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# Role of Arabidopsis *ABF1/3/4* during *det1* germination in salt and osmotic stress conditions

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## Abstract

***Key message*** **Arabidopsis *det1* mutants exhibit salt and osmotic stress resistant germination. This phenotype requires HY5, ABF1, ABF3, and ABF4.**

**Abstract** While DE-ETIOLATED 1 (DET1) is well known as a negative regulator of light development, here we describe how *det1* mutants also exhibit altered responses to salt and osmotic stress, specifically salt and mannitol resistant germination. LONG HYPOCOTYL 5 (HY5) positively regulates both light and abscisic acid (ABA) signalling. We found that *hy5* suppressed the *det1* salt and mannitol resistant germination phenotype, thus, *det1* stress resistant germination requires HY5. We then queried publically available microarray datasets to identify genes downstream of HY5 that were differentially expressed in *det1* mutants. Our analysis revealed that ABA regulated genes, including *ABA RESPONSIVE ELEMENT BINDING FACTOR 3* (*ABF3*), are downregulated in *det1* seedlings. We found that *ABF3* is induced by salt in wildtype seeds, while homologues *ABF4* and *ABF1* are repressed, and all three genes are underexpressed in *det1* seeds. We then investigated the role of *ABF3, ABF4*, and *ABF1* in *det1* phenotypes. Double mutant analysis showed that *abf3, abf4*, and *abf1* all suppress the *det1* salt/osmotic stress resistant germination phenotype. In addition, *abf1* suppressed *det1* rapid water loss and open stomata phenotypes. Thus interactions between *ABF* genes contribute to *det1* salt/osmotic stress response phenotypes.

**Keywords** DET1 · HY5 · ABI5 · Salt stress · Osmotic stress · ABF3/4/1

## Abbreviations

| | |
|---|---|
| ABF | Abscisic acid responsive element binding factor |
| ABI5 | Abscisic acid insensitive 5 |
| DET1 | De-etiolated 1 |
| HY5 | Long hypocotyl 5 |

## Introduction

How plants respond to environmental stimuli affects their growth, development, and survival. Water availability is one of the most important extrinsic factors that limit plants' ability to grow and survive. Water scarcity and high salinity induce osmotic stress, inhibiting the normal functions of the plant and consequently reducing yield. Tolerance of these stresses is one of the major challenges facing current agriculture and food production (Deinlein et al. 2014).

The plant hormone abscisic acid (ABA) controls an array of physiological processes in plants, including regulating water balance and conferring osmotic stress tolerance (Raghavendra et al. 2010). ABA also induces gene expression associated with stress response (Busk and Pages 1998), resulting in the accumulation of osmoprotectant proteins, the modification of metabolic pathways, changes in ion uptake, and scavenging of free radicals, allowing the plant cell to maintain homeostasis even under stress conditions (Bhattacharjee and Saha 2014).

In a previous study we examined the role of DE-ETIOLATED 1 (DET1, AT4G10180) in ABA signalling and

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found that *det1* mutants show genetically complex phenotypes (Fernando and Schroeder 2015). DET1 is a negative regulator of light signalling and is part of the CONSTITUTIVE PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (COP/DET/FUS) group of genes which are central repressors of photomorphogenesis. DET1 indirectly regulates levels of the transcription factor LONG HYPOCOTYL 5 (HY5, AT5G11260) via the COP1 E3 ubiquitin ligase (Huang et al. 2014; Osterlund et al. 2000). HY5 is a bZIP transcription factor that positively regulates both light and ABA signalling (Chattopadhyay et al. 1998; Chen et al. 2008). HY5 directly binds to the promoter of *ABSCISIC ACID INSENSITIVE 5 (ABI5*, AT2G36270*)* and upregulates transcription, thereby positively regulating ABA signalling (Chen et al. 2008). We previously showed that *det1* mutants are hypersensitive to ABA inhibition of seed germination and that this sensitivity requires both HY5 and ABI5. Thus, in *det1* mutants, increased levels of HY5 appear to result in upregulation of the germination inhibiting transcription factor *ABI5*, resulting in reduced germination (Fernando and Schroeder 2015; Osterlund et al. 2000). Moreover, germination of *hy5* is resistant to salt and glucose, suggesting a possible role of HY5 in ABA-mediated salt and osmotic stress response (Chen et al. 2008).

Many ABA regulated genes share a common *cis*-element, 8–10 base pairs in length, known as the ABA-responsive element (ABRE). A small class of bZIP transcription factors, highly homologous to ABI5, called ABA RESPONSIVE ELEMENT BINDING FACTORS (AREB/ABFs), bind to these regions (Busk and Pages 1998). There are 9 AREB/ABFs in Arabidopsis. The AREB/ABF subfamily of bZIP transcription factors are upregulated by water stress and ABA, and require ABA for full activation. In vegetative tissues *ABF3* and *AREB2*/*ABF4* are highly induced by ABA and osmotic stress (Yoshida et al. 2010). ABF1 (AT1G49720), AREB1/ABF2 (AT1G45249), ABF3 (AT4G34000), and AREB2/ABF4 (AT3G19290) are the main downstream transcription factors involved in ABA-mediated osmotic stress signalling in vegetative tissues (Choi et al. 2000; Uno et al. 2000; Kang et al. 2002). *ABF1*, whose gene expression levels are lower than the other *ABF* genes, also plays an important role in drought stress (Yoshida et al. 2015). ABI5 and ABF3 have some overlapping functions in terms of seed germination and root growth in response to various stress conditions (Finkelstein et al. 2005). The *AREB*/*ABF* genes have highly overlapping functions and triple mutants show ABA insensitive root growth and reduced drought tolerance (Yoshida et al. 2010). In addition, a genome wide study of HY5 target genes in Arabidopsis showed that HY5 binds to the *ABF3* promoter and that light downregulates *ABF3* expression. While *ABF1* and *ABF4* are also targets of HY5, *ABF2* is not (Lee et al. 2007). Despite these findings, the relationship between ABFs, HY5, and DET1 under abiotic stress conditions is unclear.

In this study we used genetic analysis to investigate the role of HY5 and ABI5 in the germination phenotypes of *det1* mutants in salt and osmotic stress conditions. We also examined the role of *ABF1/3/4* in *det1* germination phenotypes. This study provides an understanding of the role of DET1 and ABF1/3/4 in stress signalling during salt and osmotic stress.

## Materials and methods

### Plant materials and growth conditions

Except for *abi5-1*, which is in the Wassilewskija-2 (Ws-2) background, all *Arabidopsis thaliana* mutants in this study are in the ecotype Columbia-0 (Col-0). *det1-1, hy5* (SALK_096651C), point mutant *abi5-1* (CS8105), and their respective double mutants are as previously described (Fernando and Schroeder 2015). The *abf1* (SALK_043079), *abf3* (SALK_075836), and *abf4* (SALK_069523) T-DNA insertion mutants, previously characterized in Kim et al. (2004) and Finkelstein et al. (2005), were obtained from the Arabidopsis Biological Resource Centre (http://abrc.osu.edu). For growth analysis, sterilized seeds were plated on Linsmaier and Skoog (LS) media (Caisson) supplemented with 2% sucrose and 0.86% Phytoblend (Caisson) and stratified at 4 °C for 2 days. For germination assays, seeds were plated as above but on LS media with 0% sucrose. Plates were then transferred to a growth chamber at 20 °C and 50% relative humidity for 14 days. Long day conditions (16 h of light and 8 h of dark) were provided by fluorescent bulbs (100 µM photons $m^{-2}$ $s^{-1}$). After 14 days seedlings were transplanted to Sunshine mix number 1 (SunGro, Bellevue, WA).

### Construction of double mutants in the *det1* background

All double mutants (*det1 abf1, det1 abf3*, and *det1 abf4)* were generated using standard protocols (Weigel and Glazebrook 2002). Multiple independent F2 homozygous double mutant lines were identified for each double mutant combination based on their *det1* mutant phenotypes and PCR genotyping using the oligonucleotide primers described below. ABF1_F (5′-GGTTTTCATTATTTCAGCCTGC-3′) and ABF1_R (5′-GGGACCTAGTGGTTTTGTTCC-3′) were used to detect the wildtype *ABF1* allele while the *ABF3* wildtype allele was detected using ABF3_F2 (5′-TTTCTAATTGGACCACGTTGC-3′) and ABF3_R2 (5′-ACAGCTAACCCACCAATGTTG-3′). ABF4_F (5′-TCCTCGATTAAGCACATACGG-3′) and ABF4_R (5′-GAACAAGGGTTTTAGGGCTTG-3′) were used to detect the *ABF4*

wildtype allele. T-DNA insertions were detected using LBb1.3 (5′-ATTTTGCCGATTTCGGAAC-3′) in combination with one of the above primers for each genotype.

## Seedling analysis

For hypocotyl analysis, plants were grown under long day or dark conditions (after exposure to light for 6 h to initiate germination). Plates were scanned on a flat bed scanner after 7 days and hypocotyl length and cotyledon width were measured using NIH Image J software (Schneider et al. 2012). Chlorophyll content was measured using 7 day old whole seedlings, with 2 replicates per line of 20 seedlings each. Chlorophyll was extracted with 80% acetone overnight and A645 and A663 were measured using a Spectrophotometer (model 2100 pro, Ultrospec Biochrom). Chlorophyll content was calculated according to the Mackinney method (Mackinney 1941). Seedling experiments were repeated at least twice.

## Adult growth parameter measurements

For adult growth analysis, parameters measured were: flowering time, in terms of both number of days until the first bud became visible and total number of rosette and cauline leaves on the main inflorescence; rosette diameter at 4 weeks; total number of inflorescences, silique length, and height at 6 weeks. Adult growth experiments were performed at least twice.

## Seed germination assays

Sterilized Arabidopsis seeds from each genotype were sown on LS media, 0.86% Phytoblend and 0% sucrose supplemented with 0, 100, or 200 mM NaCl (Fisher Scientific), or 200 or 400 mM Mannitol or Sorbitol (Fisher Scientific), or 0.5, 2.5, or 5 µM ABA (Sigma). Plates were stratified at 4 °C for 2 days then transferred to 20 °C and long day conditions (16 h of light and 8 h of dark). Seed germination was scored every 12 or 24 h as percentage of seeds with radicles completely penetrating the seed coat, for up to 5–10 days (Bolle 2009). Representative graphs are shown indicating germination up to 5 days.

## Publicly available microarray gene expression data analysis

To identify genes differentially expressed between the *det1* mutant and wildtype plants we used publicly available microarray data from CATdb (a Complete Arabidopsis Transcriptome data base) (Gagnot et al. 2008). Project RS09-01_Det1 (expression profile of *det1-1* mutants during photomorphogenesis) was used as the source of data. This experiment utilized seedlings grown in the dark for 5 days on 1xMS media without sucrose, after 1 h exposure to light (100 µM photons $m^{-2}$ $s^{-1}$) to induce germination. Microarray gene expression data (*det1* dark grown seedlings vs. Col-0 dark grown seedlings) was processed using ChipEnrich to identify significantly enriched Gene Ontology (GO) terms according to the methods of Brady et al. (2007) and modified by Belmonte et al. (2013) to predict biological function. GO terms were considered to be statistically enriched at $10^{-3}$ ($P < 0.001$) when compared to the Arabidopsis genome using hypergeometric distribution. Enriched GO terms were then visualized in a heat map using Multiple Expression Viewer TMeV (Saeed et al. 2006). The 'analysis' function in ChipEnrich was then used to predict transcription factor modules within the dataset. This analysis associates transcription factors with significantly enriched DNA sequence motifs ($P < 0.001$) within the 1 kb upstream region of the transcription start site of genes belonging to the GO terms identified above. The 'network' and 'attribute' files were used to generate the network diagram in Cytoscape (version 2.6.3 http://www.cytoscape.org).

## RNA extraction and real time PCR

RNA was extracted from approximately fifty 7 day old long day or dark grown seedlings, or approximately 200 seeds imbibed in LS liquid media [0% sucrose with or without intermediate salt concentration 150 mM NaCl as in Kilian et al. (2007)] for 2 days at 4 °C, using the RNeasy plant mini kit (Qiagen) according to manufacturer's instructions. 1 µg of total RNA was used to synthesize cDNA (Maxima First Strand cDNA synthesis kit, Fermentas). Both RNA and cDNA were quantified using a Nano-drop spectrophotometer (Thermo Scientific). Quantitative RT-PCR was performed to detect the relative abundance of *ABF1, ABF3*, and *ABF4* transcript levels in wild type and *det1*. qPCR primer sequences used were as follows: *ABF1* (AT1G49720) ABF1_c131F (5′-TCAACAACTTAGGCGGCGATAC-3′) and ABF1_c340R (5′-GCAACCGAAGATGTAGTA GTCA-3′); *ABF3* (AT4G34000) ABF3_c1276F (5′-TAC GATGGAACTGGAAGCAG-3′) and ABF3_c1385R (5′-GAGGCTCCAGAAGCTGATTT-3′); *ABF4* (AT3G19290) ABF4_c1300F (5′-AACTGGAAGCCGAAATTGAAA-3′) and ABF4_c1404R (5′-ACGTTTCTTTCAGCTGCT CAT-3′). Amplified samples were normalized against *EF1α* (AT5G60390) (5′-CTGGAGGTTTTGAGGCTGGTAT-3′, 5′-CCAAGGGTGAAAGCAAGAAGA-3′) (Hossain et al. 2012; Jain et al. 2006). Real time PCR was performed using a 10-fold dilution of cDNA in a 96-well plate using iQ SYBR Green Supermix (Bio-Rad). CFX Connect Real time PCR detection system (Bio-Rad) was used for the analysis. At least three technical replicates per sample were performed and the mean values were calculated.

### Transpirational water loss assays

To assess dehydration tolerance, water loss assays were performed following a method slightly modified from Cheong et al. (2007). Rosette leaves were detached from 5 week old plants (three leaves from wild type and single mutant plants, six leaves from *det1* and double mutants) and were kept on the laboratory bench on a weighing boat. Fresh weights were measured after the indicated periods of time. Water loss was calculated as percentage of weight loss vs initial fresh weight. Two replicates were used in each experiment per genotype and the experiments were repeated at least twice.

### Measurement of stomatal index

Whole leaves of similar developmental stage (5th leaf of each plant) were detached, mounted in water, and observed immediately under an upright light microscope (Zeiss AxioVision). Five areas of $220 \times 170\ \mu m^2$ per leaf from three different plants were imaged at 40× oil immersion and Stomatal Index (SI) was calculated for each area. SI was calculated using the equation SI = number of stomata/(number of stomata + number of pavement cells) × 100%. SI was calculated individually for each leaf and the mean was calculated per genotype (Kang et al. 2009).

### Measurement of stomatal aperture

Stomatal apertures were measured in 4-week old rosette leaves following a method slightly modified from that of Li et al. (2013). Detached whole leaves (4th or 5th leaf) were floated with abaxial surfaces facing down on MES/KCl buffer (50 mM KCl, 0.1 mM $CaCl_2$, 10 mM MES, pH 6.15), with 1 µM ABA at 20 °C and 50% relative humidity, in the dark. After 2, 3, and 4 h, leaves were placed on a glass slide and mounted in the same buffer. The abaxial side of the leaves was immediately observed under an upright light microscope (Zeiss Axiovision) and images captured under 100× oil immersion. Pore width and length of at least 12 different stomata/genotype/treatment were measured using NIH Image J software (Schneider et al. 2012).

### Statistical analysis

Each experiment was repeated at least twice and the results of a single representative experiment presented here. Student's *t* tests were performed and single mutants compared to the wild type and double mutants compared to *det1*. $P \leq 0.05$ was considered to be statistically significant.

## Results and discussion

### *det1* mutants exhibit salt and mannitol resistant germination

In a previous study we observed that *det1* mutants are hypersensitive to ABA inhibition of seed germination (Fernando and Schroeder 2015). Since salt and osmotic stresses are ABA-mediated stress signalling pathways, we investigated salt and osmotic stress responses in *det1* mutants. However, as also described in Fernando and Schroeder (2016), the opposite phenotype was observed. *det1* mutants exhibited resistant germination on media supplemented with salt, mannitol, or sorbitol (Fig. 1a, S1). Mannitol and sorbitol create osmotic stress conditions while salt creates both ionic and osmotic stress (Tholakalabavi et al. 1994; Finkelstein et al. 2005). Therefore, *det1* mutants exhibit the unusual combination of ABA hypersensitive and osmotic stress resistant germination.

### *det1 hy5* and *det1 abi5* salt/mannitol germination responses

Prior studies have shown that *hy5* partially or completely rescues many *det1* seedling and adult growth phenotypes, indicating that *HY5* is epistatic to DET1 (Chory 1992; Pepper and Chory 1997). We previously found that HY5 is required for *det1* ABA sensitive germination. We proposed that on ABA containing media *det1* mutants have excess HY5, resulting in upregulation of ABI5, thus reducing germination in *det1* mutants (Osterlund et al. 2000; Fernando and Schroeder 2015). HY5 has been implicated in salt stress (Chen et al. 2008), thus we examined the role of HY5 and ABI5 in *det1* salt/mannitol resistant germination.

While others have reported that *hy5* exhibits salt resistant germination on 3% sucrose supplemented media (Chen et al. 2008; Yu et al. 2016), we found that on sucrose free media *hy5* single mutants are slightly sensitive to low concentrations of salt, but exhibit resistance to high concentrations of mannitol (Fig. 1b, S2). This appears to be a sucrose dependent phenotype, since on 0.6% sucrose *hy5* exhibited salt and mannitol resistant germination as reported (Fig. S3). In *det1 hy5* double mutants, the *det1* resistant germination phenotype was suppressed on both salt and mannitol (Fig. 1b, S2). Thus HY5 is required for the *det1* resistant germination phenotype and HY5 is epistatic to DET1 with respect to salt/osmotic stress regulation of germination.

Since HY5 is required for *det1* stress resistant germination, we examined the role of ABI5 in *det1* salt/osmotic stress response (Fig. 1c, S4). As expected, the *abi5* single

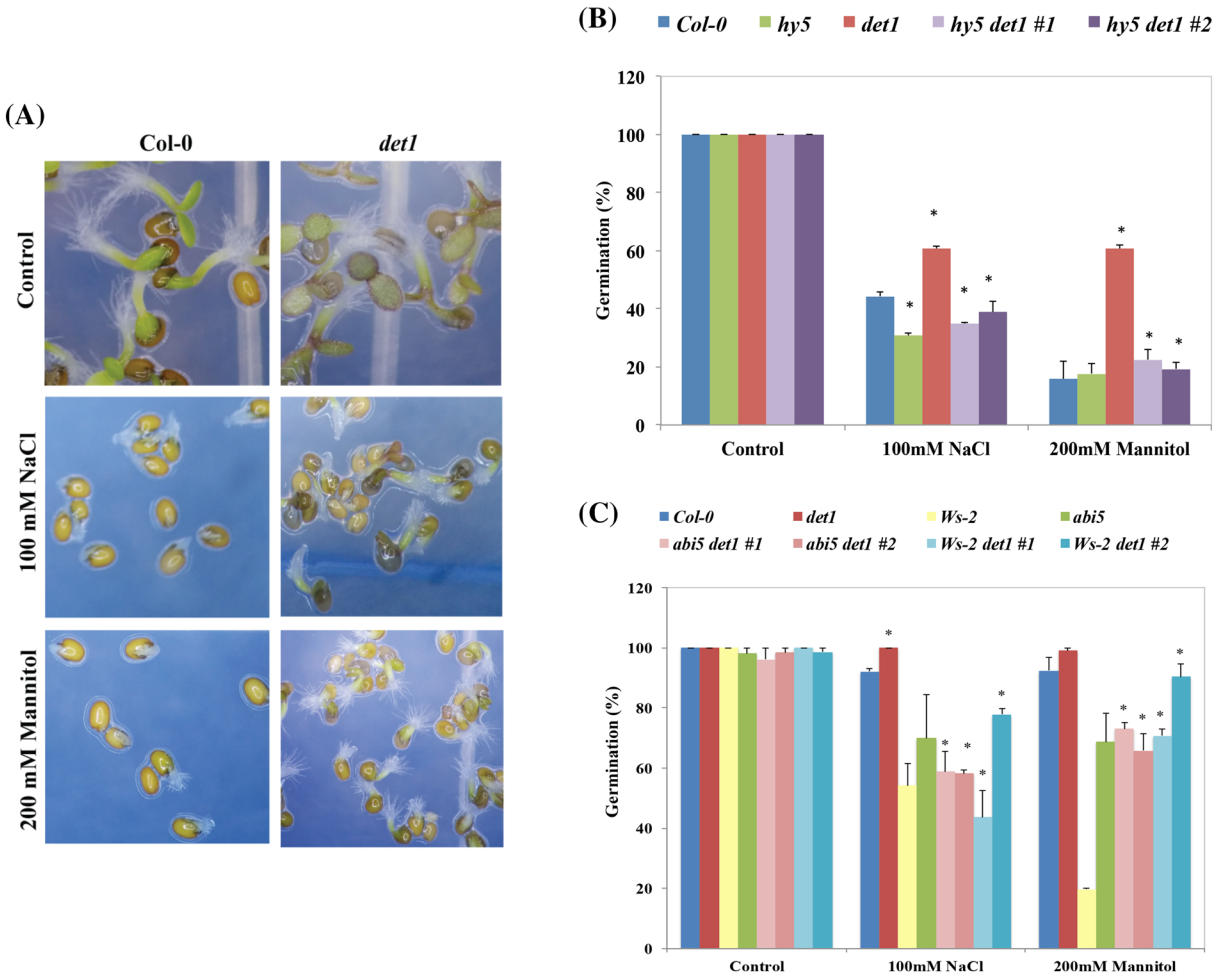


**Fig. 1** Germination in *det1 hy5* and *det1 abi5* double mutants. Representative germination and cotyledon emergence of Col-0 and *det1* on control media, 100 mM NaCl and 200 mM Mannitol after 3 days (**a**). Germination (%) of **b** *det1 hy5* and **c** *det1 abi5* on control, 100 mM NaCl, and 200 mM Mannitol after 2 days. Values are means ± SE of two replicates of 50–100 seeds. *P < 0.05 of single mutants vs. appropriate wild type and doubles vs. *det1*

mutant showed increased germination on salt and mannitol relative to its Ws-2 control. Since *abi5* is in the Ws-2 background while *det1* is in the Col-0 background, we compared *det1* segregating in the Ws-2 background (wildtype ABI5) to *det1 abi5* double mutants as an additional control to account for the differences in the ecotype. We did not observe a significant effect of the presence or absence of ABI5 on the *det1* salt phenotype, indicating that ABI5 is not required for this phenotype. The delayed germination of *det1 abi5* double mutants relative to *det1* appears to be due to the Ws-2 background rather than *ABI5* itself. Our previous developmental analysis suggested there are modifier(s) of *det1* in the Ws-2 background (Fernando and Schroeder 2015). No effect of ABI5 is observed on 200 mM mannitol but on 400 mM mannitol the *abi5 det1* double mutants exhibited less germination than the *det1* Ws-2 controls (Fig. S4b). Thus ABI5 is required for the *det1* osmotic stress germination phenotype at high concentrations. However, this result is the opposite of what might be predicted. The absence of germination inhibiting ABI5 should result in more germination, whereas in the *det1* Ws-2 background it resulted in less germination. Therefore, this may be an indirect effect. Taken together, *det1* salt/mannitol resistant germination requires HY5, but ABI5 does not seem to be directly involved in this response. Hence another gene downstream of *HY5* is likely driving the early germination phenotype in *det1* mutants (Fig. 2a).

## Identification of genes driving precocious germination in *det1*

The *det1* early germination phenotype is observed not only during salt/osmotic stress but also in control conditions and requires HY5 in all cases (Fernando and Schroeder 2015). In *det1* mutants, the germination and seedling

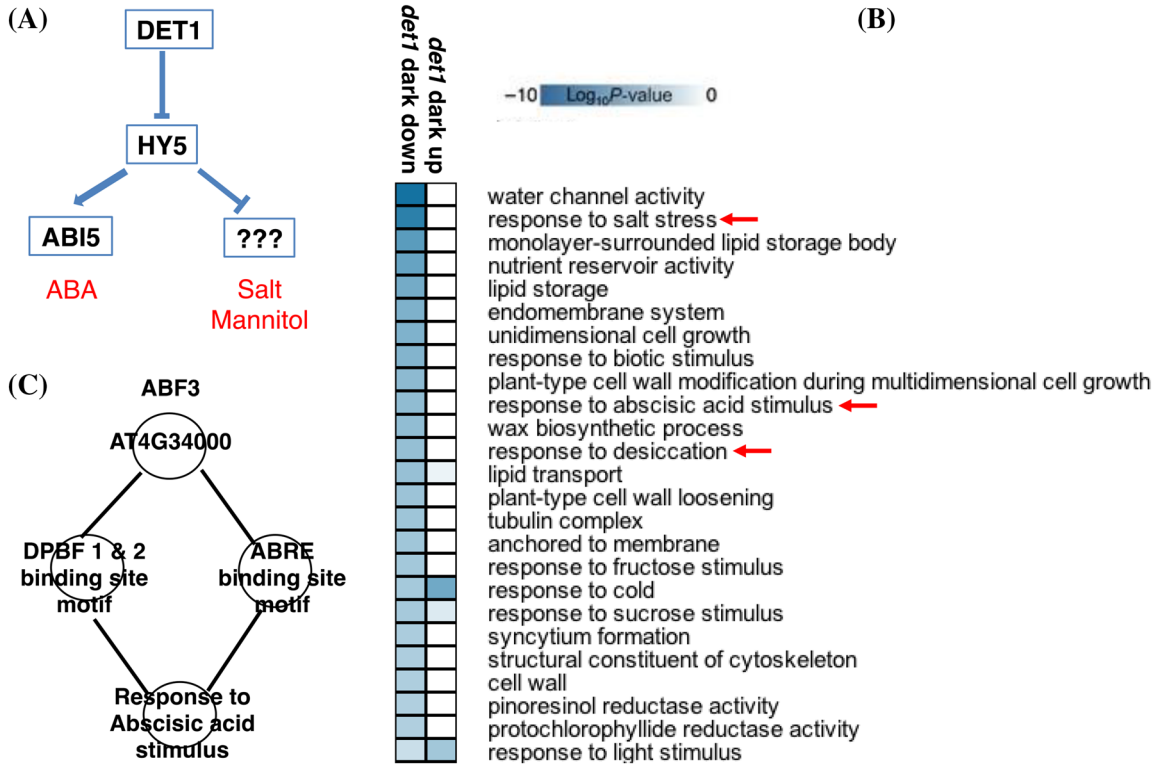


**Fig. 2** Identification of possible candidate gene(s) driving germination in *det1* mutants in salt/osmotic conditions. **a** Overall summary of germination phenotypes based on double mutant analysis. **b** Heat map of enriched GO terms (visualized in multiple expression viewer) downregulated in *det1* dark grown seedlings in publicly available microarray data. Downregulated GO terms and upregulated GO terms in dark grown *det1* seedlings are represented in left and right columns respectively. Statistically enriched terms are blue in colour. GO terms were considered to be statistically enriched at $10^{-3}$ ($P < 0.001$). The scale indicates 10 = highly statistically enriched to 0 = not statistically enriched. **c** Analysis of promoter elements and transcription factors bound to these elements in the "response to ABA stimulus" GO term revealed an ABF3 transcriptional module

photomorphogenic response are light independent phenotypes and may be molecularly similar. To identify candidate gene(s) that may be driving early germination in *det1* mutants, we examined publicly available microarray data from *det1* and wildtype dark grown seedlings available via CATdb (Gagnot et al. 2008) and performed whole genome microarray data analysis. ChipEnrich was used to identify predicted GO terms enriched in dark grown *det1* seedlings. This analysis showed that, as expected, genes associated with photosynthesis were upregulated in *det1* in the dark (Fig. S5). Downregulated genes in dark grown *det1* seedlings included a number of growth related terms, consistent with its short hypocotyl. A number of ABA related terms, including response to salt stress (GO: 0009651), response to ABA stimulus (GO: 0009737), and response to desiccation (GO: 0009269), were also significantly enriched within the downregulated GO terms (Fig. 2b). This analysis suggested that increased germination in *det1* mutants may be the result of decreased ABA signalling.

In order to determine the basis of these patterns, we analyzed predicted transcription factor—DNA sequence motif interactions within the gene sets belonging to these GO terms. In the "response to ABA stimulus" GO term we identified an ABF3 transcription factor module (Fig. 2c) where the bZIP transcription factor, ABF3, is predicted to interact with the ABRE and DPBF binding site motifs within the 1 kb upstream region of genes in this GO term. Since *ABF3* and other genes responding to ABA stimulus are underexpressed in *det1* mutants in the dark (Fig. 2b), decreased levels of *ABF3* may result in correspondingly decreased levels of downstream ABA response genes in *det1*.

Our double mutant analysis suggests that a gene downstream of *HY5* regulates *det1* salt/mannitol resistant germination (Fig. 2a). *ABF3* is a direct target of HY5 and is also repressed by light, suggesting it is negatively regulated by HY5 (Lee et al. 2007). In addition, *ABF3* is induced by both salt and mannitol (Fig. S6) (Kilian et al. 2007). Since *det1* mutants have increased levels of HY5, perhaps negative regulation of *ABF3* by HY5 results in decreased ABF3 and therefore increased germination in *det1* mutants.

Thus, we were interested in examining the role of ABF3 in *det1* germination phenotypes. ABF3 has been shown to

function redundantly with ABI5 in regulating seed germination and seedling growth under ABA mediated stress conditions (Finkelstein et al. 2005). *ABF3* homologues *ABF4* and *ABF1* also act redundantly with *ABF3* during vegetative growth (Yoshida et al. 2010), and are somewhat induced by salt and osmotic stress (Fig. S6) (Kilian et al. 2007). HY5 also binds to the promoter of *ABF4* and *ABF1* (Lee et al. 2007). Thus we also included ABF4 and ABF1 in our analysis. We did not include ABF2 in our study because *ABF2* is not a target of HY5 (Lee et al. 2007) and although *ABF2* is highly expressed in dry seeds, it does not have any effect in germination, but is mostly involved in seedling glucose responses (Kim et al. 2004).

### *ABF* mRNA levels in *det1* mutants

We then validated our dark grown seedling microarray analysis and examined the effect of light on *ABF3, ABF4*, and *ABF1* mRNA levels. As predicted, *ABF3* mRNA levels were lower in dark grown *det1* seedlings than in wild type. In light grown seedlings, wild type *ABF3* levels were lower than in the dark and did not differ significantly from *det1* (Fig. S7a). *ABF4* levels were also reduced in wild type in the light, and no significant difference was observed between *det1* and wild type in either condition (Fig. S7b). In contrast, levels of *ABF1* were upregulated in *det1* mutants in both dark and light conditions (Fig. S7c).

Previous studies have shown that *ABF* genes are induced to different extents by a variety of stress conditions. For example, Fujita et al. (2005) found that in 3 week old plants *ABF3* and *ABF4* are induced by ABA, salt, and desiccation, while *ABF1* was only induced by desiccation. Yoshida et al. (2015) found that *ABF1* and *ABF4* were induced by desiccation, salt, and ABA in both aerial and root tissues of 12 day old plants, while *ABF3* was induced by desiccation and salt but not significantly by ABA. AtGenExpress data (Kilian et al. 2007) shows that in 18 day old plants *ABF1, ABF3*, and *ABF4* are all induced to varying extents by cold, salt, desiccation and osmotic stress, with *ABF1* most highly induced by cold and *ABF3* by salt and osmotic stress. Additional AtGenExpress data, generated in 7 day old seedlings and further analyzed by Nemhauser et al. (2006), indicates that *ABF1* and *ABF4* are induced twofold and fourfold by ABA while *ABF3* was induced nearly 30 fold. However, in imbibed seeds *ABF1* and *ABF3* were not induced by ABA while *ABF4* was only upregulated by 50%. Thus the effect of stress on *ABF* gene expression seems to vary with stage and with study conditions.

To examine the effect of salt on *ABF* gene expression in the *det1* mutant background we analyzed *ABF3, ABF4*, and *ABF1* mRNA levels in *det1* seeds with or without salt treatment. All three *ABF* gene transcript levels were reduced in *det1* mutants relative to the wild type in the control as well as in salt treated seeds (Fig. 3), consistent with the increased germination in *det1* mutants. In the wild type, *ABF3* was significantly induced by salt stress, whereas there was no effect of salt on *ABF3* expression in *det1* (Fig. 3a). However, *ABF4* was repressed by salt in wildtype seeds (Fig. 3b). Although *ABF4* mRNA levels were low in *det1* seeds, it was also repressed by salt. *ABF1* was repressed by salt treatment in wild type but not in *det1* (Fig. 3c).

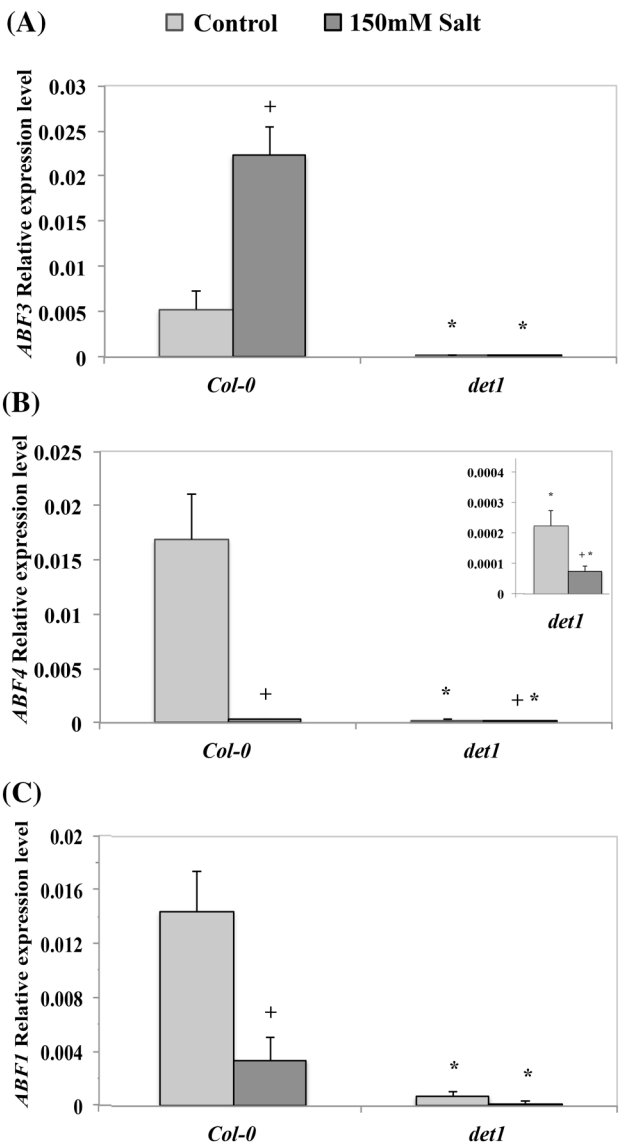


**Fig. 3** Effect of salt on *ABF* transcript levels in *det1* seeds. Real-time PCR analysis of **a** *ABF3*, **b** *ABF4*, and **c** *ABF1* mRNA levels in Col-0 and *det1* seeds imbibed in liquid media in the presence or absence of 150 mM NaCl for 48 h during cold stratification at 4 °C. Values are normalized relative to the reference gene *EF1α*. Error bars indicate SE of six technical replicates. *P ≤ 0.05 of *det1* vs. Col-0 and $^{+}$P ≤ 0.05 of + NaCl vs. − NaCl

To examine the role of *ABF3, ABF4*, and *ABF1* in *det1* phenotypes, we obtained T-DNA loss of function alleles of

the three genes (Fig. S8a), generated double mutants with *det1* (Fig. S8b), then analyzed seedling and adult developmental phenotypes, as well as stress responses with respect to germination and water loss.

## *det1 abf3* developmental phenotypes

We examined the effect of *abf3* on *det1* dark grown seedlings and found no significant effect of *abf3* on *det1* hypocotyl length or cotyledon width, nor did we detect any phenotypes in *abf3* single mutants in the dark (Fig. S9a–c). In light grown seedlings, *abf3* mutants had short hypocotyls and enhanced the *det1* short hypocotyl phenotype (Fig. S9d, e), suggesting an additive effect. *abf3* mutants also resulted in decreased cotyledon width in both the wildtype and *det1* backgrounds (Fig. S9f). The *abf3* single mutant did not show a difference in chlorophyll content relative to the wildtype, but suppressed the *det1* pale phenotype (Fig. S9g). In adults, *abf3* mutants exhibited delayed flowering time in terms of both days and number of leaves, and suppressed the *det1* early flowering phenotype (Fig. S10a, b). *abf3* mutants exhibited decreased rosette diameter but did not affect rosette width or height in *det1* (Fig. S10c, d). *abf3* suppressed the *det1* short silique phenotype though (Fig. S10e). *abf3* mutants also exhibited increased apical dominance, that is decreased inflorescence stem number, in both the wildtype and *det1* backgrounds, thus suppressing the *det1* decreased apical dominance phenotype (Fig. S10f). Thus, *abf3* suppressed the *det1* chlorophyll, flowering time, silique length, and apical dominance phenotypes and enhanced the *det1* light hypocotyl length phenotype, indicating genetic interactions between *DET1* and *ABF3* during development.

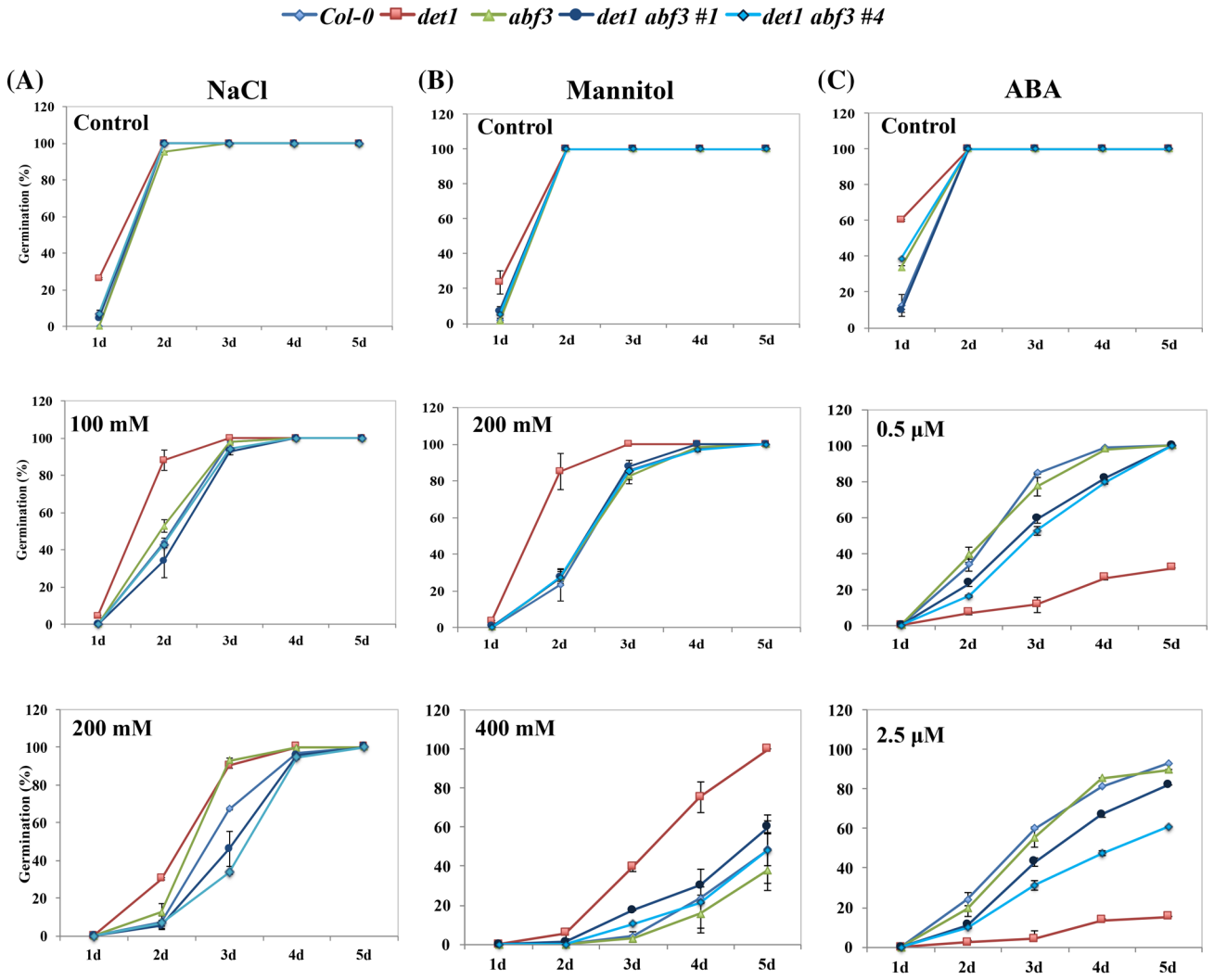


**Fig. 4** Germination in *det1 abf3* double mutants. Germination (%) on **a** 0, 100, 200 mM NaCl, **b** 0, 200, 400 mM Mannitol, **c** 0, 0.5, 2.5 µM ABA. Values are means ± SE of two replicates of 50–100 seeds

### *det1 abf3* salt/mannitol germination responses

*det1* mutants showed resistant germination on both salt and mannitol. Since *det1* mutants have low levels of *ABF3*, if *det1* phenotypes are due to the absence of ABF3, then *det1* and *abf3* should exhibit similar phenotypes. We found *abf3* germination was resistant to 200 mM salt but not 100 mM salt or mannitol relative to wildtype (Fig. 4a, b). These results are consistent with those of Kim et al. (2004) who also found that *abf3* mutants exhibited salt resistant germination. However, Finkelstein et al. (2005), using the same SALK allele, reported that *abf3* single mutants do not show significant resistance to salt. However, they reported that *abf3 abi5* double mutants show significantly enhanced germination on both ABA and salt containing media, suggesting redundant roles of ABF3 and ABI5 (Finkelstein et al. 2005). Compared to *abf3, det1* exhibited a stronger resistance phenotype in both stress conditions, indicating that lack of ABF3 cannot be the sole basis of the *det1* phenotype. We found that *abf3* suppressed the *det1* salt and mannitol resistant germination phenotypes (Fig. 4a, b), thus ABF3 is required for *det1* stress resistant germination. However it was not clear how lack of germination-inhibiting ABF3 resulted in less germination in the *det1* background. To test whether homologous genes were acting redundantly, we examined *ABF4* and *ABF1* transcript levels in the *det1 abf3* double mutants and found that both *ABF4* and *ABF1* were upregulated in the double mutants relative to *det1* (Fig. 5). Thus perhaps upregulation of homologous genes such as *ABF4* and *ABF1* correlates with decreased germination in *det1 abf3*.

We also examined ABA inhibition of germination in *det1* and *abf3* mutants and found that *abf3* did not have any significant effect on germination in the wildtype, consistent with previous reports (Finkelstein et al. 2005). In the *det1* background *abf3* suppressed the *det1* ABA sensitive germination phenotype (Fig. 4c). Thus ABF3 is required for both *det1* stress resistant and ABA sensitive germination, despite being opposite effects.

### *det1 abf4* developmental phenotypes

Since *ABF1* and *ABF4* are close homologs of *ABF3* that have been shown to act redundantly (Yoshida et al. 2015), we also examined growth and germination responses of *det1 abf4* and *det1 abf1* mutants. In contrast to *abf3, abf4* mutants had shorter hypocotyls in the dark and decreased both *det1* hypocotyl length and cotyledon width (Fig. S11a–c). In the light, *abf4* exhibited no hypocotyl phenotypes but had increased cotyledon width relative to wild type. However *abf4* enhanced the *det1* small cotyledon width phenotype in light (Fig. S11e, f). Similar to *abf3, abf4* suppressed the *det1* decreased chlorophyll phenotype (Fig. S11g). In adults,

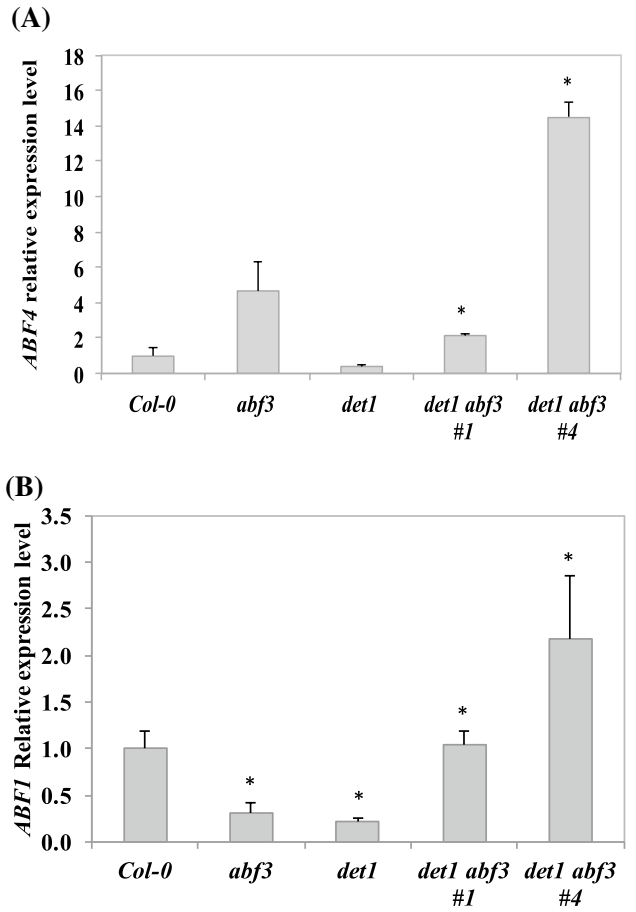


**Fig. 5** *ABF4* and *ABF1* transcript levels are upregulated in *det1 abf3*. Real-time PCR analysis of **a** *ABF4* and **b** *ABF1* mRNA levels in *det1 abf3* seeds imbibed in liquid control media for 48 h during cold stratification at 4 °C. Values are normalized relative to the reference gene *EF1α* then relative to Col-0. Error bars indicate SE of three technical replicates. *P ≤ 0.05 of single mutants vs. Col-0 and double mutants vs. *det1*

similar to *abf3, abf4* partially suppressed the *det1* early flowering phenotype (Fig. S12a, b) and decreased apical dominance phenotype (Fig. S12f). In addition, *abf4* enhanced the *det1* decreased rosette diameter and height phenotypes (Fig. S12c, d) but had no significant effect on silique length (Fig. S12e). Thus while *abf4* and *abf3* differed in their affect on several *det1* phenotypes, they exhibited common regulation of *det1* light grown seedling cotyledon width and chlorophyll content as well as adult flowering time and apical dominance phenotypes. These results are consistent with the similar patterns of *ABF3* and *ABF4* expression in *det1* light and dark grown seedlings.

### *det1 abf4* salt/mannitol germination responses

In germination assays, the *abf4* single mutant exhibited resistance to salt and mannitol relative to wild type

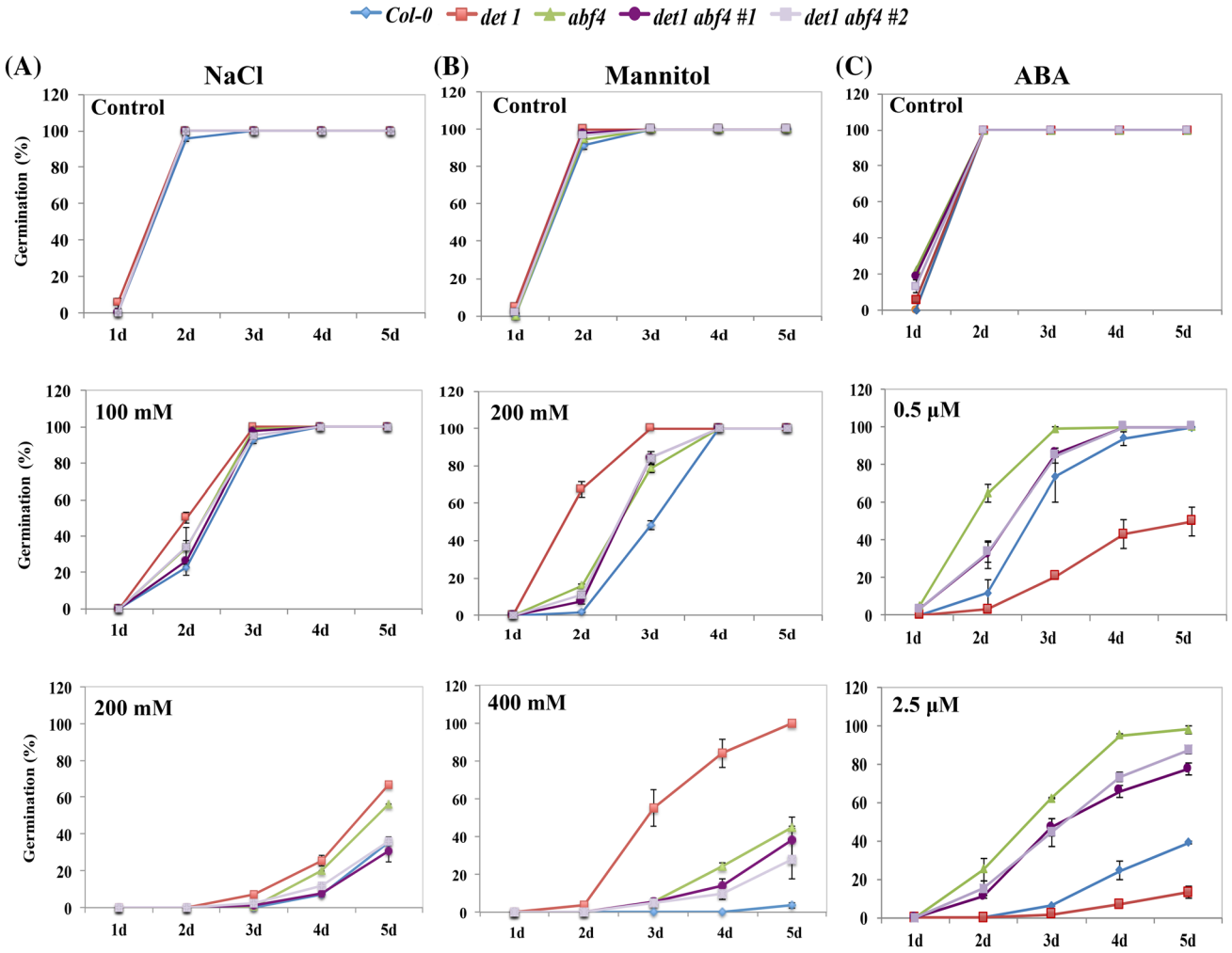


**Fig. 6** Germination in *det1 abf4* double mutants. Germination (%) on **a** 0, 100, 200 mM NaCl, **b** 0, 200, 400 mM Mannitol **c** 0, 0.5, 2.5 µM ABA. Values are means ± SE of two replicates of 50–100 seeds

(Fig. 6a, b), consistent with the results of Kim et al. (2004). Like *abf3*, this resistance phenotype was not as severe as that of *det1*. In the double mutants, on salt *det1 abf4* showed complete rescue and exhibited a germination rate similar to that of wild type (Fig. 6a). On the other hand, on mannitol *det1 abf4* mutants germinate slower than *det1* and similar to *abf4* single mutants, showing partial recue (Fig. 6b). This indicates that *abf4* suppresses *det1* and that ABF4 is required for the *det1* salt/mannitol resistant germination phenotype. However, like *abi5* and *abf3*, the fact that the loss of germination inhibiting ABF4 results in less germination in *det1* suggests this is an indirect effect. On ABA (Fig. 6c), *abf4* exhibited resistant germination, again consistent with the results of Kim et al. (2004), but in contrast to the absence of phenotype reported by Yoshida et al. (2010). The *det1 abf4* double mutants were nearly as resistant to ABA as the *abf4* single mutants, indicating that *ABF4* is required for *det1* ABA sensitive germination. Note that this effect was in the expected direction, that is, lack of ABF4 resulted in more germination in the *det1* background.

## *det1 abf1* developmental phenotypes

In the dark, *abf1* partially suppressed the *det1* hypocotyl length and cotyledon width phenotypes (Fig. S13a–c). In the light, *abf1* single mutants had significantly longer hypocotyls than Col-0 and also suppressed the *det1* hypocotyl length phenotype, but do not affect cotyledon width or chlorophyll content (Fig. S13d–g). In adults, *abf1* exhibited early flowering in terms of both days and number of leaves but suppressed the *det1* early flowering (days) phenotype (Fig. S14a, b). Even though we did not observe a height phenotype in *abf3* and *abf4, abf1* mutants were significantly

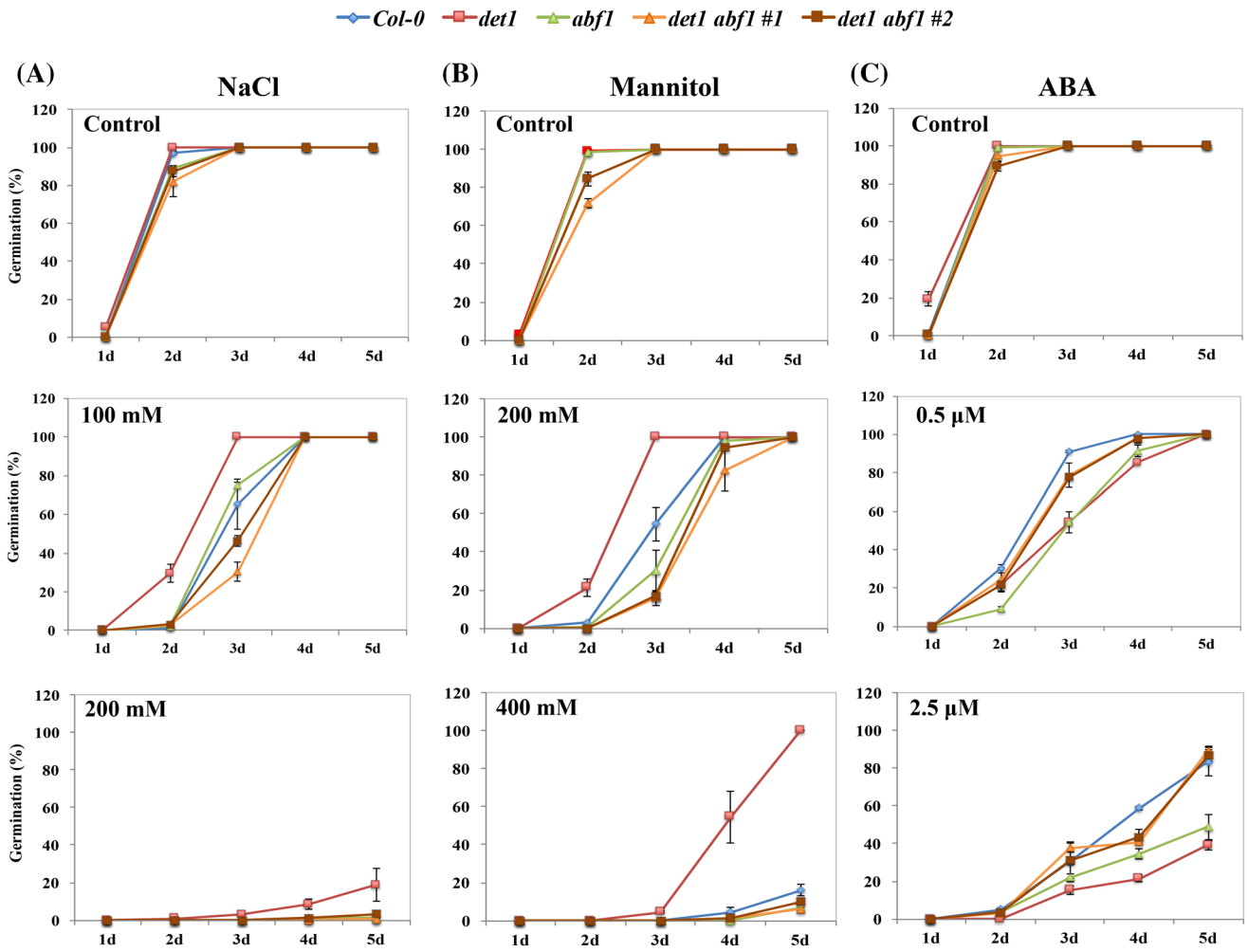


**Fig. 7** Germination in *det1 abf1* double mutants. Germination (%) on **a** 0, 100, 200 mM NaCl **b** 0, 200, 400 mM Mannitol **c** 0, 0.5, 2.5 µM ABA. Values are means ± SE of two replicates of 50–100 seeds

taller than the wild type (Fig. S14d). In addition, *abf1* suppressed a number of *det1* growth phenotypes namely decreased rosette diameter, height, and silique length (Fig. S14c–e). *abf1* single mutants exhibited decreased apical dominance but suppressed the *det1* reduced apical dominance phenotype (Fig. S14f). Overall *abf1* suppressed eight of the eleven *det1* phenotypes examined and did not enhance any *det1* phenotypes, suggesting that *ABF1* acts downstream of *DET1*.

Interestingly, both *hy5* and *abf1* suppress nearly all the *det1* developmental phenotypes examined (Chory 1992; Fernando and Schroeder 2015; Pepper and Chory 1997). HY5 is known to act downstream of DET1, and DET1 is a negative regulator of HY5 (Osterlund et al. 2000). The similarities in their effects on *det1* suggest that ABF1 may also be downstream of DET1 and negatively regulated by it. The fact that HY5 binds the *ABF1* promoter (Lee et al. 2007) suggests that DET1 regulation of ABF1 may be via HY5 and that HY5 may positively regulate *ABF1* transcription. Thus *det1* mutants with increased levels of HY5 would be predicted to exhibit increased levels of *ABF1* transcription, which is in fact what we observe in light and dark grown seedlings (Fig. S7).

## *det1 abf1* salt/mannitol germination responses

The *abf1* single mutant did not exhibit a significant germination phenotype on stress media (Fig. 7a, b), consistent with previous reports (Finkelstein et al. 2005). Similar to *abf1* suppression of *det1* seedling and adult phenotypes, germination of the *det1 abf1* double mutant was significantly delayed relative to *det1*. On 100 mM salt, germination of the double mutant was even slower than *abf1*, whereas on mannitol, the germination rate of the *det1 abf1* lines was more or less like the *abf1* single. Therefore, *abf1*

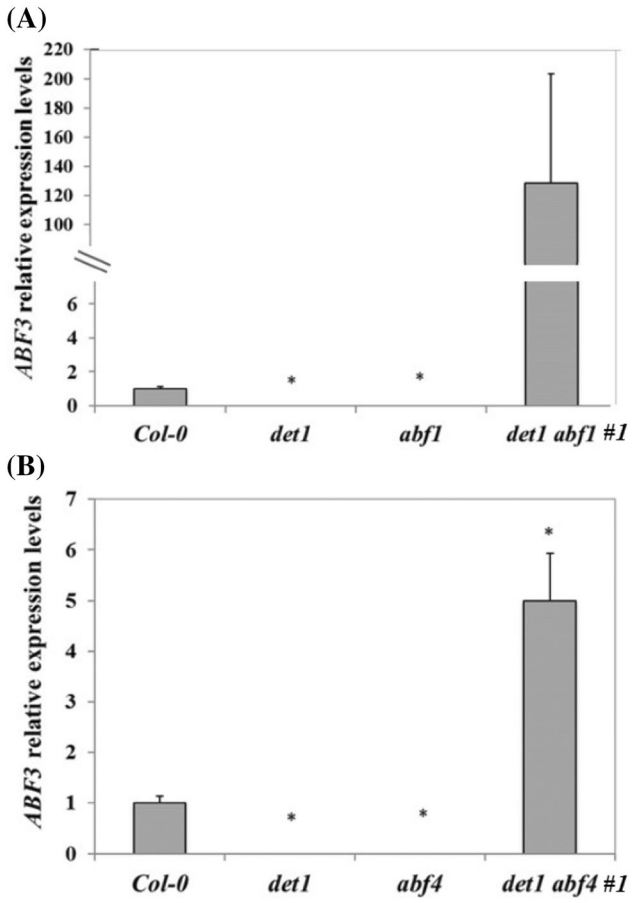


**Fig. 8** *ABF3* transcript levels are upregulated in salt treated *det1abf1* and *det1 abf4* seeds. Real-time PCR analysis of *ABF3* mRNA levels in **a** *det1 abf1* and **b** *det1 abf4* seeds imbibed in liquid media in the presence of 150 mM NaCl for 48 h during cold stratification at 4 °C. Values are normalized relative to the reference gene *EF1α* then relative to Col-0. Error bars indicate SE of three technical replicates. *P ≤ 0.05 of single mutants vs. Col-0 and double mutants vs *det1*

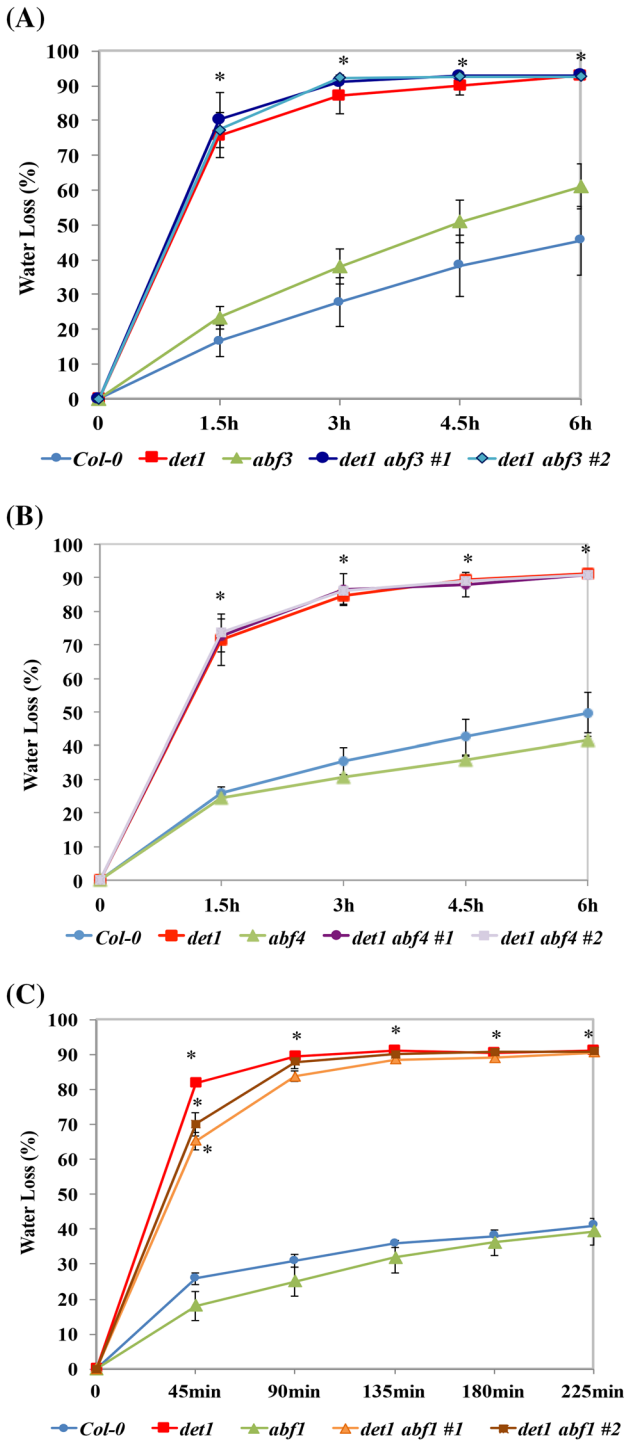


**Fig. 9** *det1* rapid water loss phenotype requires *ABF1* but not *ABF3* or *ABF4*. Water loss from detached leaves as a % loss of fresh weight. Values are means ± SE of two samples of three to six leaves, *P < 0.05 of single mutants vs. Col-0, double mutants vs. *det1*

completely suppressed the *det1* early germination phenotypes on salt and osmotic stress media. Thus, *ABF1* is epistatic to *DET1* with respect to stress germination phenotypes. However, like ABF4, this rescue was unexpected since absence of ABF1 should result in increased germination. To test whether these are additional instances of homologous gene action, we examined *ABF3* levels in the *det1 abf1* and *det1 abf4* double mutants and found that in fact *ABF3* was highly upregulated in the double mutants (Fig. 8). Thus, increased *ABF3* levels likely provide a basis for the decreased germination observed in the *det1 abf1* and *det1 abf4* double mutants.

On ABA the *abf1* single mutant, like *det1*, exhibited sensitive germination (Fig. 7c). This is in contrast to the results of Finkelstein et al. (2005), who reported that *abf1* germination on ABA was not significantly different from that of wild type. Nonetheless, *abf1* completely rescued the *det1* ABA sensitive germination phenotype, indicating that ABF1 is also required for *det1* ABA sensitivity.

## Role of *ABF* genes in the *det1* transpirational water loss phenotype

We have previously shown that *det1* mutants exhibit rapid water loss from detached leaves (Fernando and Schroeder 2015). In order to investigate the role of the *ABF* genes in this phenotype, we examined water loss in *det1 abf3, det1 abf4*, and *det1 abf1* leaves (Fig. 9). The *abf* single mutants did not show significant phenotypes in this assay, perhaps due to redundancy of function. Neither *abf3* nor *abf4* affected the *det1* rapid water loss phenotype. However, *abf1* significantly reduced water loss in *det1*, suggesting that *ABF1* is required for this phenotype. Since *abf1* was able to partially suppress the *det1* rapid water loss phenotype, we examined stomata phenotypes in order to investigate the basis of this effect.

## Stomatal phenotypes of *det1* and *det1 abf1*

Factors contributing to rapid water loss from detached leaves could include increased stomatal density (as indicated by increased stomatal index) or failure to close the stomatal pore. Therefore, we examined these features in wild type, *det1, abf1*, and *det1 abf1* leaves. We found that *det1* had a higher stomatal index than wild type (Fig. 10a). We did not however observe any clustered stomata in the 4-week old leaves, as opposed to those observed in 10 day old *det1* light grown seedlings by Kang et al. (2009). Other photomorphogenic mutants, including *cop1* and *cop10*, also exhibit clustered stomata and increased SI (Kang et al. 2009; Delgado et al. 2012). Nonetheless *det1 abf1* double mutants rescue the *det1* increased SI phenotype, indicating that ABF1 plays a role in *det1* stomatal patterning (Fig. 10a).

ABA acts as chemical signal to induce stomatal closure under water-deprived conditions (Busk and Pages 1998). We examined stomatal closure in response to ABA by treating leaves with 1 µM ABA then measuring stomatal pore length and width after 2–4 h (Fig. 10b, c). We observed that at 2 h stomatal apertures (width/length ratio) of *abf1* single mutants were reduced relative to wild type, while in *det1* the stomatal apertures were significantly larger. Eventually *det1* stomata started to close after 3 h, however this was delayed relative to the wild type. Thus, *det1* mutants have defects in regulation of the stomatal pore in response to ABA. Several studies have shown that genes that act as central repressors of photomorphogenesis are also repressors of stomatal opening. Photomorphogenic mutant *constitutive photomorphogenic 1* (*cop1*) also has impaired stomatal movements and larger stomatal apertures than wildtype (Mao et al. 2005; Kang et al. 2009; Delgado et al. 2012). Delgado et al. (2012) reported that *cop10* mutants also have larger stomata with reduced ABA response compared to wild type. Further analysis needs to be done in order to determine whether *DET1* has a role in cytoskeletal processes in the guard cells as in

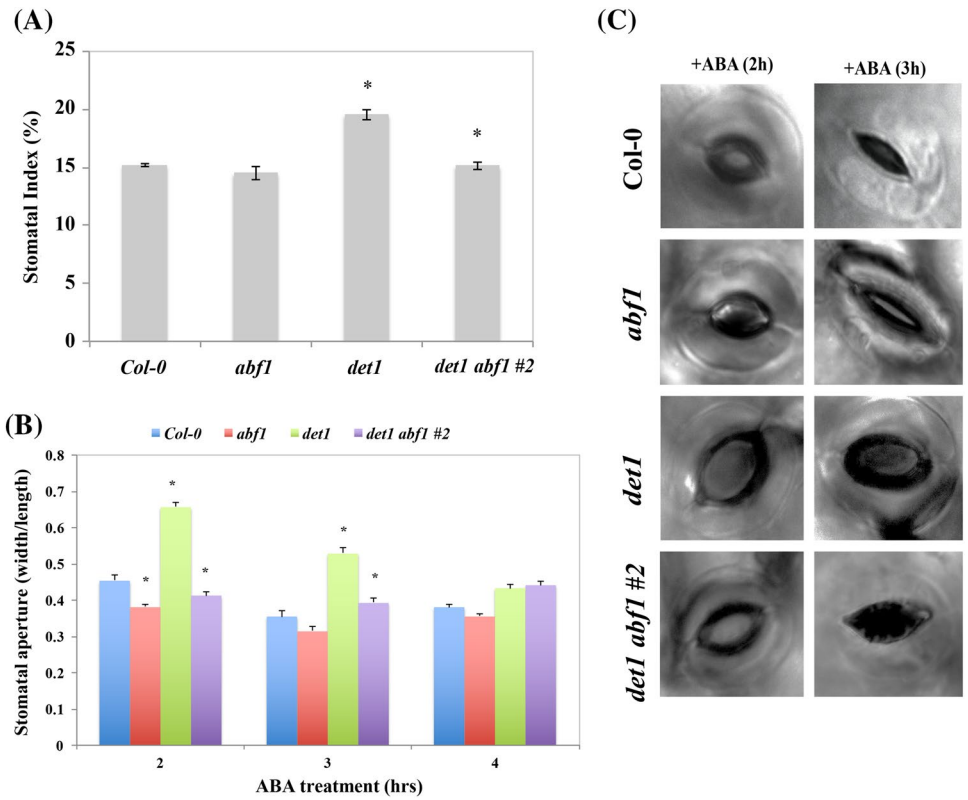


**Fig. 10** *det1* stomatal phenotypes are suppressed by *abf1*. **a** Stomatal Index [number of stomata/(number of stomata + number of pavement cells) × 100%] as mean ± SE of five areas of three independent rosette leaves of 4 week old plants. **b** Stomatal aperture as mean ± SE of 10–12 stomata/leaf treated with ABA for 2, 3 and 4 h. **c** Stomatal pore phenotypes. *P ≤ 0.05 of single mutants vs. Col-0 and double mutants vs. *det1*

the case of COP1 (Khanna et al. 2014). *det1 abf1* double mutants also rescued the *det1* delayed stomatal closure phenotype (Fig. 10b, c), indicating that ABF1 has a role in this phenotype as well. Thus both increased stomatal closure and decreased SI in the double mutants may contribute to the decreased water loss phenotype.

In conclusion, our analysis indicates that *det1* mutants show a resistant germination phenotype to salt/osmotic stress, unlike on ABA where *det1* germination was sensitive (Fernando and Schroeder 2015). The *det1* salt resistant germination phenotype requires *HY5* but not *ABI5*. We identified *ABF3, ABF4*, and *ABF1* as candidate genes acting downstream of *DET1* during stress response. We found that *abf3, abf4*, and *abf1* rescue both *det1* stress resistant and ABA sensitive germination phenotypes. *ABF* genes show interactions with *DET1* not only during germination but also during seedling and adult growth. While the *det1* rapid water loss phenotype was independent of *ABF3* and *ABF4, abf1* rescues *det1* stomatal phenotypes, resulting in reduced transpiration in *det1*. Thus *det1* mutants show a variety of phenotypes under stress conditions and *HY5* and the *ABF* genes appear to be involved in these traits.

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**Author contributions** WAK performed preliminary experiments, MFB analyzed microarray data. All other experiments were performed by VCDF. All authors contributed to experimental design, manuscript writing, and editing.

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