Aleksza et al., 2017; Yoshiba et al., 1999). Here we evaluated the role of ABRE binding transcriptional factors on proline accumulation. We measured proline after 24, 48, 72, and 96 h of ABA application (50 µM) in Col-0 and *abf1 abf2 abf3 abf4*. Shoot proline concentration in Col-0 significantly increased already at 24 h of ABA treatment (Supporting information Table 1). Proline levels increased rapidly with time in Col-0. At 96 h of ABA application, shoot proline was six-fold higher in Col-0 compared to non-treated plants. In contrast, *abf1 abf2 abf3 abf4* showed a steady increase in proline with the duration of ABA treatment (Fig. 2a). In *abf1 abf2 abf3 abf4*, a significant difference in proline concentration between ABA treated and non-treated samples were only observed after 72 h . However, the shoot proline levels in Col-0 were significantly higher at all sampling times compared to *abf1 abf2 abf3 abf4* (Supporting information Table 1). Proline concentration was two and three-fold higher in Col-0 compared to *abf1 abf2 abf3 abf4* at 72 and 96 h of ABA application. Shoot proline concentration did not differ between Col-0 and *abf1 abf2 abf3 abf4* in non-treated conditions (Fig. 2a and Supporting information Table 1).

Savouré et al. (1997) showed that *P5cs1* mRNA expression in Arabidopsis was activated upon ABA treatment. We evaluated the relative expression of *P5cs1* in Col-0 and *abf1 abf2 abf3 abf4* after ABA application. *P5cs1* expression was significantly upregulated in Col-0 upon ABA treatment. In Col-0, the expression was seven, fifteen, eighteen, and twenty-one fold higher at 24, 48, 72, and 96 h in treated samples. Also, *abf1 abf2 abf3 abf4* showed a significant increase in the expression of *P5cs1* after 48 h. However, the expression levels in Col-0 was around four-fold higher at 24 and 48 h six-fold higher at 72 and 96 h compared to *abf1 abf2 abf3 abf4* (Fig. 2b and Supporting information Table 1).

ABFs contributes to proline biosynthesis under acute dehydration

We also measured proline and *P5cs1* expression under acute dehydration stress. Fifteen days old Arabidopsis seedlings were placed above the parafilm, and samples were collected after 1, 2, and 3 h of dehydration. Shoot proline concentration significantly increased in Col-0 after dehydration (Supporting information Table 2). Proline levels were 1.6, 2.5, and 5 times higher in

dehydrated samples in Col-0 (Fig. 3a). Higher proline levels were detected in dehydrated shoots of *abf1 abf2 abf3 abf4* compared to control plants, although the significant differences can be observed only after 3 h (Supplementary Table 2). However, the shoot proline concentration was significantly higher in Col-0 compared to *abf1 abf2 abf3 abf4* after 2 h (25%), and 3 h (63%) of acute dehydration (Figure 3a).

Similar to ABA treatment, we observed significant upregulation of *P5cs1* upon dehydration. The relative expression of *P5cs1* in Col-0 was 2.5 to 4 fold higher compared to *abf1 abf2 abf3 abf4* in dehydrated tissues (Figure 3a and Supporting information Table 2).

Shoot proline concentration differences between wild type and *abf1 abf2 abf3 abf4* was detected at the early stage of drought stress

We induced terminal drought to 21 d old seedlings and measured shoot proline concentration at 4, 5, 6, and 7 d after stress treatment. Compared to plants grown under well-watered conditions, proline concentration increased significantly in drought-stressed plants after 5 d. At 5 d, shoot proline was significantly higher (2 fold) in wild type compared to *abf1 abf2 abf3 abf4*. However, the shoot proline rose to a similar level between wild type and *abf1 abf2 abf3 abf4* at 6 d and 7 d (Fig. 4a). The result indicates that AREBs/ABFs might be critical for early drought response concerning proline biosynthesis. We can argue that the ABA-independent pathway compensated the inhibition of ABA-dependent proline biosynthesis under drought in *abf1 abf2 abf3 abf4*.

***abf1 abf2 abf3 abf4* showed physiological damage to drought stress**

We also evaluated the morphological and physiological response of *abf1 abf2 abf3 abf4* to drought stress. The wild type showed lower wilting symptoms compared to *abf1 abf2 abf3 abf4*. Shoot fresh and dry weight were measured from both control and drought conditions. The growth of *abf1 abf2 abf3 abf4* was severely affected by drought compared to Col-0 (Fig. 4b). Shoot fresh and dry weight significantly reduced in both Col-0 and *abf1 abf2 abf3 abf4*. Fresh weight and dry weight were three and two times higher in Col-0 compared to *abf1 abf2 abf3 abf4* after one week of drought (Fig. 4c and d).

The extent of cell membrane integrity or cell death was estimated by measuring EL (Bajji et al., 2002). EL increased significantly under drought in both Col-0 and *abf1 abf2 abf3 abf4*. However, the extent of cell membrane damage was more significant (two-fold higher) in *abf1 abf2 abf3 abf4* compared to Col-0 (Fig. 4e). We also estimated cellular hydration through RWC (Ghoulam

et al., 2002). Tissue water status decreased in both wildtype and quadruple mutant under drought. RWC was 31% percent higher in Col-0 compared to *abf1 abf2 abf3 abf4* (Fig. 4f). Oxidative stress can be estimated by determining lipid peroxidation in the tissues quantified as MDA concentration (Hodges et al., 1999). MDA levels were measured from the fresh tissues harvested one week after a drought. MDA levels significantly increased under drought conditions. MDA concentration in *abf1 abf2 abf3 abf4* was 78% higher compared to Col-0 (Fig. 4g).

The growth rate of *abf1 abf2 abf3 abf4* reduced under moderate drought

Plant growth rate and rosette morphology was recorded from 16 to 41 d after sowing. Two sets of wild type and *abf1 abf2 abf3 abf4* were grown in 20 biological replicates in a completely randomized design. One set was grown under well-watered condition (60% VMC). Water stress was applied to another set by withholding watering at 24 d after seeding (10% VMC). Rosette images recorded in the GROWSCREEN-FLURO setup were analyzed to derive different growth-related parameters, including PLA. The PLA of wild type was around 15% lower compared to *abf1 abf2 abf3 abf4* from 4 w after seeding. In contrast, PLA reduced significantly in *abf1 abf2 abf3 abf4* compared to wild type (Fig. 5a and b). Plants were rewatered at 36 d after seeding when *abf1 abf2 abf3 abf4* when further growth was ceased in *abf1 abf2 abf3 abf4*. At 29 d and 36 d, PLA was 17% and 54% higher in wild type compared to *abf1 abf2 abf3 abf4*. After rewatering, both wild type and mutants recovered at a similar rate where PLA was 45% higher at 41 d in wild type (Fig. 5a and b). Averaged over the drought period, PLA significantly reduced in both wild type and *abf1 abf2 abf3 abf4*. However, the wild type plants were significantly bigger than *abf1 abf2 abf3 abf4* (Supporting information Fig. S5a). We also estimated the RGR per day for plants grown under control and water-stressed conditions. Under the water stress, RGR decreased sharply in wild type and mutants compared to the control conditions. Although the RGR was marginally higher in mutants in well-watered pots, wild type maintained remarkably higher shoot growth rate compared to *abf1 abf2 abf3 abf4* under moderate water stress (Fig. 5c and d). At 41 d, plants were harvested for fresh and dry biomass determination. Fresh and dry weight of mutant was marginally higher (15% and 18% respectively) under control condition. But, the shoot biomass strongly reduced in mutants under moderate drought condition compared to wild type. Shoot fresh weight and dry weight was 47% and 44% higher in wild type compared to *abf1 abf2 abf3 abf4* (Supporting information Fig. S5b).

The rosette circumference of wild type was measured significantly smaller compared to *abf1 abf2 abf3 abf4* from 30 d after sowing in control conditions (Fig. 6a). Averaged over the drought period the rosette circumference decreased in wild type and mutant (Fig. 6c). Though, at later stages of drought the circumferce and calculated circular size of rosette was significantly larger in wild type compared to *abf1 abf2 abf3 abf4* (Fig. 6b and Supporting information Fig. S6). Also, moderate drought resulted in more compact rosette and petiole size in mutant which can be explained by reduced growth rate. Rosette stockiness and excentricity remain unaffected under moderate drought in wild type and *abf1 abf2 abf3 abf4* (Supporting information Fig. S7 and S8).

Discussion

Proline accumulation in plants in response to osmotic stress like drought and salinity (Forlani et al., 2019; Szabados and Savouré, 2010; Verbruggen and Hermans, 2008; Verslues and Sharma, 2010). Stress-inducible proline accumulation is regulated in an ABA-dependent manner (Strizhov et al., 1997). The role of ABA in proline biosynthesis has been demonstrated in several studies where ABA deficient mutants aba1 and aba2 accumulated less proline upon stress treatment. External ABA application was able to rescue the proline phenotype in aba1 and aba2 (Liu and Zhu, 1997; Verslues and Bray, 2006; Yoshiba et al., 1995). However, the transcription factors modulating osmotic stress-induced proline accumulation are not well characterized. In this study, we evaluated the role of ABF transcription factors in ABA-mediated proline accumulation. In Arabidopsis, four members of AREB/ ABFs (ABF1, AREB1/ ABF2, ABF3, and AREB2/ ABF4) induced by ABA and osmotic stress (Yoshida et al., 2015, 2010). We measured proline in quadruple mutants of ABF1, AREB1/ ABF2, ABF3, and AREB2/ ABF4 (*abf1 abf2 abf3 abf4*) under external ABA application and acute dehydration. ABA application highly induced proline accumulation in wild type. Similar results were obtained when non-osmotically stressed Arabidopsis seedling were treated with 50 µM ABA (Finkelstein and Lynch, 2000; Savouré et al., 1997). Shoot proline level was significantly lower in *abf1 abf2 abf3 abf4* compared to wild type under external ABA treatment. However, we also observed a significantly higher (around two folds) proline in *abf1 abf2 abf3 abf4* after 72 h of ABA treatment. We also estimated shoot proline concentration under acute dehydration, where plants were removed from the soil and kept on parafilm. Proline levels increased significantly in the seedlings exposed to dehydration. Unlike ABA treatment, *abf1 abf2 abf3 abf4* showed a better response to dehydration concerning proline accumulation. However, we observed different dynamics of proline accumulation under drought treatment. Proline level significantly increased only after 5 d of terminal drought compared to the control condition. At 5 d, wild type accumulated two times

higher proline concentration compared to *abf1 abf2 abf3 abf4*. However, the shoot proline content swelled to a comparable level between wild type and *abf1 abf2 abf3 abf4* at later stages of drought. The observation was in line with previous findings that proline biosynthesis is regulated under ABA-dependent and ABA-independent pathway during osmotic stress (Savouré et al., 1997; Sharma and Verslues, 2010; Szabados and Savouré, 2010; Verslues and Bray, 2006). Our data implies that the ABA-dependent pathway might dominate drought-inducible proline biosynthesis at the earlier stage of hydraulic stress. At later stage, both ABA-dependent and ABA-independent pathways are critical for proline accumulation All together, this study submits firm evidence that AREBs/ABFs are the key regulators of proline accumulation under ABA signaling.

In higher plants, P5CS1 catalyzes the rate-limiting step of conversion of glutamate to proline (Hu et al., 1992; Székely et al., 2008). Several studies showed that *P5cs1* mRNA expression level was positively correlated to proline accumulation under stress conditions (Aleksza et al., 2017;1995; Hayashi et al., 2000; Sripinyowanich et al., 2013; Yoshiba et al., 1995). *P5cs1* expression was triggered by ABA application. Our findings are in line with the previous observation that exogenous ABA application induced *P5cs1* expression in higher plants (Sripinyowanich et al., 2013; Zarattini and Forlani, 2017). The expression of *P5cs1* was six-folds lower in *abf1 abf2 abf3 abf4* after 96 h of ABA application. *P5cs1* transcript levels were also upregulated under dehydration treatment. In contrast to the ABA application, the *P5cs1* expression was only two to four times higher in wild type compared to *abf1 abf2 abf3 abf4*. It was also noteworthy that proline accumulation and *P5cs1* expression were not strongly responsive to ABA, but we observed a slow but steady increase over time. This observation points out that ABFs are one of the key regulators of *P5cs1* under ABA signaling. Recently, Muzammil et al. (2018) showed that polymorphism in the ABRE motif in the promoter of *HvP5cs1* explained the expression differences in cultivated and wild barley under drought. They found that the GUS activity driven by wild barley promoter significantly reduced in *abf1 abf2 abf3 abf4* protoplast compared to wild type through heterologous expression of promoter:GUS construct. In Arabidopsis, there are three ABRE and related motifs present within 450 bp upstream of the *P5cs1* gene. ABFs bind to ABRE motifs and regulate the transcription of target genes (Hobo et al., 1999; Nakashima et al., 2009; Shen, Qingxi; Zhang, Pengnian; Ho, 1996; Shinozaki et al., 2003; Yoshida et al., 2015, 2010). AREB1/ ABF2 and AREB2/ ABF4 regulated the ABA-inducible expression of rd29B which contains ABRE motifs in the promoter region (Uno et al., 2000). Choi et al., (2000) showed that ABF1, AREB1/ ABF2, ABF3, and AREB2/ ABF4

activated the transcription of ABRE dependent protein in plants treated with ABA and osmotic stress. Previous studies showed that knock-out lines of ABA-insensitive (*abi1* and *abi2*), which is characterized as group A protein phosphate 2Cs (PP2C) showed reduced proline accumulation upon ABA treatment (Strizhov et al., 1997; Verslues and Bray, 2006). It was also discovered that AREBs/ABFs activity was suppressed in the *abi1* and *abi2* mutant (Uno et al., 2000). PP2C is the negative regulators of ABA and dephosphorylate SNF1 related reporter like kinases 2 (SnRK2s) in the absence of ABA (Wang et al., 2018). Under osmotic stress, ABA receptor binds to PP2C, and SnRK2 is activated, which plays a major role in post-translation phosphorylation of ABA-responsive genes including AREBs/ABFs for full activation (Furihata et al., 2006; Kobayashi et al., 2005; Yoshida et al., 2015, 2010). We can assume that ABA-dependent inhibition of ABI1 and ABI2 by ABA receptors is critical for AREBs/ABFs mediated transcriptional regulation of *P5cs1* for ABA-dependent proline biosynthesis under osmotic stress.

Previous studies demonstrated that proline plays a vital role in drought adaptation (Fu et al., 2018; Muzammil et al., 2018; Székely et al., 2008; Vendruscolo et al., 2007). Also, transgenic plants with high proline levels showed tolerance to osmotic stress (Hong et al., 2000; Vendruscolo et al., 2007). The protection of macromolecules and cellular hydration are the primary functions of proline during osmotic stress (Szabados and Savouré, 2010; Verbruggen and Hermans, 2008). We observed that *abf1 abf2 abf3 abf4* showed higher electrolyte leakage, indicating significant membrane damage compared to wild type. Also, the tissue turgidity was significantly lower in *abf1 abf2 abf3 abf4*. Low proline accumulating mutants had reduced RWC, indicating poor osmotic adjustment (Székely et al., 2008; Verslues and Bray, 2004). Likewise, wild type also showed lower lipid peroxidation compared to *abf1 abf2 abf3 abf4*. Retarded plant growth *abf1 abf2 abf3 abf4* compared to wild type under moderate and terminal drought can be linked to higher oxidative damage and reduced osmotic adjustment in the mutant. Proline accumulation or external proline accumulation correlated with reduced oxidative damage during osmotic stress (Alia et al., 1993; Ghaffari et al., 2019; Székely et al., 2008; Verslues and Bray, 2004). Proline is directly involved in non-enzymatic ROS scavenging and activation of the antioxidant pathway (Alia et al., 2001; Signorelli et al., 2015, 2014). The increased sensitivity of *abf1 abf2 abf3 abf4* observed in our study should not be linked to proline alone as AREBs/ABFs control the regulation of a large number of osmotic stress-responsive genes. Microarray study showed a

plethora of differently regulated genes regulating redox balance, photosynthesis, amino acid metabolism, signaling pathway, dehydrin, secondary metabolism, and several other pathways between wild type and *abf1 abf2 abf3 abf4* under drought and salinity (Yoshida et al., 2015).

Conclusion

Our study provided novel insights into the role of AREBs/ABFs in proline biosynthesis in Arabidopsis. Proline accumulation and *P5cs1* transcription were impaired in the quadruple mutant of four AREBs/ABFs (*abf1 abf2 abf3 abf4*) compared to Col-0 under external ABA treatment and acute dehydration. The dynamics of proline accumulation under terminal drought indicated that ABA-dependent pathway mediates the earlier response to proline accumulation under water stress. It is also noteworthy that *abf1 abf2 abf3 abf4* did not show complete loss of ABA responsiveness to external ABA application concerning the proline phenotype. This study provides strong evidence that AREBs/ABFs are the most important (but not the only) regulators of ABA-dependent *P5cs1* transcription and subsequent proline accumulation under osmotic stress. Therefore, it paves the path to explore and engineer AREBs/ABFs mediated regulation of proline accumulation in crop plants.

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Table1: Summary statistics of shoot proline concentration and *P5cs1* mRNA expression under ABA treatment. Means sharing a letter are not significantly different by Tukey-adjusted comparisons ($P<0.05$).

Mean values of shoot proline (µg/ g Fresh weight)							
	-ABA		+ABA				
Hour	*abf1 abf2 abf3 abf4*	Col-0	*abf1 abf2 abf3 abf4*	Col-0	Genotype	Treatment	Interaction
24	90^{a}	87^{a}	120^{a}	183^{b}	**	***	*
48	112^{ab}	92^{a}	170^{bc}	233^{c}	ns	***	ns
72	118^{a}	107^{a}	209^{b}	448^{c}	*	***	*
96	131^{a}	135^{a}	259^{b}	847^{c}	***	***	***
Mean values of relative *P5cs1* expression (fold change to control)							
24	1.01^{a}	1.16^{a}	1.89^{a}	6.82^{b}	*	**	*
48	1.06^{a}	1.03^{a}	4.61^{b}	15.26^{b}	**	***	**
72	1.00^{a}	1.00^{a}	3.32^{b}	18.39^{c}	***	***	***
96	1.04^{a}	1.03^{a}	3.52^{b}	21.00^{c}	**	***	**

Table 2: Summary statistics of shoot proline concentration and *P5cs1* mRNA expression under acute dehydration treatment. Means sharing a letter are not significantly different by Tukey-adjusted comparisons ($P<0.05$).

Mean values of shoot proline (µg/ g FW)							
	Control		Dehydration				
Hour	*abf1 abf2 abf3 abf4*	Col-0	*abf1 abf2 abf3 abf4*	Col-0	Genotype	Treatment	Interaction
1	54.2^{a}	60.7^{a}	80.3^{ab}	94.8^{b}	**	**	**
2	62.2^{a}	66.8^{a}	114.7^{ab}	168.7^{b}	*	***	ns
3	61.5^{a}	58.3^{a}	185.1^{b}	286.0^{c}	*	***	*
Mean values of relative *P5cs1* expression (fold change to control)							
1	1.1^{a}	1.1^{a}	1.22^{a}	4.1^{b}	**	**	**
2	0.9^{a}	1.1^{ab}	2.7^{bc}	6.9^{c}	***	***	***
3	1.14^{a}	1.0^{a}	3.5^{b}	13.5^{c}	***	***	***

Table3: Summary statistics for the morphological and biochemical response to drought stress. Means sharing a letter are not significantly different by Tukey-adjusted comparisons (P<0.05). FW, fresh weight

Trait	Control		Drought		Genotype	Treatment	Interaction
	Col-0	*abf1 abf2 abf3 abf4*	Col-0	*abf1 abf2 abf3 abf4*			
Fresh weight (g)	177.5^{a}	165.9^{a}	56.6^{b}	21.5^{b}	*	***	ns
Dry weight (g)	11.85^{a}	12.24^{a}	7.55^{b}	3.86^{c}	*	***	*
MDA (nmol/ g FW)	10.0^{a}	10.8^{a}	13.3^{a}	23.7^{b}	**	***	**
Electrolyte leakage (%)	5.3^{a}	5.1^{a}	7.46^{a}	14.69^{b}	**	***	**
Relative water content (%)	81.5^{a}	80.9^{a}	50.1^{b}	38.1^{c}	**	***	*
Proline (µg/g FW)	45.0^{a}	44.2^{a}	3338.6^{b}	3012.8^{b}	ns	***	ns

Figure legend

Authors Contribution

AS, SS, JL, AN: Conceptualization and methodology

AS, DKC: Experimentation, data analysis and interpretation

FF: Shoot growth experiment in GROWSCREEN-FLURO system

AS: Drafting of the article

AN, SS, JL: Critical revision and editing of the article

AS, DKC, FF, SS, JL, AN: Final approval of the article

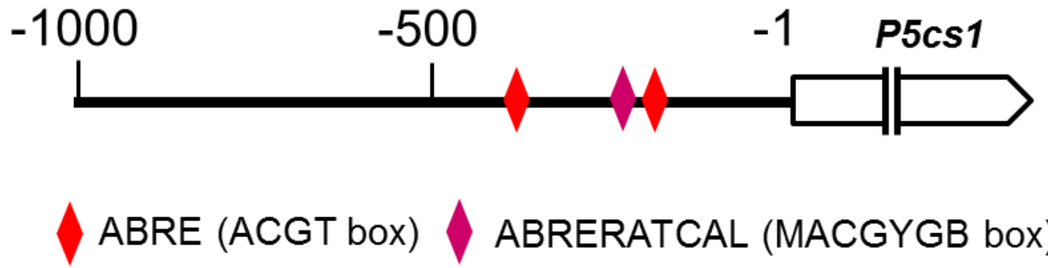
AN, JL: Provision of study materials

AN, JL: Obtaining of funding

AN, SS: Supervision

Acknowledgment

We are grateful to Dr. Yamaguchi-Shinozaki for providing the seeds of the quadruple mutant (*abf1 abf2 abf3 abf4*). We acknowledge the German Research Society (DFG) research training group (GRK2064) for funding the study.

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 -950 GTGAAATTTGATAACAAGTAAGATTTGTTTCTTAACACAAATCTAAAATT
 -900 TGTTTTCTAATTAGGTTTGAGAGAGAGAGAGAAAGAAACGCTTTGTATGA
 -850 TACACATCTAGGCTATGAATGAAGGCAGCGGACAAAGCGGTCTAATTTGT
 -800 CTGCGGTTTAGTCCATCTCATTTTTGGGGTGGACAATAAACCGCTGCGGA
 -750 CCAAGTTTATTTGTATGTAAAAACGGTCCGCAGATGGTCCGCAACGATTT
 -700 TCTTCTATTTTTTTAAGTCCAGACCACTGCGGACTATAATTGATGAATGA
 -650 TAAATAAAAAACGGTCTGATCCGTTGACGGTTTTGTCCGCCCCAACCGCC
 -600 ATAACCATTCAAACCCCTAATTATTTCATCAGATAACATTATACACTAAT
 -550 AATCATTGCACTCAAATATGTCACACAATCATATAATAAAATAATAACAA
 -500 TGATTAAAATGAAAAAATTGTTGTGGCGCCGCATAAAATAGAAATCGTGA
 -450 GAGACGACGTCATCTAAAAATTGCCTTGCTGTCCACTTTTCACTTTGTCC
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 -350 TATTTATTTCCCAAAATACCCATCACCTATAGCGCCACAATCCTCTACAT
 -300 CACACCCTAATCTCATTACCATACACCACCCAACGAACACGCGCCACTTC
 -250 ATTTGTTAGTATCTAAAATACCAAACCTACCCTTAGTTCCACACGTGGCG
 -200 TTTCCTGGTTTGATAACAGAGCCTGAGTCTCTGGTGTCGCTGGTGTTTAT
 -150 AAACCCCTTCATATCTTCCTTGGTGATCTCCACCTTTCCCTCACCTGATA
 -100 TTTATTTTCTTACCTTAAATACGACGGTGCTTCACTGAGTCCGACTCAGT
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      ATG
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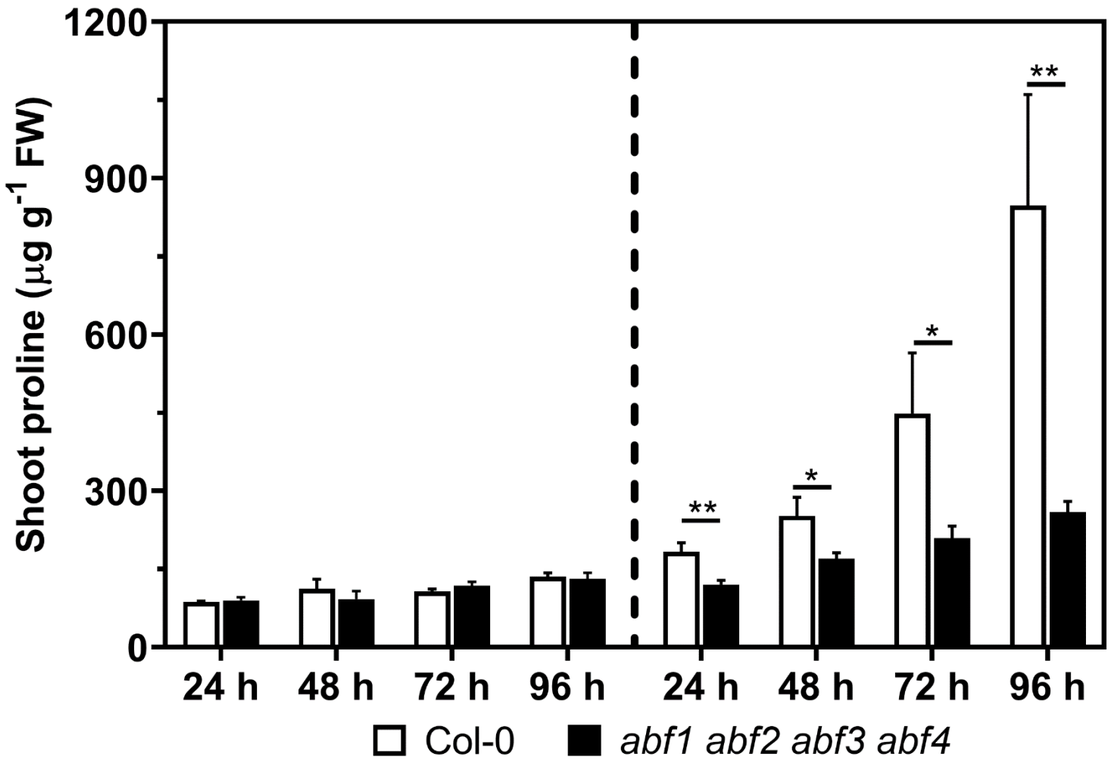
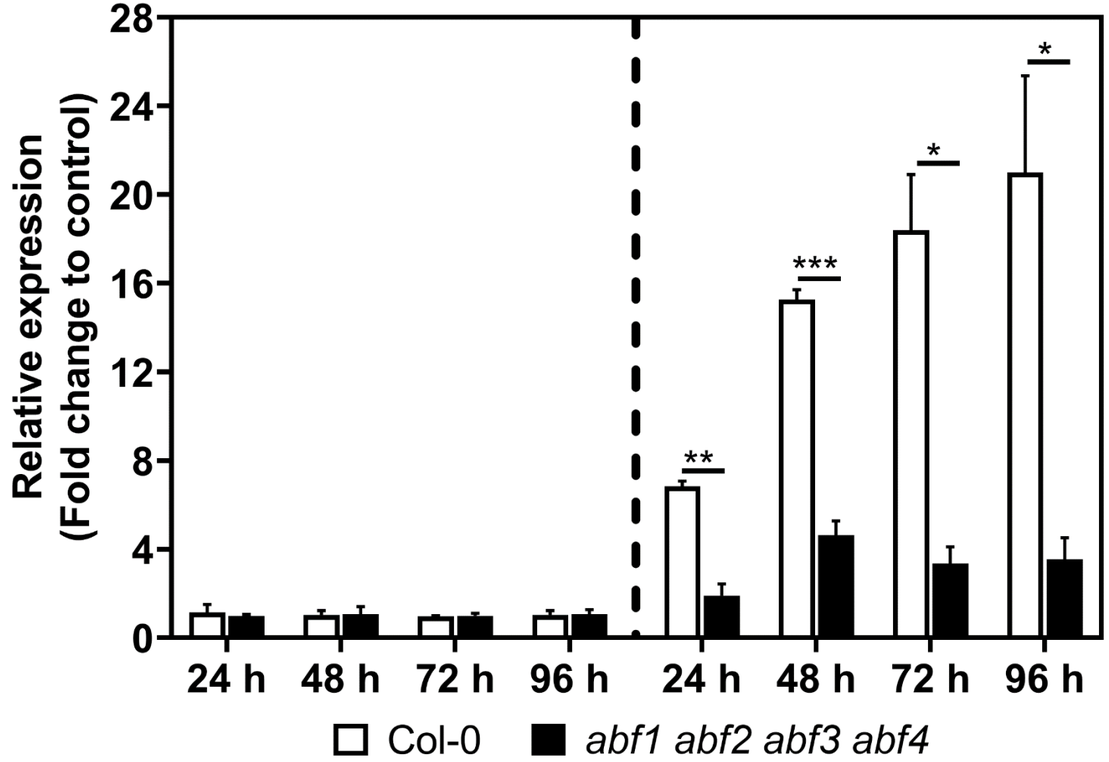
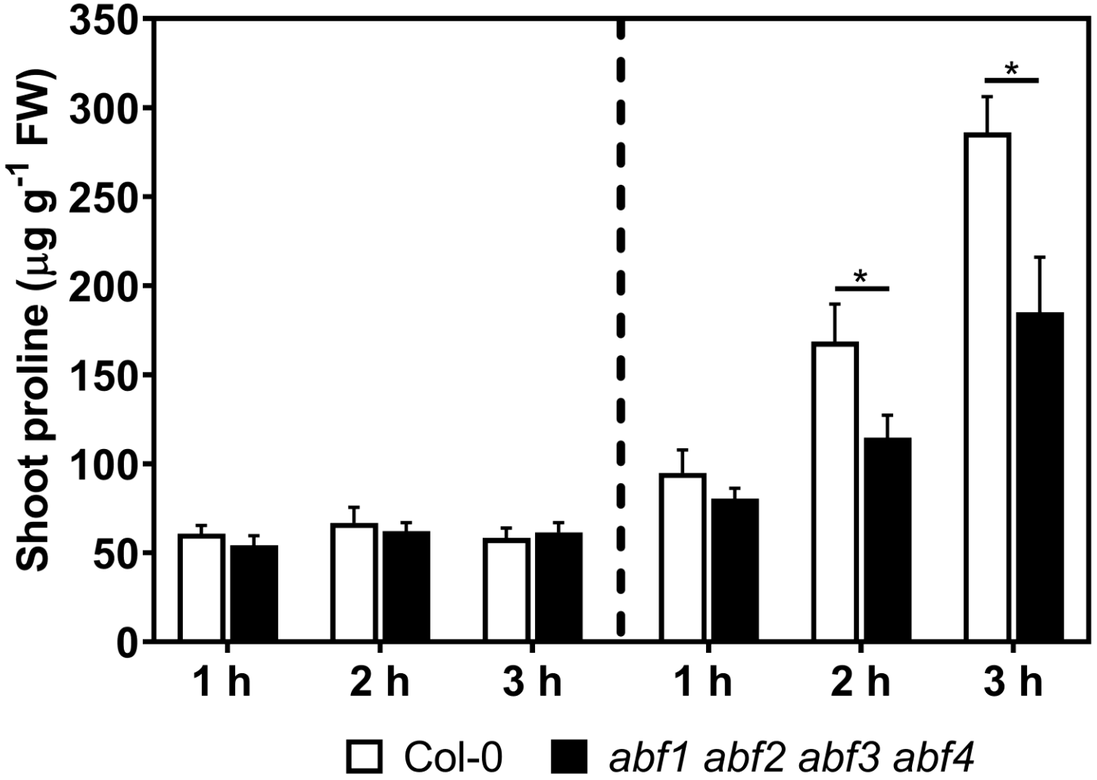
a


Control
+ABA
Shoot proline (µg g-1 FW)
1200
900
600
300
0
*
**
*
**
24 h
48 h
72 h
96 h
24 h
48 h
72 h
96 h
Col-0
abf1 abf2 abf3 abf4

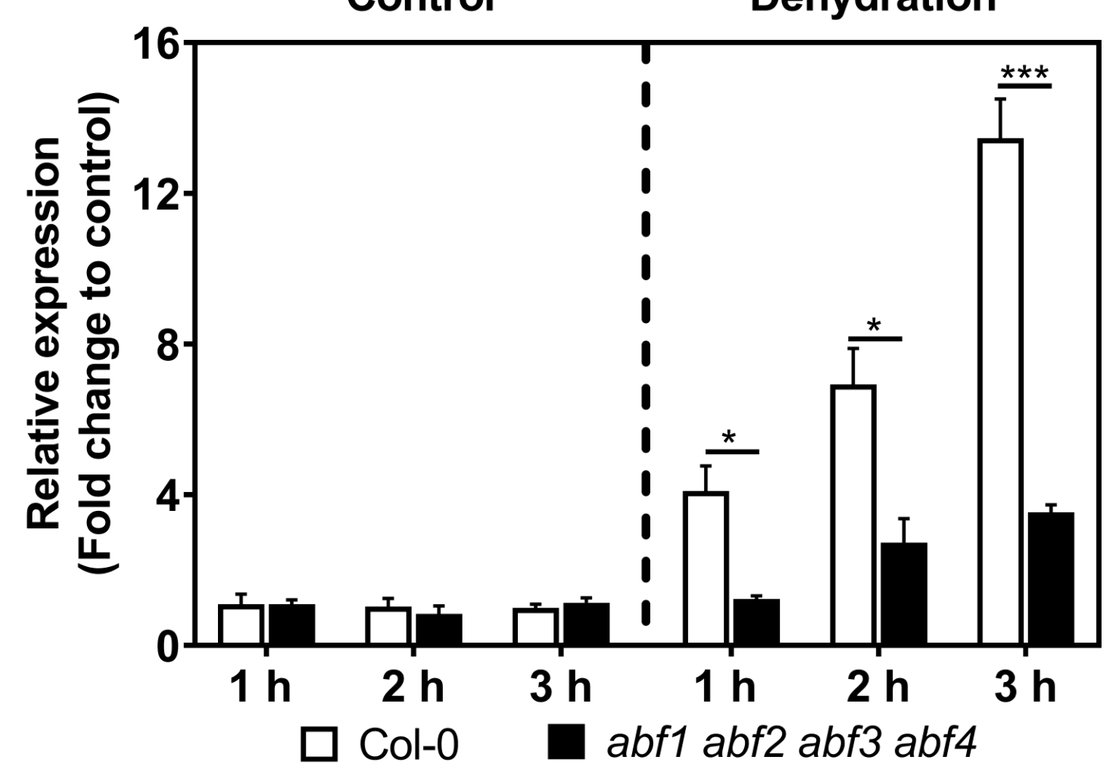
b

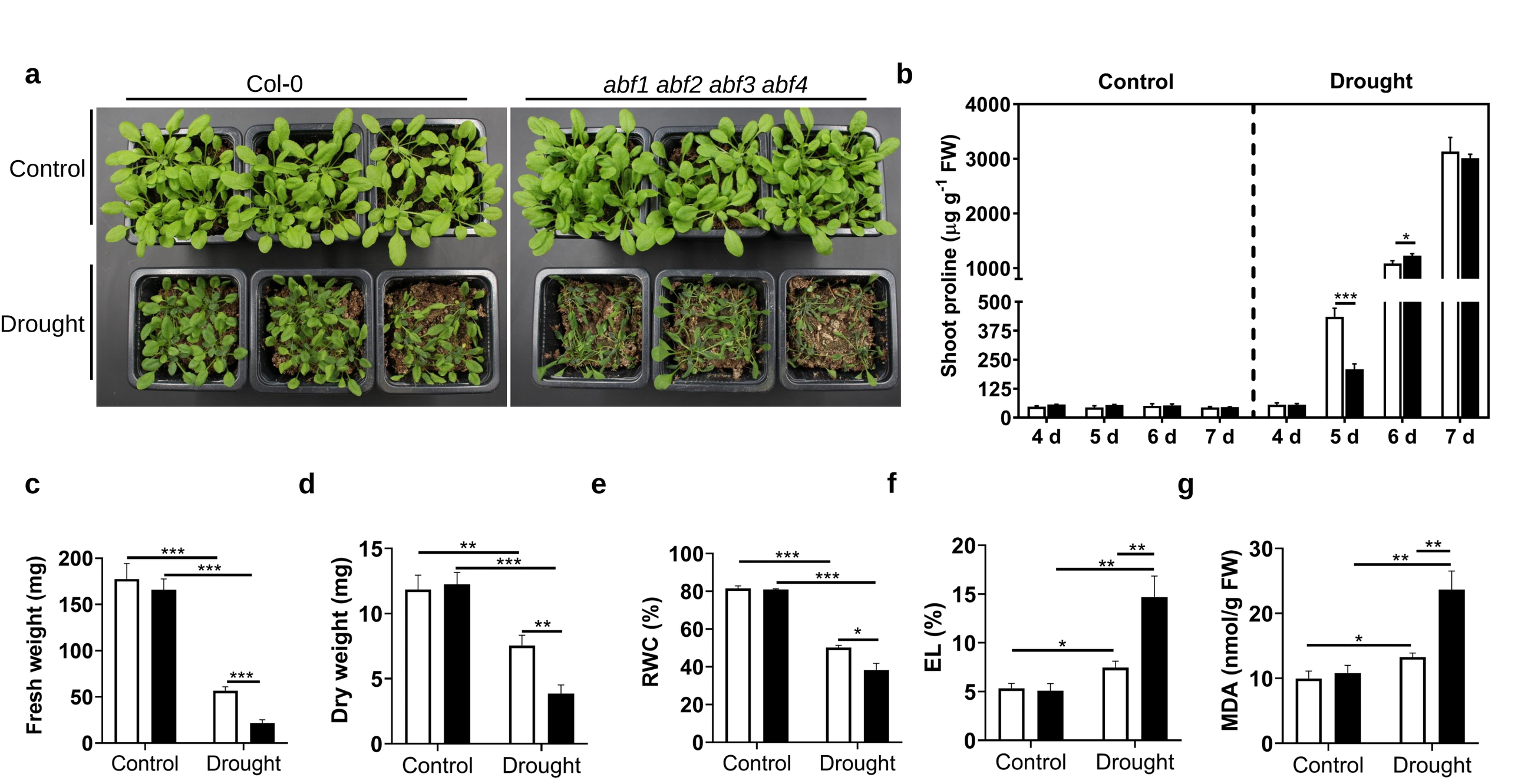

Control
+ABA
Relative expression
(Fold change to control)
28
24
20
16
12
8
4
0
**
***
*
*
24 h
48 h
72 h
96 h
24 h
48 h
72 h
96 h
Col-0
abf1 abf2 abf3 abf4

a


Control
Dehydration
Shoot proline (μg g-1 FW)
350
300
250
200
150
100
50
0
*
*
1 h
2 h
3 h
1 h
2 h
3 h
Col-0
abf1 abf2 abf3 abf4

b


Control
Dehydration
Relative expression
(Fold change to control)
16
12
8
4
0
*
*
***
1 h
2 h
3 h
1 h
2 h
3 h
Col-0
abf1 abf2 abf3 abf4


a
Col-0
abf1 abf2 abf3 abf4
Control
Drought
b
Control
Drought
Shoot proline (μg g-1 FW)
4000
3000
2000
1000
500
375
250
125
0
4 d
5 d
6 d
7 d
***
*
c
Fresh weight (mg)
200
150
100
50
0
d
Dry weight (mg)
15
10
5
0
e
RWC (%)
100
80
60
40
20
f
EL (%)
20
15
10
5
g
MDA (nmol/g FW)
30
20
10
**
Col-0
abf1 abf2 abf3 abf4

Control

Drought

a

b

c

d

Projected leaf area (cm^2)

Relative growth rate (% d^{-1})

Before drought

During drought

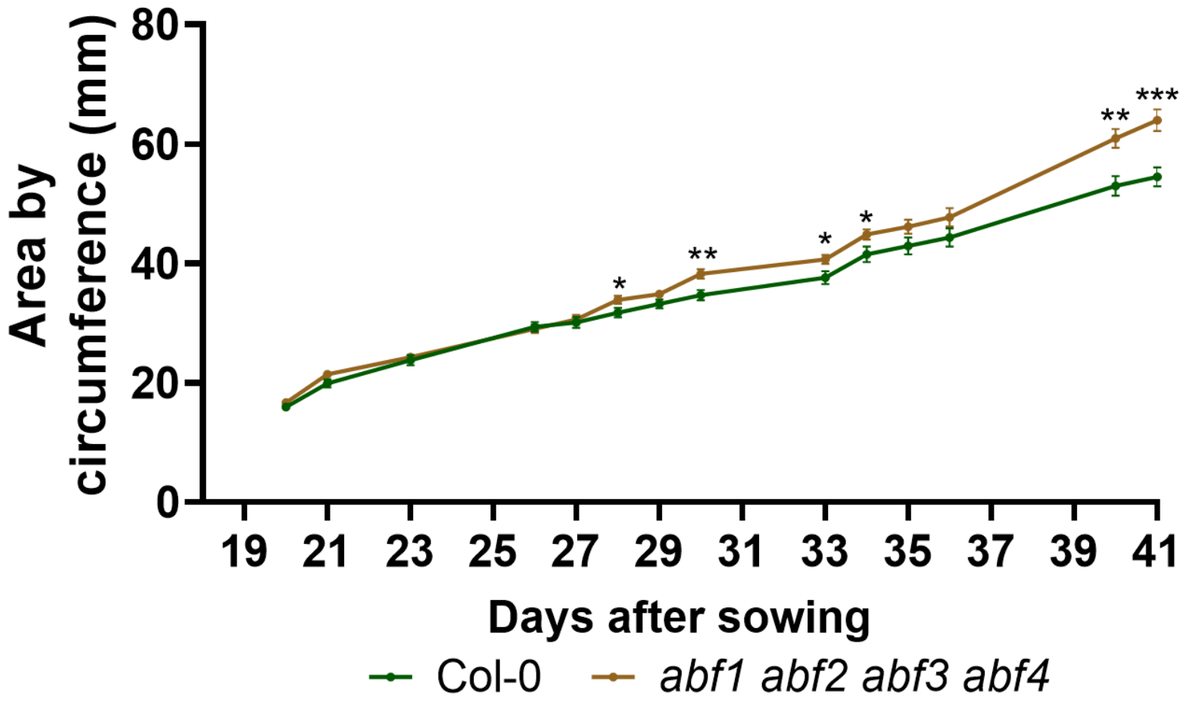
After rewatering

Days after sowing

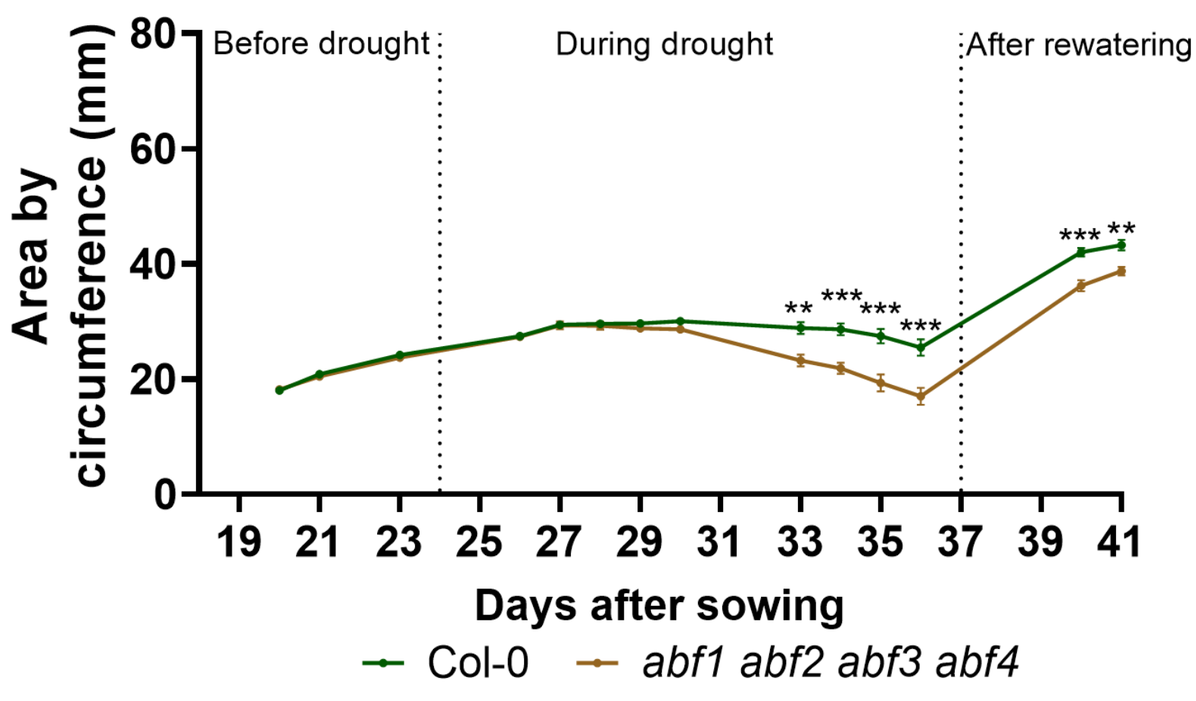
Col-0

abf1 abf2 abf3 abf4

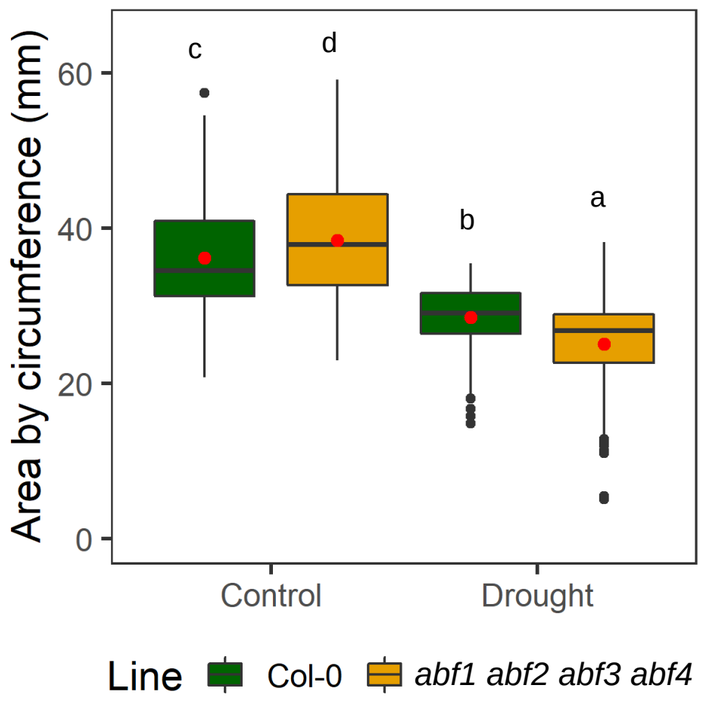
a

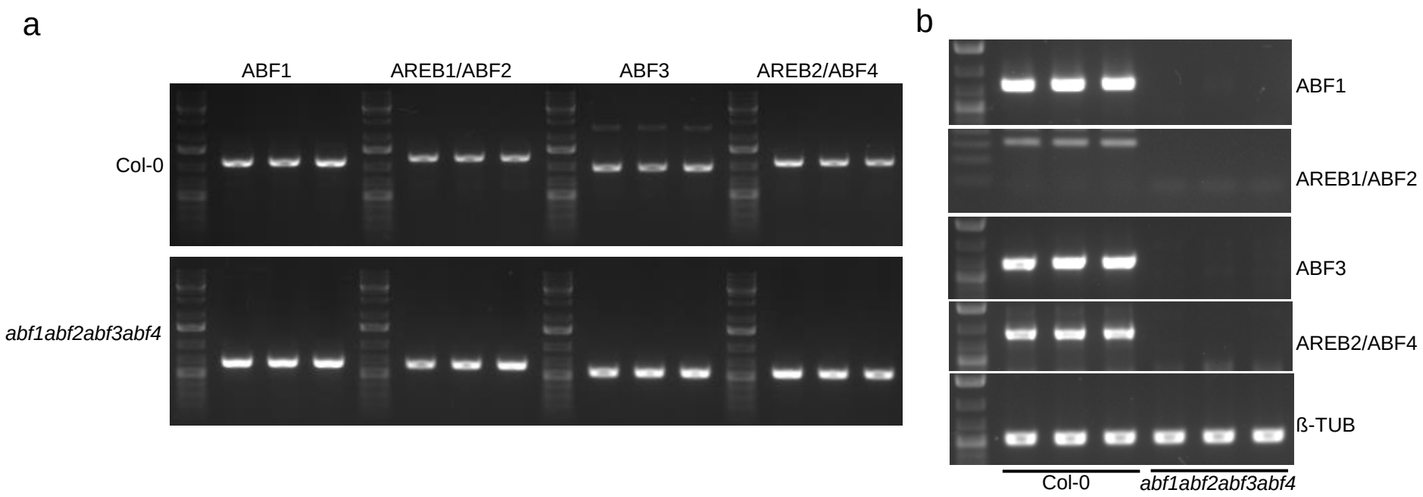

Area by circumference (mm)
0
20
40
60
19
21
23
25
27
29
31
33
35
37
39
41
Days after sowing
Col-0
abf1 abf2 abf3 abf4

b


Before drought
During drought
After rewatering
Area by circumference (mm)
0
20
40
60
19
21
23
25
27
29
31
33
35
37
39
41
Days after sowing
Col-0
abf1 abf2 abf3 abf4

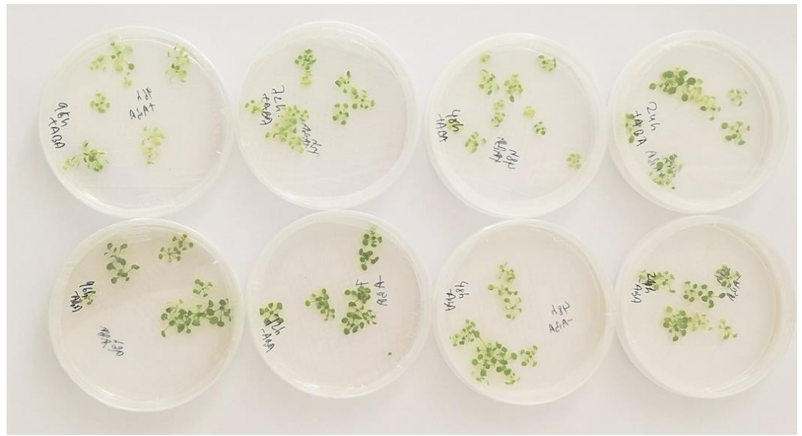
c


Area by circumference (mm)
0
20
40
60
c
d
b
a
Control
Drought
Line
Col-0
abf1 abf2 abf3 abf4



Supporting information Fig. S1: Genotyping of ABF mutants. A) PCR products amplified from genomic DNA of Col-0 and *abf1 abf2 abf3 abf4* using the genotyping primers for SALK_132819, SALK_002984, SALK_096965 and SALK_069523. B) The presence/ absence analysis of gene expression of four ABFs (ABF1, AREB1/ABF2, ABF3 and AREB2/ABF4) was evaluated by semi-quantitative PCR. ß-TUB1 was used as reference gene. Fifteen days old seedlings were dehydrated for 1h before RNA extraction.

a



b

Control

Dehydration

Supporting information Fig. S2: Experimental setup for (a) external ABA application and (b) acute dehydration

```
ABF3          M - - - - - - - G S R L N F K S F V D G V S - - - E - - - - Q Q P T V G T S L P L T R Q N S V F S L T F D
AREB2/ABF4    M - - - - - - - G T H I N F N N L G G G G H P G G E G S S N Q M K P T G S V M P L A R Q S S V Y S L T F D
ABF1          M - - - - - - - G T H I D I N N L G G D T S R G N E - - - - - - - - - - - S K P L A R Q S S L Y S L T F D
AREB1/ABF2    M V Q I Q L L G G S R F - C R K M D G S M N L G N E - - - - P P G D G G G G G G L T R Q G S I Y S L T F D

ABF3          E F Q N S W G G G I G K D F G S M N M D E L L K N I W T A E E S H S M M G N N T S Y T N I S N G N S G N T
AREB2/ABF4    E L Q N T L G G P - G K D F G S M N M D E L L K S I W T A E E A Q A M A M T S A P A - - - - - - - - - - - -
ABF1          E L Q S T L G E P - G K D F G S M N M D E L L K N I W T A E D T Q A F M T T T S S - - - - - - - - - - - -
AREB1/ABF2    E F Q S S V - - - - G K D F G S M N M D E L L K N I W S A E E T Q A M A S G V V P V - - - - - - - - - - - -

ABF3          V I N G G G N N I G G L A V G V G G E S G G F F T G G S L Q R Q G S L T L P R T I S Q K R V D D V W K E L
AREB2/ABF4    - - - - - - - - - - - A T A V A Q P G - A G I P P P G G N L Q R Q G S L T L P R T I S Q K T V D E V W K C L
ABF1          - - - - - - - - - - - - - - - V A A P G P S G F V P G G N G L Q R Q G S L T L P R T L S Q K T V D E V W K Y L
AREB1/ABF2    - - - - - - - - - - - - - - - - - - - - - L G G G Q E G L Q L Q R Q G S L T L P R T L S Q K T V D Q V W K D L

ABF3          M K E D D I G N G V V N G G - - - - - - - - - - - - - T S G I P Q R Q Q T L G E M T L E E F L V R A G V V R E
AREB2/ABF4    I T K D G N M E G S S G G G G - - - - - - - - - - - - E S N V P P G R Q Q T L G E M T L E E F L F R A G V V R E
ABF1          N S K - - - - E G S N G N T G - - - - - - - - - - - T D A L - - E R Q Q T L G E M T L E D F L L R A G V V K E
AREB1/ABF2    S K - - - - - V G S S G V G G S N L S Q V A Q A Q S Q S Q S Q R Q Q T L G E V T L E E F L V R A G V V R E

ABF3          E P Q - - - P V E S V T N F N G G F Y G F G S N G - G L G T A S N G F V A N Q P Q D L S G - - - - N G V A
AREB2/ABF4    D N C V Q Q M G Q V N G N N N N G F Y G N S T A A G G L - - - G F G F G Q P N Q N S I T - - - - - - F N G
ABF1          D N T - - - - - Q Q N E N S S S G F Y A N N G A A - G L - - - E F G F G Q P N Q N S I S - - - - - - F N G
AREB1/ABF2    E A Q V A A R A Q I A E N N K G G Y F G N D A N T - G F - - - S V E F Q Q P S P R V V A A G V M G N L G A

ABF3          V R Q D L L T A Q T Q P L - - - - - - Q M Q Q P Q M V Q Q P Q M V Q Q P Q Q L I Q T Q E R P - - - - - - - -
AREB2/ABF4    T N D S M I L N Q P P G L G L K M G G T M Q Q Q Q - - Q Q Q Q L L Q Q Q Q Q Q M Q Q L N Q P H P Q Q R L P
ABF1          N N S S M I M N Q A P G L G L K V G G T M Q Q Q Q - - Q P H Q - - - - - - - - - Q Q L Q Q P H - - Q R L P
AREB1/ABF2    E T A N S L Q V Q G S S L P L N V N G A R T T Y Q - - Q S Q Q - - - - - - - - - - - - - Q Q P - - - - - - -
```

```
ABF3          - - - F P K Q T T I A F S N T V D V V N R S Q P A T Q C Q E V K P S I L G I H N H P M N N N L L Q A V D F
AREB2/ABF4    Q T I F P K Q A N V A F S A P V N I T N K - - - - - - - - - - - - - G F A G A A N N S I N N N N G L A S Y G
ABF1          P T I F P K Q A N V T F A A P V N M V N R - - - - - - - - - - - - - G L F E T S A D G P A N S N M G - - - - -
AREB1/ABF2    - - I M P K Q P G F G Y G T Q M G Q L N S - - P G I R - - - - G G G L V G L G D Q S L T N N V G F V Q G A

ABF3          K T G V T - - - - V A A V S P G S Q M S P D L T P K S A L D A S L - S P V P Y M F G - - - R V R K T G A V
AREB2/ABF4    G T G V T - - - - V A A T S P G T S - - - - - - - - - S A E N N S L - S P V P Y V L N - - - R G R R S N T G
ABF1          G A G G T - - - - V T A T S P G T S - - - - - - - - - S A E N N T W S S P V P Y V F G - - - R G R R S N T G
AREB1/ABF2    S A A I P G A L G V G A V S P V T P L S S E G I G K S N G D S S S L S P S P Y M F N G G V R G R K S G T -

ABF3          L E K V I E R R Q K R M I K N R E S A A R S R A R K Q A Y T M E L E A E I A Q L K E L N E E L Q K K Q V E
AREB2/ABF4    L E K V I E R R Q R R M I K N R E S A A R S R A R K Q A Y T L E L E A E I E K L K K T N Q E L Q K K Q A E
ABF1          L E K V V E R R Q K R M I K N R E S A A R S R A R K Q A Y T L E L E A E I E S L K L V N Q D L Q K K Q A E
AREB1/ABF2    V E K V V E R R Q R R M I K N R E S A A R S R A R K Q A Y T V E L E A E V A K L K E E N D E L Q R K Q A R
                                  Basic leucine zipper (bZIP) domain
ABF3          I M E K Q K N Q L L E P L R Q P W G M G - - - - - - - - - - - - - - - - - - C K R Q C L R R T L T G P W
AREB2/ABF4    M V E M Q K N E Q L K - E T S K R P W G - - - - - - - - - - - - - - - - - - S K R Q C L R R T L T G P W
ABF1          I M K T H N S E V I T - F F L Y L S K G I F E A A S I A G Q K T M L E K N P Y R S V V R R - - - - - - -
AREB1/ABF2    I M E M Q K N Q E T E - M R N L L Q G G - - - - - - - - - - - - - - - - - - P K K K - L R R T E S G P W
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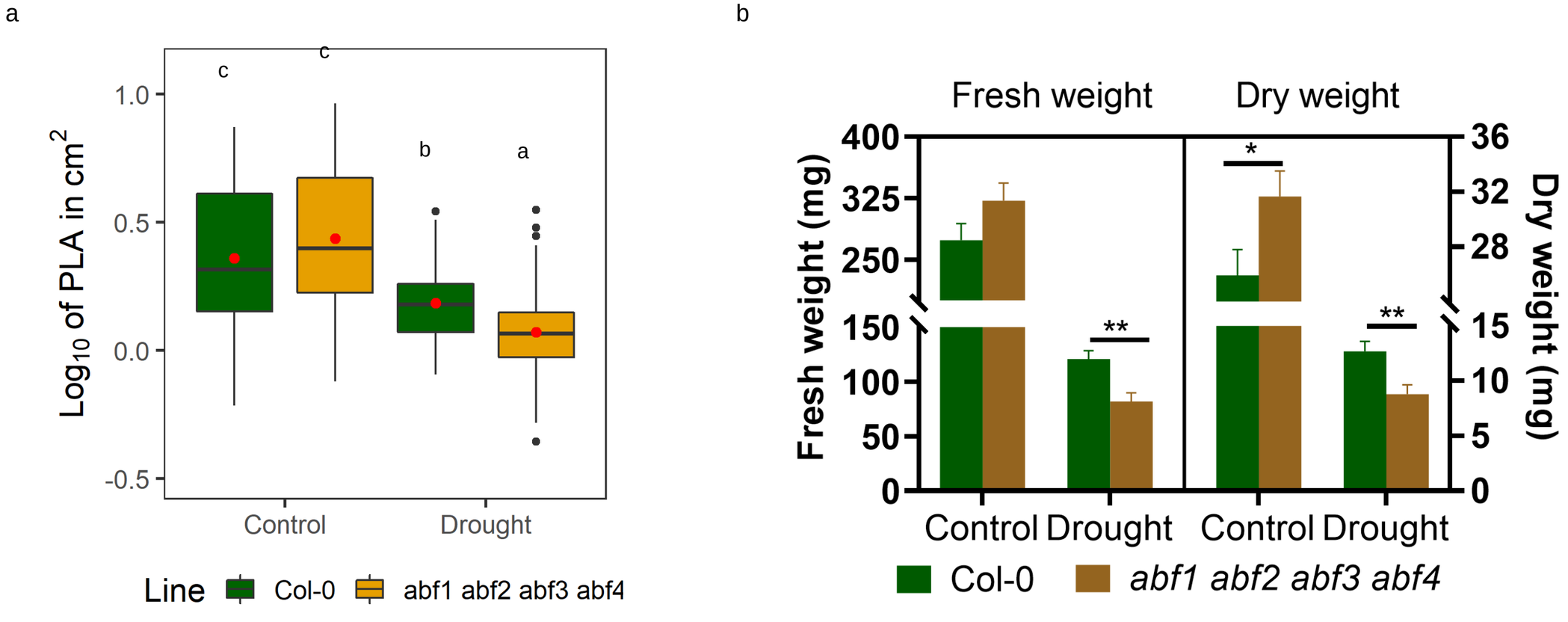
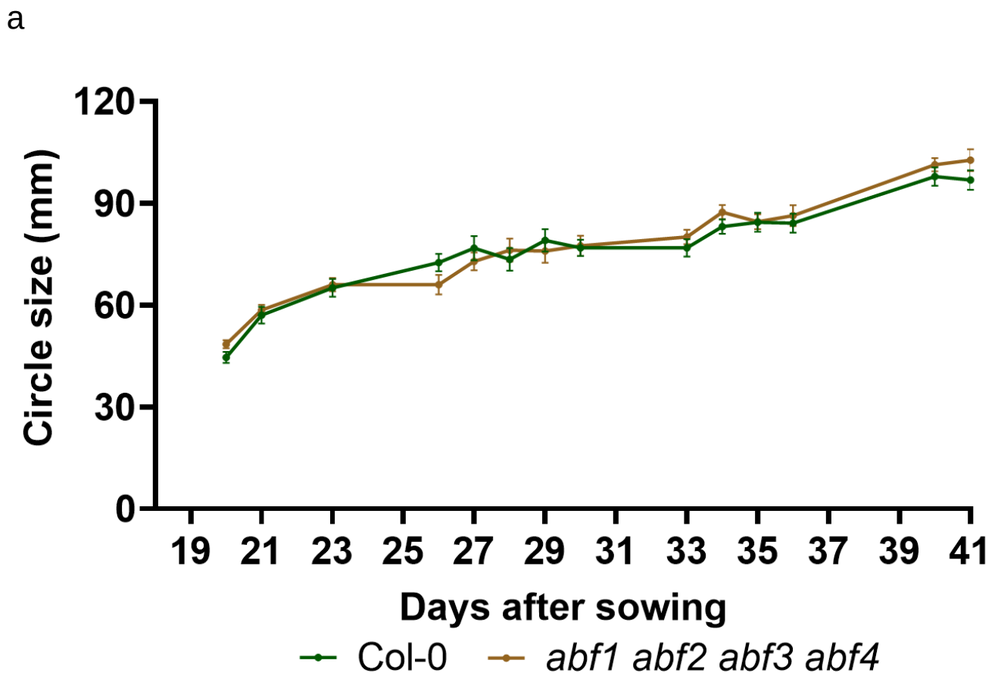
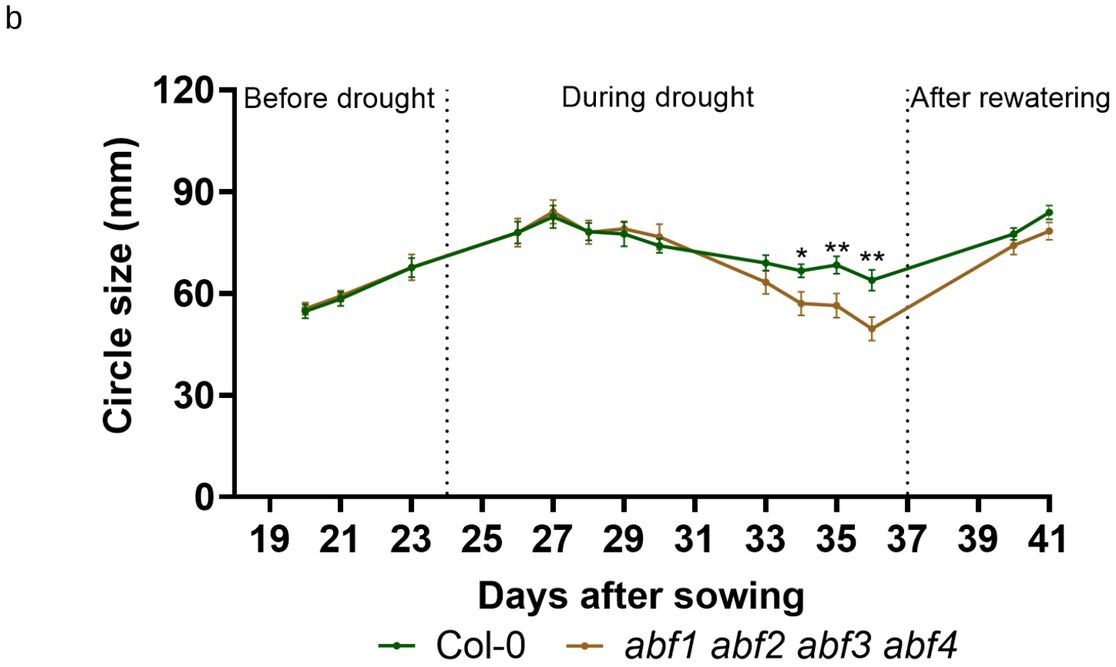
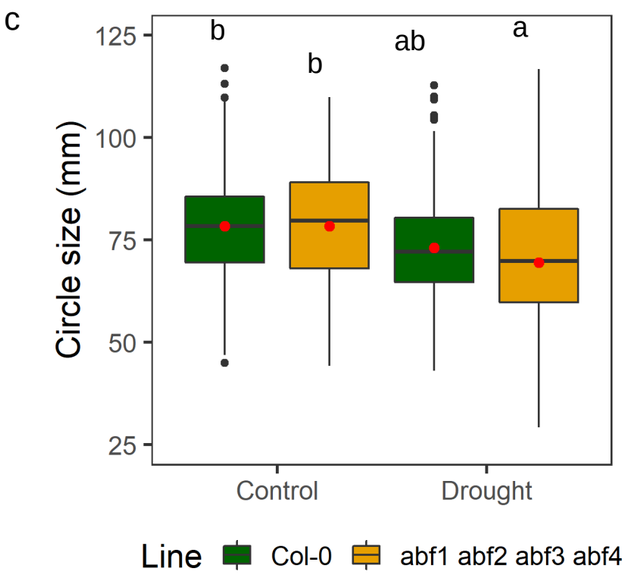
Supporting information Fig. S3: Protein sequence alignment of four ABA responsive element binding factors in Arabidopsis. Basic leucine zipper domain is highlighted in yellow.



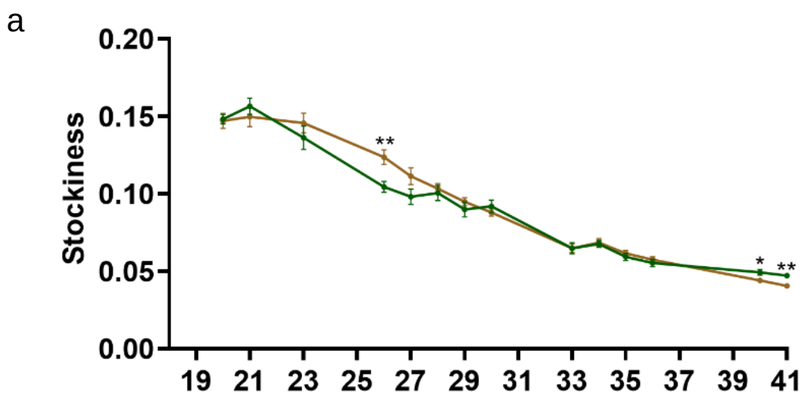
Supporting information Fig. S4: Shoot growth under constant drought stress. Watering was stopped at 24 d after seeding and moderate drought (10% volumetric moisture content) was maintained until 36 d. PLA was recorded every day after drought treatment, and the plants were re-watered when no further increase in PLA was observed under drought conditions. Plants were harvested 5 days after re-watering. (a) Boxplot for projected leaf area between 26 d and 36 d (duration of drought). Red dot indicate mean of the distribution (n = 180). Index above the bars indicates a significant difference between the genotypes ($P < 0.05$) using TukeyHSD test. (b) Effect of moderate water stress on shoot fresh and dry weigh. Bar indicates mean ± standard error (n = 20). Asterisks indicate significance difference between genotypes (*$P < 0.05$, **$P < 0.01$) using student's *t*-test.

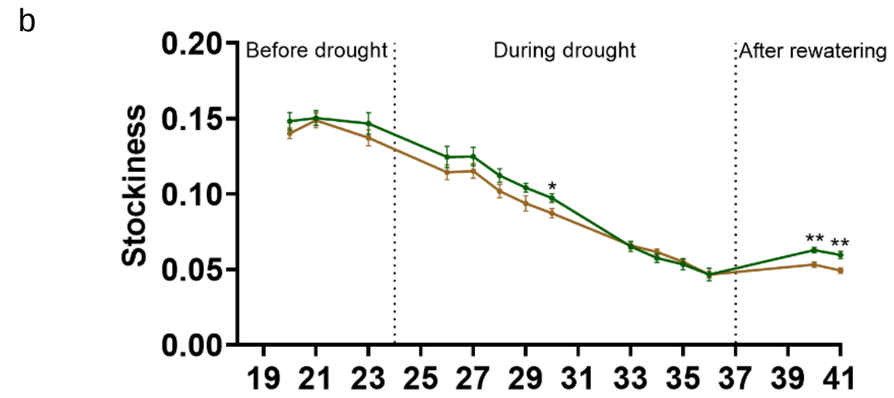


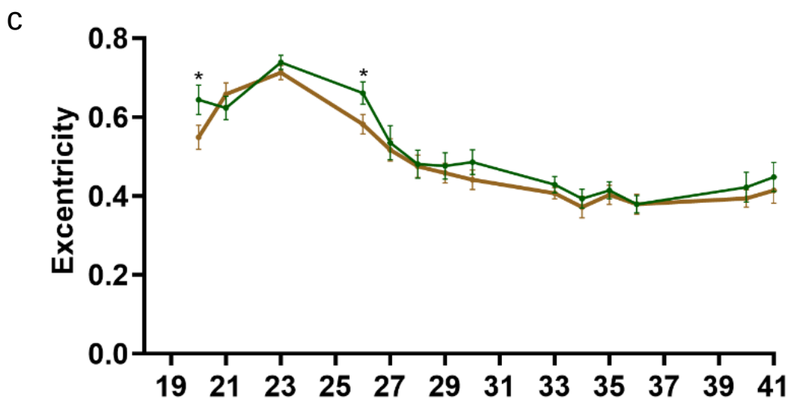


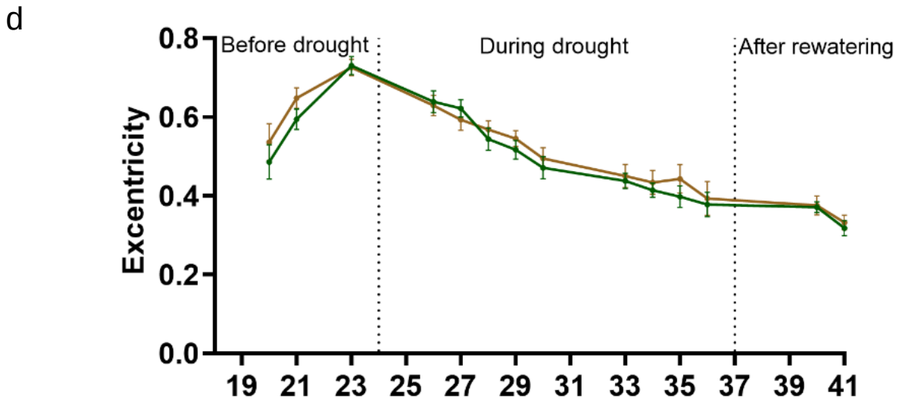


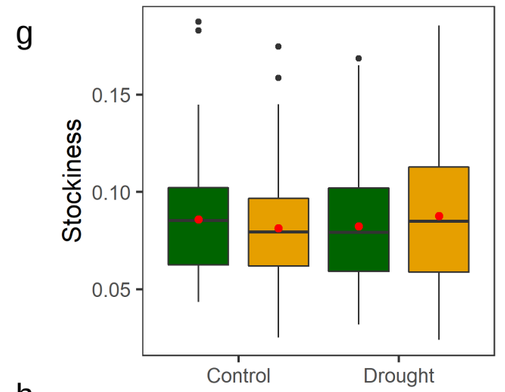
Supporting information Fig. S5: Rosette morphology under constant drought stress. Watering was stopped at 24 d after seeding and moderate drought (10% volumetric moisture content) was maintained until 36 d. PLA was recorded every day after drought treatment, and the plants were re-watered when no further increase in PLA was observed under drought conditions. Estimated circle size of rosette (a) control condition and (b) under drought conditions. The line graph represents mean ± SE (n = 20). Asterisks indicate significance difference between genotypes (*P<0.05, **P < 0.01, ***P < 0.001) using student's t-test. (c) Boxplot for estimated circle size of rosette between 26 d and 36 d (duration of drought). Red dot indicate mean of the distribution. Indexed letters above the bars indicate a significant difference between the genotypes (P < 0.05) using TukeyHSD test.

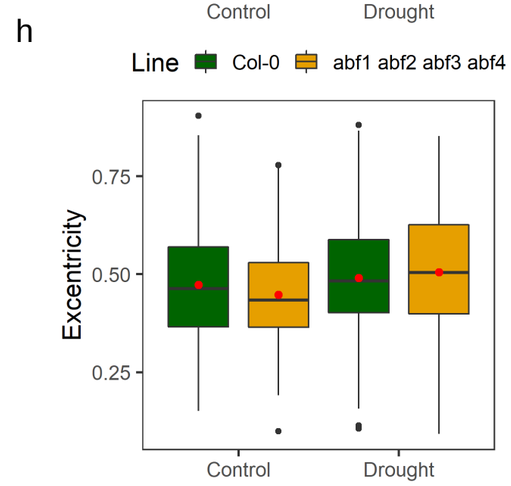


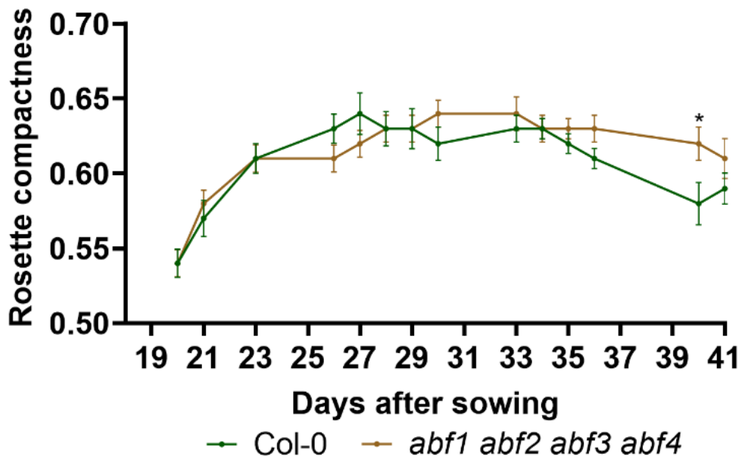


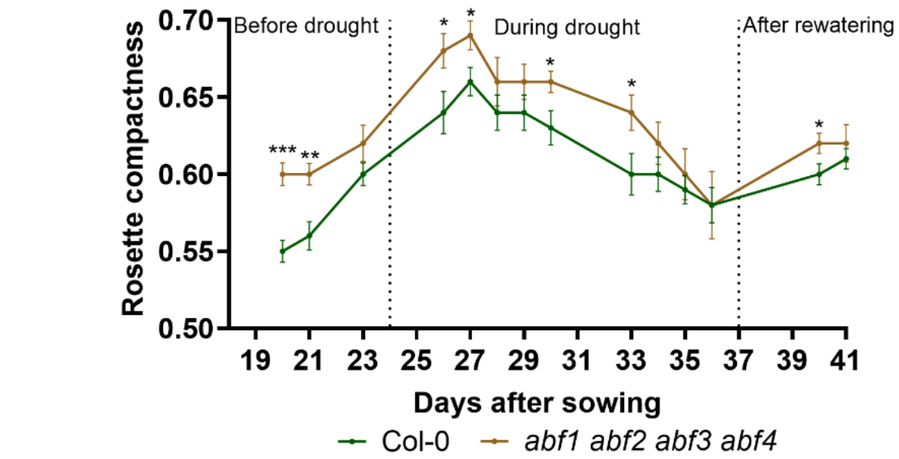


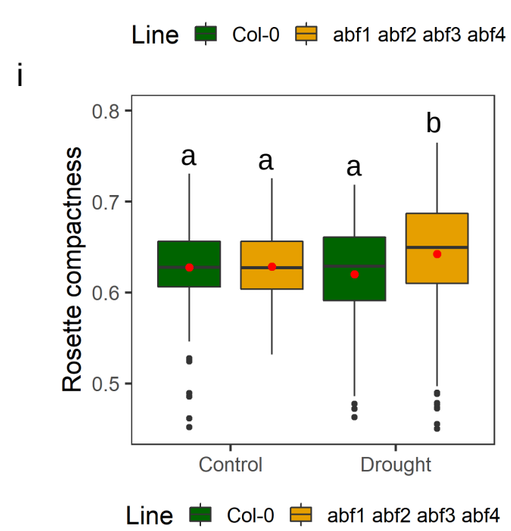












Supporting information Fig. S6: Rosette morphology under constant drought stress. Watering was stopped at 24 d after seeding and moderate drought (10% volumetric moisture content) was maintained until 36 d. PLA was recorded every day after drought treatment, and the plants were re-watered when no further increase in PLA was observed under drought conditions. Stockiness (a) control condition and (b) under drought condition. Excentricity under (c) control condition and (d) drought condition. Rosette compactness (c) control condition and (d) drought condition. The points on the line graph represents mean ± standard error (n = 20). Asterisks indicate significance difference between genotypes (*$P < 0.05$, **$P < 0.01$) using student's *t*-test. Boxplot for estimated (g) rosette compactness (h) rosette stockiness (i) rosette exentricity between 26 d and 36 d (duration of drought). Red dot indicate mean of the distribution. Indexed letters above the bars indicate a significant difference between the genotypes ($P < 0.05$) using TukeyHSD test.

Supporting information Table S1: List of primers used in the study

Primers used for genotyping of SALK lines			
Primer name	Primer sequence (5 → 3)	Gene	Remarks
S132819-F	CCGGTAAGGGTTCTTCTCAAG	AT1G49720	SALK_132819
S132819-R	AGAGGCAACAGACTTTAGGGG		
S002984-F	TAATGGGAAATCTTGGTGCAG	AT1G45249	SALK_002984
S002984-R	TCTTTTGCATTTCCATGATCC		
S096965-F	ACACTGTTATTAACGGCGGTG	AT4G34000	SALK_096965
S096965-R	CTTTCTCCAGAACTGCACCTG		
S069523-F	TCCTCGATTAAGCACATACGG	AT3G19290	SALK_069523
S069523-R	GAACAAGGGTTTTAGGGCTTG		
SALK_LB 1.3	ATTTTGCCGATTTCGGAAC		Left border primer
Primers used for semi-quantitative PCR			
abf1-RT-F	AGAGGCAACAGACTTTAGGGG	AT1G49720	
abf1-RT-R	CCGGTAAGGGTTCTTCTCAAG		
areb1-RT-F	GGTGGTCTTGTGGGACTTGGA	AT1G45249	
areb1-RT-R	CTTCAAGCTCCACGGTGTAAG		
abf3-RT-F	GCTGTTGGTGTGGGAGGAGAA	AT4G34000	Yoshida *et al.* 2015
abf3-RT-R	GGGCGCTCTTTGGAGTCAGAT		
areb2-RT-F	GGGTTTTAGGGCTTGGATGCT	AT3G19290	
areb2-RT-R	TTCACAGGCGCAGAAAATGCT		
bTub1-F	ATCCCACCGGACGTTACAAC	AT1G75780	
bTub1-R	TTCGTTGTCGAGGACCATGC		
Primers used for qPCR			
elf4a-F	TCAGAAGGAGAGAGACGCCA	AT3G19760	Feng *et al.* 2016
elf4a-R	CACGGTTGTTGGGGAGATCA		
p5cs1-F	AAGAGCCCCATATCAGGATTCTTCT	AT2G39800	
p5cs1-R	GAGCGATGTTGAAGGTCTTTACACA		

Feng X.J., Li J.R., Qi S.L., Lin Q.F., Jin J.B. & Hua X.J. (2016) Light affects salt stress-induced transcriptional memory of P5CS1 in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **113**, E8335–E8343.

Yoshida T., Fujita Y., Maruyama K., Mogami J., Todaka D., Shinozaki K. & Yamaguchi-Shinozaki K. (2015) Four Arabidopsis AREB/ABF transcription factors function predominantly in gene expression downstream of SnRK2 kinases in abscisic acid signalling in response to osmotic stress. *Plant, Cell and Environment* **38**, 35–49.

Supporting information Table S2: Summary statistics of proline concentration and P5cs1 mRNA expression in shoot under ABA treatment. Means sharing a letter are not significantly different by Tukey-adjusted comparisons ($P < 0.05$). The asterisks indicated significant genotype, treatment and genotype by treatment interaction effect (ns, non-significant; $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***).

Hour	-ABA		+ABA		ANOVA		
	abf1 abf2 abf3 abf4	Col-0	*abf1 abf2 abf3 abf4*	Col-0	Genotype	Treatment	Interaction
Mean values of shoot proline concentration (µg/ g Fresh weight)							
24	90^{a}	87^{a}	120^{a}	183^{b}	**	***	*
48	112^{ab}	92^{a}	170^{bc}	233^{c}	ns	***	ns
72	118^{a}	107^{a}	209^{b}	448^{c}	*	***	*
96	131^{a}	135^{a}	259^{b}	847^{c}	***	***	***
Mean values of relative P5cs1 expression (fold change to control)							
24	1.01^{a}	1.16^{a}	1.89^{a}	6.82^{b}	*	**	*
48	1.06^{a}	1.03^{a}	4.61^{b}	15.26^{b}	**	***	**
72	1.00^{a}	1.00^{a}	3.32^{b}	18.39^{c}	***	***	***
96	1.04^{a}	1.03^{a}	3.52^{b}	21.00^{c}	**	***	**

Supporting information Table S3: Summary statistics of shoot proline concentration and P5cs1 mRNA expression in shoot under acute dehydration treatment. Means sharing a letter are not significantly different by Tukey-adjusted comparisons (P<0.05). The asterisks indicated significant genotype, treatment and genotype by treatment interaction effect (ns, non-significant; P < 0.05, *; P < 0.01, **; P < 0.001, ***).

Mean values of shoot proline (µg/ g FW)							
Hour	Control		Dehydration		ANOVA		
	abf1 abf2 abf3 abf4	Col-0	*abf1 abf2 abf3 abf4*	Col-0	Genotype	Treatment	Interaction
1	54.2^{a}	60.7^{a}	80.3^{ab}	94.8^{b}	**	**	**
2	62.2^{a}	66.8^{a}	114.7^{ab}	168.7^{b}	*	***	ns
3	61.5^{a}	58.3^{a}	185.1^{b}	286.0^{c}	*	***	*
Mean values of relative P5cs1 expression (fold change to control)							
1	1.1^{a}	1.1^{a}	1.22^{a}	4.1^{b}	**	**	**
2	0.9^{a}	1.1^{ab}	2.7^{bc}	6.9^{c}	***	***	***
3	1.14^{a}	1.0^{a}	3.5^{b}	13.5^{c}	***	***	***

Supporting information Table S4: Summary statistics of shoot proline concentration under drought stress. Means sharing a letter are not significantly different by Tukey-adjusted comparisons ($P < 0.05$). The asterisks indicated significant genotype, treatment and genotype by treatment interaction effect (ns, non-significant; $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***).

Day	Control		Drought		ANOVA		
	abf1 abf2 abf3 abf4	Col-0	*abf1 abf2 abf3 abf4*	Col-0	Genotype	Treatment	Interaction
4	57^{a}	47^{a}	55^{a}	55^{a}	ns	ns	ns
5	55^{a}	44^{a}	209^{b}	435^{c}	***	***	***
6	53^{a}	51^{a}	1226^{c}	1085^{b}	***	***	*
7	45^{a}	44^{a}	3013^{b}	3132^{b}	ns	***	ns

Supporting information Table S5: Summary statistics for the morphological and biochemical response to drought stress. Means sharing a letter are not significantly different by Tukey-adjusted comparisons ($P<0.05$). The asterisks indicated significant genotype, treatment and genotype by treatment interaction effect (ns, non-significant; $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***).

Trait	Control		Drought		ANOVA		
	Col-0	*abf1 abf2 abf3 abf4*	Col-0	*abf1 abf2 abf3 abf4*	Genotype	Treatment	Interaction
Fresh weight (g)	177.5^{a}	165.9^{a}	56.6^{b}	21.5^{b}	*	***	ns
Dry weight (g)	11.85^{a}	12.24^{a}	7.55^{b}	3.86^{c}	*	***	*
Malondialdehyde (nmol/ g fresh weight)	10.0^{a}	10.8^{a}	13.3^{a}	23.7^{b}	**	***	**
Electrolyte leakage (%)	5.3^{a}	5.1^{a}	7.46^{a}	14.69^{b}	**	***	**
Relative water content (%)	81.5^{a}	80.9^{a}	50.1^{b}	38.1^{c}	**	***	*