The role of DET1 in *Arabidopsis thaliana* seed germination responses on ABA, salt and osmotic stress conditions

By

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ABSTRACT

Plant development is tightly linked to the environment. Environmental signals interact with endogenous hormonal cues, allowing plants to optimize their response to external stress. The plant hormone abscisic acid (ABA) is involved in a variety of stress tolerance mechanisms. These pathways have been widely studied in the model plant *Arabidopsis thaliana* (Arabidopsis). In this thesis I investigate the effect of light, ABA, and ABA mediated salt and osmotic stress on Arabidopsis seed germination, examining the role of light signaling component DE-ETIOLATED 1 (DET1) in these mechanisms. DET1 is a negative regulator of the light response promoting transcription factor ELONGATED HYPOCOTYL 5 (HY5). *det1* mutants showed a variety of germination phenotypes on control, ABA, and salt/osmotic stress conditions. Germination of *det1* was sensitive to ABA but resistant to salt and osmotic stress. *det1* ABA sensitive germination appeared to be due to an upregulation of a germination inhibiting transcription factor ABSCISIC ACID INSENSITIVE 5 (ABI5) by HY5. On the other hand, we propose that in salt/osmotic stress conditions, HY5 downregulates another transcription factor, ABSCISIC ACID BINDING FACTOR 3 (ABF3), which inhibits seed germination, thus *det1* mutants exhibited resistant germination. In addition, *DET1* exhibits genetic interactions with genes in the ABA signalling pathway during development. Both *hy5* and *abf1* mutants suppressed *det1* phenotypes not only during vegetative development but also during germination, suggesting that a pathway whereby DET1 negatively regulates HY5 and HY5 positively regulates ABF1. Moreover components of distinct CULLIN4 DAMAGED DNA BINDING PROTEIN 1 (CUL4-DDB1) E3 ligase complexes both suppressed and enhanced *det1* phenotypes. Finally, the *det1* rapid water loss phenotype was independent of all genes considered except for *ABF1*. In conclusion, DET1 plays an important role in Arabidopsis response to stress

conditions and, by acting as a repressor of HY5, has a role in the integration of light and ABA signalling pathways.

Dedicated to my beloved ammi and thaththi (mom and dad)

for their unconditional love, strength and encouragement

without which I wouldn't have been able to

come this far in my life.

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LIST OF ABBREVIATIONS

ABF1	Abscisic Acid Binding Factor 1
ABF3	Abscisic Acid Binding Factor 3
ABF4/AREB2	Abscisic Acid Binding Factor 4/ABA Responsive Element Binding Factor 2
ABI5	Abscisic Acid Insensitive 5
bHLH	basic Helix-Loop- Helix
bZIP	basic Leucine Zipper
CDD	COP1-DET1-DDB1
Col	*Arabidopsis thaliana* ecotype Columbia
COP1	Constitutive Photomorphogenic 1
CSN	COP9 Signalosome
CUL4	Cullin 4
DCAF	DDB1-CUL4 Associated Factor
DDB1A/B	Damaged DNA Binding Protein 1 A and B
DDB2	Damaged DNA Binding Protein 2
DET1	De-Etiolated 1
DWA1/2	DDB1 Binding WD40 Hypersensitive to ABA 1 and 2
HY5	Long Hypocotyl 5
LS media	Linsmaier and Skoog medium

PIF	Phytochrome Interacting Factor
qRT- PCR	quantitative Reverse Transcriptase Polymerase Chain Reaction
SE	Standard error
T-DNA	Transfer DNA
Ub	Ubiquitin

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Chapter1. Literature Review

1. Literature Review

Plant development is affected by a variety of factors during the life cycle of the plant. Seed germination, seedling development, and adult growth are important phases of the developmental cycle that transform a seed in to a fully-grown adult plant. Environmental factors, such as light, temperature, water, and gravity, as well as endogenous hormonal cues, are major regulators of plant growth. Environmental signals interact with the endogenous developmental programs, resulting in physiological changes during plant development. Genetic studies have been carried out to determine the signal transduction pathways involved in these processes. Light is a key factor that regulates development, and will be discussed in detail in the next section.

In addition to external stimuli, endogenous hormonal cues play important roles in plant development because many of the plant's physiological responses are regulated by phytohormones. Seed germination and seedling development are two such processes regulated by multiple hormonal pathways often modified by light (Lau and Deng 2010). The effect of hormones, with special emphasis on role of Abscisic acid (ABA), is discussed in detail in the ABA section of this chapter.

These environmental and hormonal responses converge in order to complete the life cycle of the plant and adapt it to its environment. The intersection of light and ABA signalling, particularly with respect to seed germination, is the focus of the third and final section of the literature review.

This study mainly focused on the effect of light and ABA on seed germination in Arabidopsis and the genetic interactions between genes involved in the integration of the light and ABA signalling pathways. Specifically, I investigated the role of light signalling component DET1 during seed germination in a variety of conditions. Chapter 2 describes the genetic

interactions of DET1 and intermediate genes in ABA signalling pathway. The focus of chapter 3 is the role of DET1 in seed germination under salt and osmotic stress conditions. Finally in chapter 4 we examine the interactions of several CUL4 DDB1 E3 ligase complexes with DET1 during stress responses. The outcome of this research study will be an understanding of the role of DET1 in light and ABA signalling pathways.

1.1 Effect of light on plant development

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1.1.1 Abstract

Light is one of the most important factors regulating plant growth and development. Depending on the availability of light, seedlings undergo two different developmental programs - photomorphogenesis in the presence of light, and skotomorphogenesis in the absence of light. In the model plant *Arabidopsis thaliana* mutants in light signalling pathways have been identified that misregulate this response. The mechanisms behind light and dark growth have been studied extensively and recent studies have revealed how light signals are perceived and transmitted to downstream components. This review provides insight into light-perceiving photoreceptors and other positive and negative regulators of light signalling as well as interactions between these components. Genetic and biochemical evidence for the basis of light signalling mechanisms are discussed as well as the importance of light signalling in plant development.

1.1.2 Introduction

Among all the external stimuli affecting plant development, light has an especially important role in photosynthesis, chloroplast biogenesis, germination, seedling development, floral induction, phototropism, and shade avoidance. Thus light acts not only as an energy source but, in addition, functions as a trigger for growth and development (Chory 1993, Deng 1994, Dong et al 2015).

The effect of light on plant development can be clearly detected during seedling growth. Seedling growth in the dark (skotomorphogenesis) has a developmentally arrested etiolated phenotype with elongated hypocotyls (embryonic stems), small folded cotyledons (embryonic leaves), and undeveloped chloroplasts. In contrast, seedling growth in the light (photomorphogenisis) results in short hypocotyls, open cotyledons, and developed chloroplasts (Figure 1.1a) (Chory 1993). These distinct phenotypes have been used in genetic studies to examine light signal transduction pathways. Mutants have been identified in the model plant *Arabidopsis thaliana* that show the opposite phenotypes to those exhibited in wildtype plants. These light signalling mutants are broadly divided into two classes, mutants showing light grown phenotypes in the dark and mutants showing dark grown phenotypes in the light (Figure 1.1b). These phenotypes are a consequence of defects in either positive or negative regulators of light signalling (Quail 1991, Chory 1993).

Arabidopsis thaliana (family Brassicaceae) is an excellent model plant to study the genetic basis of the effects of external environmental factors. It has a small genome (125 Mb), which was the first plant genome to be fully sequenced. In addition, its small size, rapid growth, and extensive collections of mutants and other molecular resources make Arabidopsis an excellent model plant. Its ability to produce large numbers of progeny and ease of *Agrobacterium* mediated transformation have also contributed to making Arabidopsis a popular genetic model (Sivasubramanian et al 2015).

Although the major positive and negative regulators of light signalling in Arabidopsis were discovered more than 20 years ago, direct biochemical interactions between these components were revealed only recently. This review focuses on recent advances in our understanding of light signalling in *Arabidopsis thaliana,* with emphasis on interactions between

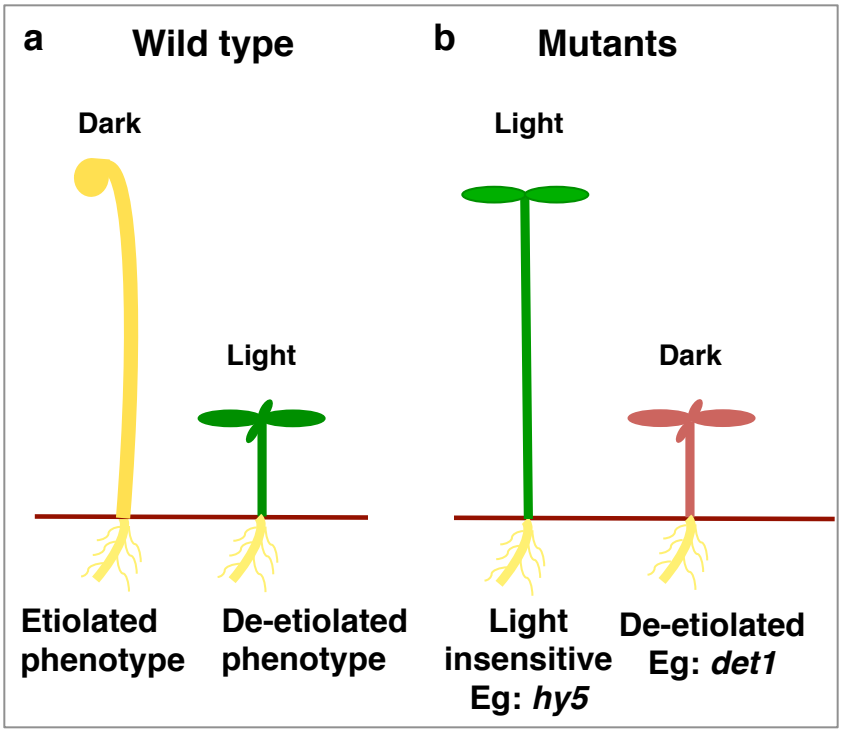

Figure 1.1 Dark and light grown wildtype seedling phenotypes (a) and light signalling mutants (b).

key regulatory components of light signalling.

1.1.3 Positive regulators of light signalling

When a component of the light signalling pathway that is involved in perception of the light signal or transduction of the light signal to downstream components is compromised, such mutants will exhibit a seedling phenotype that is insensitive, or exhibits reduced response, to light. That is, these seedlings will show an etiolated phenotype in the light, with elongated hypocotyls and reduced cotyledon expansion (Figure 1.1b). This group of mutants can be categorized into photoreceptor mutants and mutants in photomorphogenesis-promoting transcription factors.

1.1.3.1 Photoreceptors

Initiation of light signalling occurs via perception of light by photoreceptors. Plants have evolved several photoreceptors to sense and respond to a broad range of light frequencies in the environment. The major photoreceptors are the far-red and red light detecting phytochromes, blue/UV-A light sensing cryptochromes and phototropins, and UV-B detecting receptors, such as UVR8 (Galvão and Fankhauser 2015).

Arabidopsis has five phytochrome isoforms (phyA-E). Far red perception is mediated by phyA, while phyB-E initiate red light signalling, with phyB acting predominantly (Wang and Wang 2015). Phytochromes occur in a biologically active Pfr form and an inactive Pr form. Pfr and Pr are photoconvertible, where Pr is transformed into Pfr upon red light (R) absorption and Pfr transformed into Pr upon far-red (FR) light absorption. This conformational change in phytochromes is an important regulatory switch that mediates transduction of light signals to downstream components (Furuya 1993). For phyB, conversion to the Pfr form reveals a masked nuclear localization signal that results in nuclear import in the presence of light. PhyA nuclear localization requires FHY1 (FAR RED ELONGATED HYPOCOTYL 1) and FHL (FHY1 LIKE) (Wang and Wang 2015). Phys are homodimeric chromoprotein complexes that contain a phytochromobilin (PΦB) chromophore. Phys consist of chromophore binding, dimerization and kinase domains (Burgie et al 2014). Pr to Pfr conversion occurs after light activates the bilin chromophore, which undergoes isomerization and thereby a confirmation change in hairpin and helical spine structure, which stabilizes the Pfr form (Burgie et al 2016).

Cryptochromes are involved in blue light mediated regulation of seedling development and photoperiodic initiation of flowering. Analysis of cryptochrome 1 and 2 (*cry1 cry2)* mutants has shown that CRY1 and CRY2 have both unique and overlapping functions in these responses.

CRY2 is always nuclear localized while CRY1 is either nuclear or cytoplasmic. Upon blue light absorption, the main chromophore in CRYs is rapidly photoreduced, resulting in conformational changes that facilitate interactions with downstream signalling components (Galvão and Fankhauser 2015, Liu et al 2016).

1.1.3.2 Photomorphogenesis-promoting transcription factors

LONG HYPOCOTYL 5 (HY5) was one of the first positive regulators of photomorphogenesis to be characterized. The *hy5* mutants were initially identified in a screen for insensitivity to light inhibition of hypocotyl elongation (Figure 1.1b) (Koornneef et al 1980). *hy5* mutants are deficient in red, far-red, and blue light responses and act downstream from the photoreceptors (Chory 1992, Ang and Deng 1994). In addition, *hy5* mutants have defects in chlorophyll accumulation and lateral root formation (Pepper and Chory 1997, Oyama et al 1997).

HY5 encodes a nuclear localized basic leucine zipper transcription factor that promotes photomorphogenesis in a broad range of wavelengths (Oyama et al 1997). Studies of photoreceptor mutants and overexpression lines have shown that both phytochromes and cryptochromes promote HY5 accumulation in the nucleus. The key photoreceptor responsible for HY5 accumulation in R light is phyB while phyA plays a more important role in FR light. CRY1 and CRY2 are involved in HY5 accumulation under blue light conditions (Osterlund et al 2000).

Chromatin immuno-precipitation and whole genome expression analysis have shown that HY5 specifically binds to the promoters of a large number of genes of which 10% encode transcription factors. In addition, 24% of light regulated genes are HY5 targets, including both light induced and light repressed genes, indicating that HY5 has a dual role in transcriptional regulation of light signalling as an activator as well as a repressor (Lee et al 2007).

CALMODULIN 7 (CAM7) has a critical role in transcriptional regulation of *HY5* during seedling development in a broad spectrum of light conditions. CAM7 directly interacts with the *HY5* promoter and upregulates *HY5* transcription. HY5 also activates its own gene expression, therefore both HY5 and CAM7 positively regulate *HY5* transcription (Abbas et al 2014).

Other positive regulators of photomorphogenesis include HY5 HOMOLOG (HYH), a G-box binding bZIP transcription factor which shows functional redundancy with HY5. Unlike *hy5*, *hyh* mutants exhibit resistance to inhibition of hypocotyl elongation only in blue light thus HYH acts as the main positive regulator of blue light signalling mediated by CRY1 and CRY2. HYH protein levels were significantly lower in *hy5* mutants indicating that HY5 is essential for HYH protein accumulation. *hyh* mutants flower earlier than wild type but *hy5 hyh* double mutants did not show an additive effect on flowering time phenotypes (Holm et al 2002).

HFR1, a bHLH transcription factor, is another positive regulator of photomorphogenesis. The *hfr1* mutant has elongated hypocotyls in FR light. HFR1 is responsible for phyA mediated FR and CRY1 mediated blue light signalling (Jang et al 2005, Yang et al 2005, Casal et al 2014).

LAF1, a Myb transcriptional activator, is involved in transmitting phyA signals to downstream signalling components. Interestingly HY5, HFR1, and LAF1 have the ability to bind with each other and decrease degradation of each other (Casal et al 2014).

1.1.4 Negative regulators of light signalling

In Arabidopsis mutants have been identified that resemble light grown plants even when grown in the dark, that is, exhibit short hypocotyls, open cotyledons, and light regulated gene expression (Figure 1.1b) (Chory et al 1989). These mutants are referred to as *constitutive photomorphogenic (cop), de-etiolated (det),* or *fusca (fus*). Subsequent cloning of the genes

associated with these loci revealed the identity of these central repressors of photomorphogenesis. The COP/DET/FUS proteins are components of three distinct protein complexes: (i) the COP1-SPA complex, (ii) the COP9 signalosome (CSN) and the (iii) the COP10-DET1-DDB1 (CDD) complex (Lau and Deng 2012).

In addition to the *COP/DET/FUS* genes, a group of phytochrome interacting basic helix-loop-helix (bHLH) transcription factors were later identified as another class of negative regulators of light signalling. These transcription factors are called PHYTOCHROME INTERACTING FACTORS (PIFs) (Leivar et al 2008, Leivar and Monte 2014).

1.1.4.1 COP1

COP1 is a 76 kDa protein that targets positive regulators of light signalling for degradation. Ubiquitination is a mechanism whereby ubiquitin (Ub) tags are added to proteins. One of the many functions of ubiquitination is targeting proteins for subsequent proteolytic degradation via the 26S proteasome pathway. COP1 interacts with SUPPRESSOR OF PHYA-105 (SPA) proteins to form a RING E3 ubiquitin ligase which, in the dark, targets photomorphogenesis promoting transcription factors such as HY5, HYH, HFR1, and LAF1 for degradation via the 26S proteasome system, leading to skotomorphogenesis (Zhu et al 2015).

COP1 has 3 distinct domains that facilitate interaction with other proteins, namely a RING finger domain, a coiled–coil domain, and WD-40 repeat domain. COP1 interacts with most of its substrates via the WD-40 domain (Yi and Deng 2005). COP1 is capable of auto-ubiquitination and SPA1 has no specific role for this self-ubiquitination. However COP1 E3 ligase activity is strictly impaired in *spa1* mutants and thus, SPA1 is required for ubiquitination of other substrates (Seo et al 2003).

COP1 interacts with phyA, phyB, CRY1, and CRY2 in response to photoperception by the photoreceptors. Two mechanisms appear to contribute to the repression of COP1 in the light. One is the slow translocation of COP1 from the nucleus to the cytosol and the other is rapid inhibition of COP1 by the photoreceptors (von Arnim et al 1997, Lu et al 2015). Recent studies have shown that both phyA and phyB co-localize with SPA1 in the nucleus. Photoactive phyA and phyB interact with SPA1 in a light dependent manner and prevent COP1-SPA interaction and thereby formation of the active COP1-SPA complex (Figure 1.2) (Lu et al 2015, Sheerin et al 2015). In addition, both CRY1 and CRY2 regulate COP1 activity in blue light by interacting with SPA1 and preventing COP1-SPA E3 ligase function (Lau and Deng 2012, Liu et al 2016).

Another recently identified COP1 repressor, COP1 SUPPRESSOR 2 (CSU2), directly interacts with COP1 and inhibits its E3 ligase activity. *CSU2* loss of function suppresses the *cop1* mutant phenotype (Xu et al 2015b).

In contrast to its negative regulatory role in visible light signalling, COP1 acts as a positive regulator in UV-B signalling. Upon exposure to UV-B, the UV photoreceptor UVR8 undergoes a conformational change enabling interaction with COP1. As a result, *HY5* expression is increased, which leads to activation of UV-B induced genes. Thus, COP1 has a role in plant UV-B tolerance (Lau and Deng 2012, Kong and Okajima 2016).

1.1.4.2 The COP9 signalosome

The COP9 signalosome (CSN) consists of eight subunits, six of which were identified as *cop/det/fus* mutants. The CSN regulates the activity of CULLIN RING E3 ligases and thereby

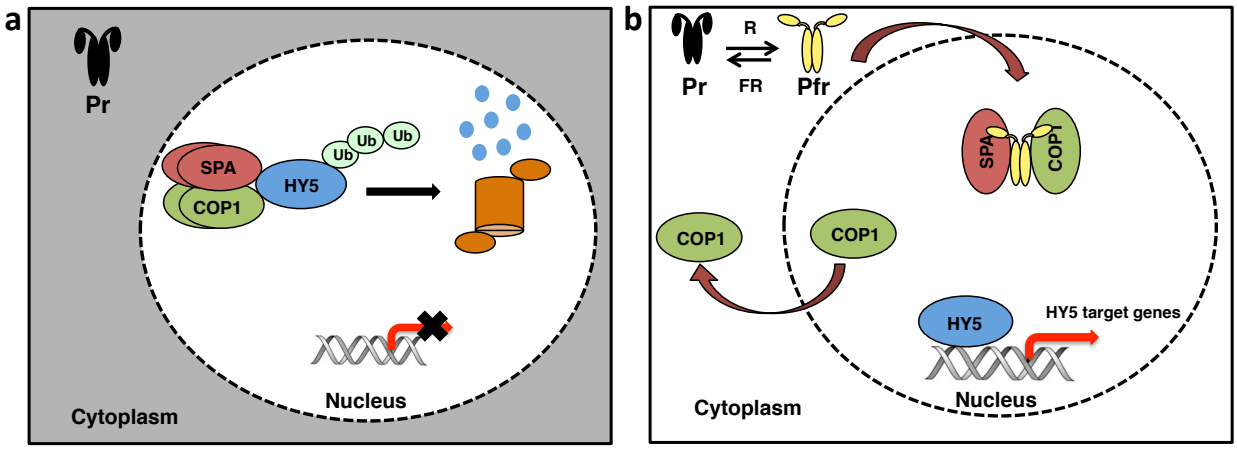

Figure 1.2. COP1 regulation of HY5 levels in (a) dark and (b) light
In the dark, nuclear localized COP1-SPA targets HY5 for degradation via the 26S proteasome and prevents photomorphogenesis. In the light, the active Pfr form of phytochrome enters the nucleus and inhibits COP1-SPA interaction. In addition, COP1 is slowly exported from the nucleus. This results in HY5 accumulation, expression of HY5 target genes and light growth (Lau and Deng 2012, Xu et al 2015a).

plays an important role in regulation of ubiquitin/proteasome mediated protein degradation. The CSN removes the ubiquitin-like modifier Nedd8 from CUL based E3 ligases (Lau and Deng 2012, Dong et al 2015). Loss of function *csn* mutants show a constitutive photomorphogenic phenotype because the CSN is essential for COP1 nuclear translocation and nuclear retention. Thus, a number of genes including light regulated genes are mis-regulated in *csn* mutants (Chamovitz 2009, Wang et al 2009).

1.1.4.3 The COP10/DET1/DDB1 (CDD) complex

In the CDD complex, COP10 and DET1 form a complex with CUL4 via DAMAGED DNA BINDING protein 1 (DDB1).

1.1.4.3.1 COP10

COP10 is an ubiquitin conjugating enzyme (E2) variant (Suzuki et al 2002). COP10 is also required for COP1 mediated degradation of HY5 (Osterlund et al 2000). COP10 directly interacts with COP1, the CSN, and proteasome subunits and forms a stable complex with DDB1 and DET1 (CDD complex). The CDD complex promotes ubiquitin chain formation and enhances E2 activity. COP10 itself has no E2 activity but can enhance the activity of other E2s in the presence or absence of the CDD complex (Yanagawa et al 2004). The CDD complex interacts with CUL4 and shows E3 ligase activity, however the target proteins were unknown until recently. The only known direct target of the CDD complex is HFR1 (Chen et al 2006, Shi et al 2015).

1.1.4.3.2 DET1

De-etiolation refers to inhibition of hypocotyl elongation and induction of leaf expansion and differentiation. The de-etiolated mutants in *Arabidopsis,* such as *det1*, resemble light grown plants when grown in complete darkness. Hence *det1* mutants exhibit short hypocotyls, expanded cotyledons with noticeable leaf primordia and initiation of chloroplast development in the dark. In addition, *det1* mutants express light regulated genes in the dark, such as photosynthesis related genes (Chory et al 1989). Thus, DET1 acts as a negative regulator of seedling de-etiolation response. *det1* mutants can continue to grow for extended periods in the dark, developing leaves and flowers. Light grown adult *det1* plants are small with increased number of inflorescence stems as well as reduced fertility (Chory et al 1989, Pepper et al 1994). DET1 is also involved in spatial patterning of light regulated gene expression and chloroplast development (Chory and Peto 1990). In addition, pleiotropic defects in *det1* mutants, including morphological defects and

abnormal gene expression in the dark and light, can be restored by increased peroxisome function. TED3 is the Arabidopsis homologue of the yeast and mammalian peroxisomal protein PEX2 and *ted3* gain of function mutants can rescue *det1* phenotypes. This indicates that both DET1 and peroxisomes play important roles in photomorphogenesis (Hu et al 2002).

hy5 mutants suppress *det1* dark grown seedling phenotypes as well as *det1* light grown adult phenotypes such as size, flowering, apical dominance, and fertility phenotypes. This suggests that HY5 acts downstream from DET1, consistent with the lack of HY5 degradation in *det1* mutants (Chory 1992, Pepper and Chory 1997, Osterlund et al 2000, Fernando and Schroeder 2015). DET1 represses *CHLOROPHYLL A/B BINDING PROTEIN 2 (CAB2)* gene expression in the dark but activates it in the light. DET1 regulation of *CAB2* expression was found to be via HY5 and the circadian regulator CCA1 (Maxwell et al 2003).

DET1 is nuclear localized and interacts directly or indirectly with a number of other proteins. DET1 interacts physically and genetically with DAMAGED DNA BINDING PROTEIN 1 A/B (DDB1A/B) and COP10 to form the CDD complex (Pepper et al 1994, Schroeder et al 2002, Yanagawa et al 2004, Ganpudi and Schroeder 2013). The CDD complex in turn interacts with CUL4 to form an active E3 Ub ligase. However, a direct target of the complex was not known until the recent discovery that HFR1 degradation mediated by the CUL4-CDD complex (Chen et al 2006, Shi et al 2015). In addition, COP1 nuclear retention and HY5 degradation require the activity of the CDD and CSN complexes (von Arnim et al 1997, Osterlund et al 2000, Wang et al 2009). Thus, *det1* mutants have increased levels of HY5 protein but DET1 does not appear to directly interact with HY5 (Osterlund et al 2000, Lau et al 2011). Also there is no evidence of direct interaction between COP1 and DET1, therefore the basis of this mechanism is still not clear (Chen et al 2010).

In contrast, DET1 was found to directly interact with the SINAT5 E3 ligase and block degradation of LATE ELONGATED HYPOCOTYL (LHY), a component of the Arabidopsis circadian clock. SINAT5 interacts with both LHY1 and DET1 but ubiquitinates only LHY. Thus, DET1 may influence flowering time in Arabidopsis by affecting protein abundance of LHY via inhibition of the SINAT5 E3 ligase (Song and Carré 2005, Park et al 2010).

In addition to its role in E3 ligase complexes, DET1 has been shown to be involved in transcriptional regulation (Lau et al 2011, Huang et al 2014). DET1 acts as a transcriptional co-repressor of the Arabidopsis circadian clock. CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LHY1 are MYB transcription factors with partially overlapping functions that act as transcriptional repressors in the morning phase of the central loop of circadian clock. DET1 directly interacts with CCA1 and LHY1 to repress CCA1/LHY1 target genes. DET1 is essential for CCA1 transcriptional repression activity and for functioning of the plant circadian clock (Lau et al 2011). Moreover, DET1 has a possible role in chromatin remodelling via binding to non-acetylated histone 2B (H2B) tails in nucleosomes (Benvenuto et al 2002).

DET1 not only represses photomorphogenesis but also represses flowering by altering the photoperiod and autonomous pathways. DET1 delays flowering particularly under short day conditions. Flowering is controlled by multiple signalling components including GIGANTIA (GI) and FLOWERING LOCUS T (FT). GI functions as a flowering inducer and activates *FT* transcription. DET1 directly interacts with GI and delays flowering by inhibiting the interaction between GI and the *FT* promoter. Thus, DET1 does not affect GI protein stability but functions as a repressor of *FT* transcription (Kang et al 2015). In addition, DET1 binds directly to MULTICOPY SUPPRESSOR OF IRA1 4 (MSI4), which is part of a CUL4-DDB1 complex that alters the expression of *FLOWERING LOCUS C (FLC)*. FLC inhibits floral transition by

suppression of floral inducers like FT. The CUL4-DDB1-MSI4 E3 ligase associates with POLYCOMB REPRESSIVE COMPLEX 2 containing histone methyltransferase CLF (CLF-PCR2) and represses *FLC* expression. *det1* exhibits altered *FLC* promoter methylation and expression (Pazhouhandeh et al 2011, Kang et al 2015).

A recent study showed that DET1 suppresses accumulation of DELLA proteins, which are negative regulators of Gibberellic acid (GA) signalling. Light and GA antagonistically regulate seedling growth and DELLAs play a role in promoting skotomorphogenesis in the dark. Therefore, DET1's role in repression of photomorphogenesis may be partly through negative regulation of DELLA protein levels in the dark (Li et al 2015). DELLAs inhibit another negative regulator of photomorphogenesis, PIFs (discussed below) (Davière and Achard 2016).

Other roles of DET1 include the recent discovery that DET1 is involved in stabilization of PHYTOCHROME INTERACTING FACTORS (PIFs) as well as mediates degradation of HFR1 (Dong et al 2014, Shi et al 2015). These topics will be discussed in more detail later in this chapter.

1.1.4.3.3 CUL4 / DDB1A/B E3 ligase complexes in light signalling

Cullin proteins are the scaffolding subunits of E3 ligase complexes, where the N-terminus of the cullin binds to an adaptor protein and the C-terminus binds to the RING finger protein RBX1. The adaptor protein functions to connect the cullin to specific substrate receptors that enable interaction with the substrate to be ubiquitinated. For instance, in CUL4 E3 ligases, DDB1 acts as the adaptor and interacts with a number of different substrate receptors. These substrate receptors commonly have roughly seven WD40 domains thus are called DWD (DDB1 binding WD40) proteins or DDB1 CUL4 ASSOCIATED FACTORS (DCAFs). There are certain

proteins, such as DET1 and COP10, which lack a WD40 domain but still interact with DDB1. CUL4-DDB1-DCAF complexes are involved in a wide array of functions in plants including repression of photomorphogenesis, facilitating damaged DNA repair, and response to abiotic stress (Biedermann and Hellmann 2011) (Table 1.1).

DDB1 was first identified in mammals as part of the DDB1-DDB2 complex that binds to UV damaged DNA and is involved in nucleotide excision repair of damaged DNA. DDB1 is a highly conserved protein in eukaryotes. In Arabidopsis DDB1 exists as two homologues, DDB1A and DDB1B, which exhibit 91% amino acid identity with each other (Schroeder et al 2002). Although the two proteins are not biochemically different, DDB1A and DDB1B show distinct functions in the light and dark and the double mutant is embryonic lethal (Schroeder et al 2002, Bernhardt et al 2010, Ganpudi and Schroeder 2013).

Distinct DDB1 complexes appear to interact with each other genetically and biochemically. DDB2 is mainly involved in the global genomic repair pathway as a substrate receptor for CUL4-DDB1 (Ganpudi and Schroeder 2011). In Arabidopsis however *DDB2* also genetically interacts with *DDB1A* and *DET1*. *DDB2* interactions with *DET1* were shown to be *DDB1A* independent for some adult phenotypes while in some dark grown seedling phenotypes the interactions were *DDB1A* dependent (Al Khateeb and Schroeder 2007). In addition, DET1 is essential for the degradation of DDB2 via the CUL4-DDB1 E3 ligase during UV damage repair. Removal of DDB2 after damaged DNA lesion recognition is an important step, allowing the repair machinery to access the damaged lesions. Thus DET1 and DDB2 work together in UV damaged DNA repair (Castells et al 2011).

Table 1.1 Selected CUL4-DDB1 E3 ligase complexes and their main functions (Biedermann and Hellmann 2011, Yu et al 2016a).

DCAF protein	targets	Functions
COP/SPA1-4	HY5, HYH, HFR1, LAF1	Repression of photomorphogenesis
DET1/COP10	HFR1	Repression of photomorphogenesis
DDB2		DNA repair
MSI4	PRC2	Flowering time
DWA1/2	ABI5	Negative regulation of ABA signalling, salt tolerance
DWA3	?	Negative regulation of ABA signalling
ABD1	ABI5	Negative regulation of ABA signalling
DDA1	PYL8	Negative regulation of ABA signalling

1.1.4.4 PHYTOCHROME INTERACTING FACTORs (PIFs)

The basic Helix-Loop-Helix (bHLH) type transcription factors PHYTOCHROME INTERACTING FACTORs (PIFs) act directly downstream of phytochromes to negatively regulate photomorphogenesis and promote skotomorphogenesis. PIFs (PIF1/PIF3-LIKE 5, PIF3, PIF4, PIF5/PIL6, PIF6/PIL2, PIF7, and PIF8) accumulate in the dark to promote dark growth. In the presence of light, activated phytochromes, in the nuclear Pfr form, interact directly with PIFs. Phytochromes then phosphorylate the PIFs, targeting them for ubiquitination and degradation via the proteasome (Figure 1.3). While there is redundancy among the PIFs, *pif3* mutants have short hypocotyls in red light while PIF4 and PIF5 are also involved in negative regulation of light signalling. PIF1 regulates seed germination and hypocotyl elongation. Furthermore, *pifq* (which

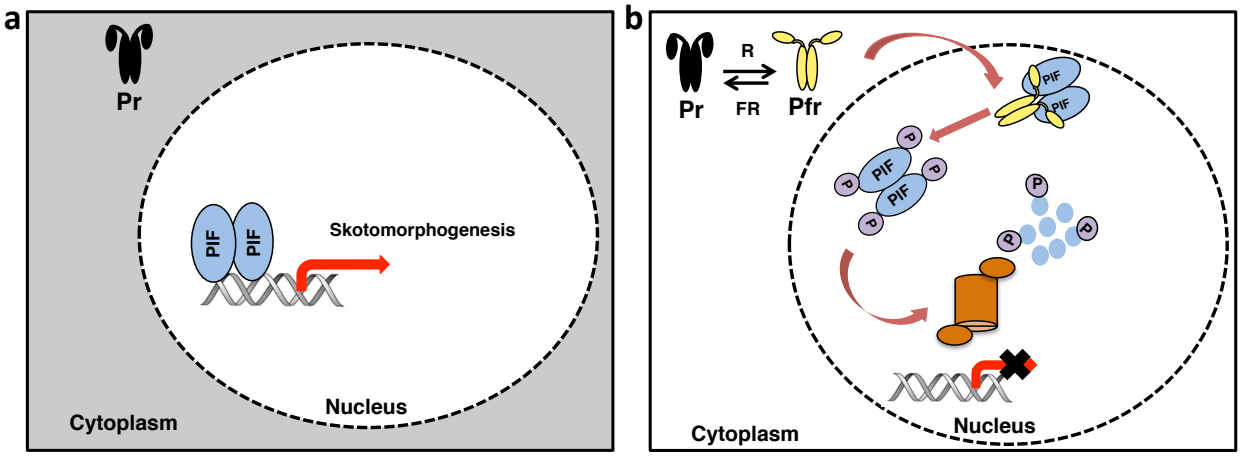

Figure 1.3. Interaction between light and PIFs in light signalling
(a) In the dark phytochromes are in the biologically inactive Pr form and are localized in the cytosol. Homo and heterodimers of PIFs bind to light regulated genes, preventing their expression and repressing photomorphogenesis. (b) In the light, the active Pfr form of phytochromes move to the nucleus to bind and rapidly phosphorylates PIFs. The phosphorylated PIFs are degraded via the 26S proteasome. As a result photomorphogenesis occurs (Xu et al 2015a).

lacks PIF1, PIF3, PIF4 and PIF5) shows a constitutive photomorphogenic phenotype (Leivar and Monte 2014).

Microarray expression profile analysis indicated that DET1 represses photomorphogenesis by regulating a number of transcription factors including PIFs. DET1 directly interacts with PIFs and positively regulates *PIF3* transcription. Thus, lack of PIF3 enhances the *det1* de-etiolated phenotype while overexpression can partially restore seedling de-etiolation in the dark. In addition, DET1 and other components of the CDD complex affect the protein stability of PIFs at the post-transcriptional level. DET1 positively regulates only *PIF3* at the gene expression level but positively regulate all the PIFs at the post-translational level. Moreover, both *det1* and *cop1* mutants have significantly reduced PIF3 protein levels, suggesting that the DET/COP/FUS group of genes repress photomorphogenesis in part by mediating protein

stability of PIFs and upregulating the function of PIF3 in the dark (Lau and Deng 2012, Dong et al 2014, Dong et al 2015).

Interactions between the two main classes of repressors of photomorphogenesis, COP/DET/FUS and PIFs, are just beginning to unravel. COP1 and PIFs have additive roles in the dark. PIFs enhance the substrate recruitment and ubiquitination functions of COP1, and PIF1 interacts with COP1, SPA1, and HY5 (Xu et al 2014). PIF1 functions via three different mechanisms to regulate COP1 degradation of HY5. Firstly, PIF1 enhances COP1 affinity for HY5, improving substrate recruitment. In addition, PIF1 promotes COP1 auto-ubiquitination and also facilitates transubiquitination of HY5 by COP1. Thus, negative regulation of photomorphogenesis by PIFs is not an independent mechanism but acts by affecting the protein stability of HY5 via regulation of COP1-SPA E3 ligase activity. Therefore, PIF1 and COP1 act as cofactors and synergistically repress light growth in the dark (Xu et al 2014).

1.1.5 Light signalling overview

In summary, different wavelengths of light are perceived by photoreceptors, with phyA mediating FR perception, phyB red light, and CRY1 and CRY2 blue (Figure 1.4). In general light absorption results in changes in photoreceptor conformation and/or localization, which facilitates interactions between the photoreceptors and downstream negative regulators. The photoreceptors then inhibit the negative regulators, resulting in disruption of COP1 activity and degradation of the PIFs. In the absence of light the negative regulators positively reinforce each other, with DET1 stabilizing the PIFs and the PIFs promoting COP1 degradation of HY5 and other photomorphogenesis promoting transcription factors. Thus in the dark the negative regulators promote skotomorphogenesis and inhibit photomorphogenesis, while the light

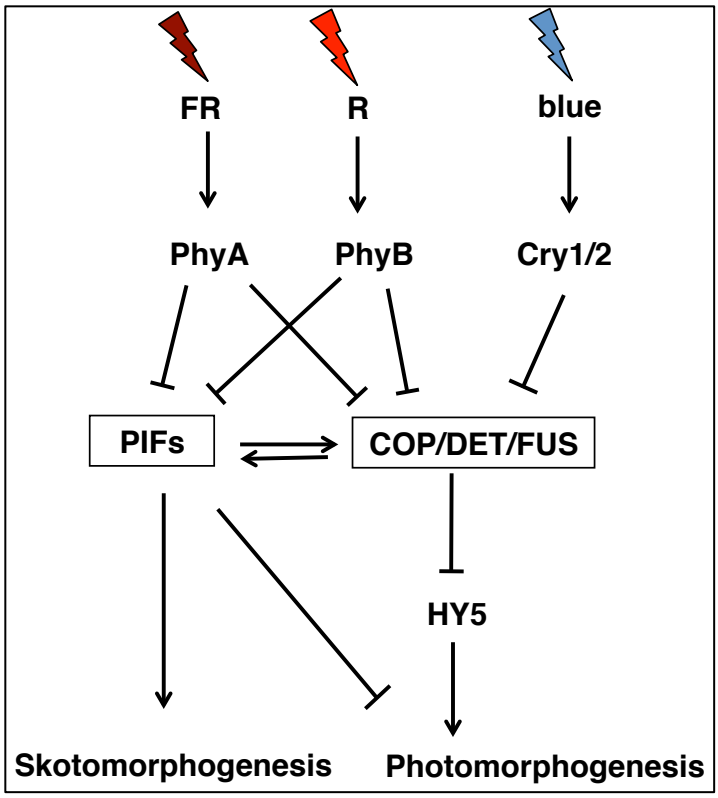

Figure 1.4. Light perception and signalling pathways.
Arrows indicate positive regulation and T-bars indicate negative regulation.

inactivation of the negative regulators by the photoreceptors allows light development to proceed (Huang et al 2014, Dong et al 2015). Light influences nearly all aspects of growth and development in plants and our understanding of this critical process is finally becoming illuminated. The knowledge gained through genetic and biochemical studies in the model plant Arabidopsis can be transferred to agriculturally more important crops, allowing us to optimize light use during crop production.

1.2 Role of ABA in salt, osmotic and desiccation tolerance in Arabidopsis

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1.2.1. Abstract

How plants respond to various environmental stimuli is an important phenomenon that affects survival. Abscisic acid (ABA) is a phytohormone with roles at various stages of plant development. ABA also plays a major role in mediating physiological responses to environmental stresses like salt, osmotic and cold stress. Plant responses to environmental stress have been widely studied in the model plant *Arabidopsis thaliana* and ABA signalling mechanisms elucidated. In general the adaptive responses of plants to various stress conditions can be either ABA dependent or ABA independent. This review will focus on the role of ABA in stress signalling and crosstalk between ABA and abiotic stress tolerance. We will discuss the intrinsic mechanisms that confer stress tolerance via ABA as well as how ABA regulated gene products play a role in salt and drought stress tolerance at different stages of the life cycle. Also we will discuss recent advances on the contribution of ABA to stomatal development and regulation of stomatal aperture and therefore the importance of ABA in desiccation tolerance. Understanding ABA signalling mechanisms in abiotic stress will provide avenues for improving plant performance.

1.2.2. Introduction

Due to their sessile nature, plants cannot avoid environmental stresses, thus they have evolved mechanisms to overcome the detrimental effects of stress. For example, plant endogenous developmental programs are modified such that structural and metabolic changes assist in overcoming adverse environmental conditions such as salinity and drought. Failure to adapt to adverse environmental conditions can significantly reduce yield by impacting plant development and productivity. Abiotic stress conditions initiate a number of molecular, biochemical and physiological changes at both the cellular and whole plant level (Wang et al 2003). One major biochemical change in response to stress is elevation of abscisic acid (ABA) levels, which in turn triggers expression of a cascade of stress responsive genes (Shinozaki and Yamaguchi-Shinozaki 2007). Cellular ABA levels are induced by environmental stimuli such as light, water and salinity stress (Cutler and Krochko 1999).

The plant hormone ABA has been identified as a key regulator of multiple stresses. In general the adaptive responses of plants to various stress conditions can be either ABA dependent or ABA independent. However there is no clear boundary between these two pathways and there is lot of crosstalk between the pathways and the components involved. This review will focus on recent advancements in ABA mediated stress signalling and the role of ABA in abiotic stress tolerance in the model plant *Arabidopsis thaliana*.

1.2.3 The phytohormone abscisic acid (ABA)

ABA, a sesquiterpenoid ($C_{15}H_{20}O_4$) with a 15-carbon ring (Figure 1.5), has a variety of biological functions and is found ubiquitously across several kingdoms, including cyanobacteria, sponges, algae, lichens, mosses and mammals (Wasilewska et al 2008, Cutler et al 2010,

Roychoudhury et al 2013, Mehrotra et al 2014). Discovered in the 1960s and initially named dormin or abscissin, ABA is now established as a widely occurring and important plant growth regulator. Although it was initially identified as an abscission-promoting hormone, later scientists discovered that this was partly due to an indirect effect of inducing ethylene biosynthesis (Cracker and Abeles 1969). ABA is an important regulator of plant growth, including embryo and seed development, seedling establishment, vegetative and reproductive growth as well as promoting seed dormancy (Barrero et al 2005, Fujii and Zhu 2009). Seed maturation and promotion of dormancy are important in preventing pre-harvest sprouting. In addition, ABA has the ability to antagonize the germination promoting effects of gibberellin, regulate guard cells and regulate stress responsive gene expression under water-deprived conditions. ABA also has a role in plant pathogen responses in a patho-system dependent manner (Cutler et al 2010, Wasilewska et al 2008).

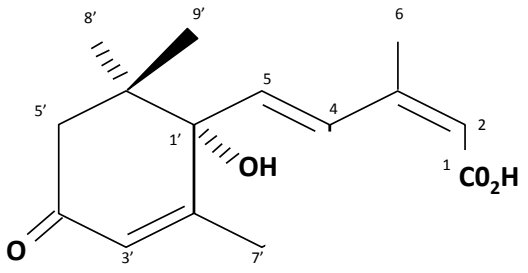

Figure 1.5 Structure of phytohormone abscisic acid S-(+)-ABA

The molecular structure of ABA has several important features that facilitate its biological functions. The side chain with the two double bonds (Figure 1.5) and ABA's stereocentre are two such important features. Exposure to UV light changes the conformation from the active (2-cis, 4-trans ABA) to the inactive (2-trans, 4-trans) form, which does not show

hormonal activity (the configuration of the two isomers is based on the position of the double bond, in relation to the ring) (Cutler et al 2010).

1.2.3.1 ABA signalling in plants

Although ABA has a broad range of functions in plant growth and development, its main function is to regulate plant water balance and osmotic stress tolerance (Raghavendra et al 2010). Thus, understanding ABA signalling is essential to improving plant performance. Genetic screens done in Arabidopsis identified many downstream ABA signalling components. Recent findings in the field of ABA signalling reveal a unique hormone perception mechanism (Figure 1.6) where ABA binds to the ABA receptors Regulatory Components of ABA Receptor/Pyrabactin Resistance Protein1/PYR-like Proteins (RCAR/PYR1/PYLs) (Cutler et al 2010). RCAR/PYR/PYL proteins belong to the START-domain superfamily and have soluble ligand binding properties. RCAR/PYR/PYL receptors are found in the cytoplasm as well as in the nucleus. ABA binding to RCAR/PYR/PYLs leads to inactivation of type 2C protein phosphatases (PP2Cs) such as ABSCISIC ACID INSENSITIVE 1 (ABI1) and its close homolog ABI2 (Nishimura et al 2010). All 14 members of the RCAR family of proteins bind to ABA and interact with PP2Cs. Except for RCAR7/PYL13, all the other RCAR members are positive regulators of ABA signalling. Among the 80 PP2Cs identified in Arabidopsis, six out of nine clade A PP2Cs acts as negative regulators of ABA signalling (Nishimura et al 2007). These phosphatases and RCAR/PYR1/PYLs function as co-receptors and form a high affinity ABA binding site (Cutler et al 2010). Inactivation of PP2Cs causes suppression of PP2C mediated dephosphorylation of Sucrose non-fermenting Kinase-1-Related protein kinase 2s (SnRK2s), which are important positive regulators of ABA signalling (Liang and Zhang 2014). As a result,

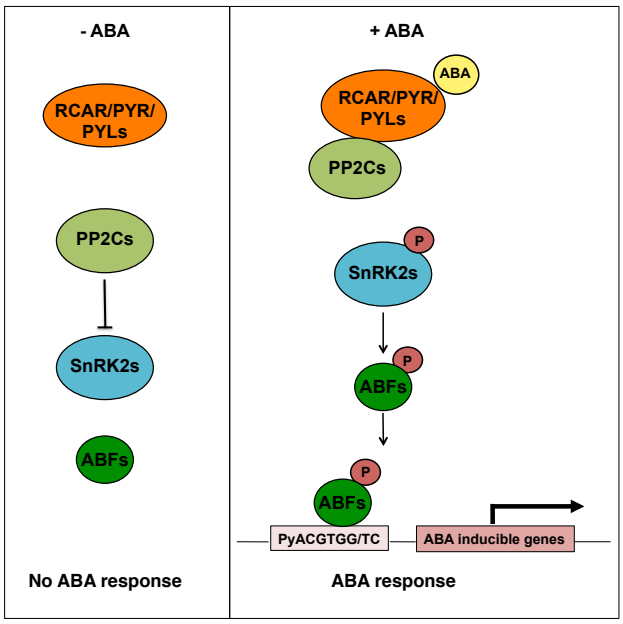

Figure 1.6 Main components in the core ABA signal transduction pathway
In the absence of ABA, PP2Cs inactivate and dephosphorylate SnRK2 kinases, preventing downstream ABA responsive gene expression. In the presence of ABA, binding of ABA to the RCAR/PYR/PYL receptor prevents inhibition of SnRK2s by PP2Cs. Phosphorylated SnRK2s consequently phosphorylate ABFs, which upregulate transcription of ABA inducible genes.

activated SnRK2s directly phosphorylate ABA dependent transcription factors and ion channels (Reviewed in (Cutler et al 2010, Raghavendra et al 2010)). Table 1.2 summarizes the major positive and negative regulatory elements in the ABA signaling pathway. SnRK2s phosphorylate ABA responsive element Binding Factors (ABFs), which are basic leucine zipper transcription factors that bind to ABA Responsive Elements (ABRE) (PyACGTGG/TC), the major *cis*-element in the promoter region of downstream genes that are induced by ABA (Busk and Pages 1998, Lumba et al 2014).

Therefore, the ABA signalling complex/ABA signalosome is comprised of three major components: (a) RCAR/PYR/PYLs; (b) PP2Cs; and (c) SnRK2s, assembled as a double negative regulatory system (Mehrotra et al 2014). In the absence of ABA, PP2Cs dephosphorylate SnRKs

inhibiting kinase activity and thereby preventing downstream gene expression (Figure 1.6). Several studies showed that these core components are essential for ABA signalling. For instance Fujita et al (2009) showed ABA signalling is completely blocked and *ABF* genes showed reduced expression in the *snrk2.2/2.3/2.6* triple null mutant but not in single or double mutants. In addition, reduced phosphorylation of other bZip transcription factors such as ABSCISIC ACID INSENSITIVE 5 (ABI5), which is a germination inhibiting transcription factor, was also observed (Finkelstein et al 2002, Nakashima et al 2009).

In guard cells (Figure 1.7), ABA binds to the PYR/PYL/RCAR receptor-PP2C and blocks its phosphatase activity. Consequently activated protein kinase SnRK2.6/OPEN STOMATA 1 (OST1) phosphorylates and regulates the key target ion channels, SLOW ANION CHANNEL ASSOCIATED 1 (SLAC1) and K^+ CHANNEL IN ARABIDOPSIS THALIANA 1 (KAT1). SnRK2.6/OST1 acts as a positive regulator of stomatal closure where it activates anion channel SLAC1 and inhibits cation channel KAT1 (Geiger et al 2009, Sato et al 2009, Vahisalu et al 2008).

1.2.3.2 ABA binding proteins and alternate ABA receptors

Identification of putative ABA receptors using forward genetic approaches were not successful for a long time due to genetic redundancy. However, biochemical approaches leading to purification and analysis of high affinity ABA binding proteins have been successful in identification of potential ABA receptor classes (McCourt and Creelman 2008, Cutler et al 2010). Some of these potential ABA receptors are cytosolic while others are on the cell surface indicating there are extracellular as well as intracellular sites of ABA perception. Different studies indicate that there can be multiple ABA receptors at different locations of the cell (Guo et

al 2011).

Table 1.2 Major positive and negative regulators of ABA signalling

Signalling component	**Regulation**	**Expressed**	**References**
Group A PP2Cs **1.** ABA INSENSITIVE 1 and 2 (ABI1/2) 2. ABA HYPERSENSITIVE GERMINATION 1 (AHG1) **3.** HYPERSENSITIVE TO ABA 1 and 2 (HAB1/2)	Negative regulators	Various tissues and developmental stages	(Gosti et al 1999, Nishimura et al 2007, Saez et al 2004, Umezawa et al 2010, Yoshida et al 2006)
SnRK2 subgroup II **1.** SRK2D/SnRK2.2 **2.** SRK2I/SnRK2.3 **3.** SRK2E/OST1/SnRK2.6	Positive regulators	Seeds and vegetative tissues Seeds and vegetative tissues Expressed in guard cells and involved in stomatal closure	(Nakashima et al 2009, Yoshida et al 2006)

1.2.3.2.1 ChlH / ABAR (H SUBUNIT OF THE CHLOROPLAST MAGNESIUM CHELATASE / ABA RECEPTOR)

The primary function of ChlH is chlorophyll synthesis. ChIH was initially identified as an ABA binding protein in broad bean (*Vicia faba*) and the Arabidopsis protein was named as ABAR. Later it was found that binding of ABA to ChIH/ABAR depends on the stereochemistry and it specifically binds to only (+) –ABA and mediate ABA responses. Although, ChIH/ABAR is localized in the chloroplast envelope, it functions as a negative regulator of ABA signalling in the nucleus. The cytosolic C-terminus of the ABAR interacts with WRKY transcription factors (WRKY 18, 40, 60) and acts as transcription repressors and represses ABA responsive genes in the nucleus. Binding of ABA with ChlH/ABAR promotes interaction with WRKYs and prevents them from repressing downstream genes such as ABI5 and DREB2 (Shen et al 2006, Shang et al 2010, Yan et al 2013, Wang and Zhang 2014). Thus, it has been proposed that ChlH mediates nuclear-chloroplast signalling. However, another research group has not been able to reproduce these results using *wrky* loss of function mutants. Also barley ChIH does not bind to ABA as well as its loss of function mutants do not show any impaired ABA responses. Despite its ABA binding properties in Arabidopsis, it is not confirmed whether ChIH functions as an ABA receptor (Reviewed in (Cutler et al 2010, Guo et al 2011). ChlH/ABAR also mediates ABA induced stomatal closure and ABA inhibition of blue light mediated stomatal opening. In addition, ChIH/ABAR has a role in ABA mediated fruit ripening in peach and strawberry (Reviewed in (Wang and Zhang 2014).

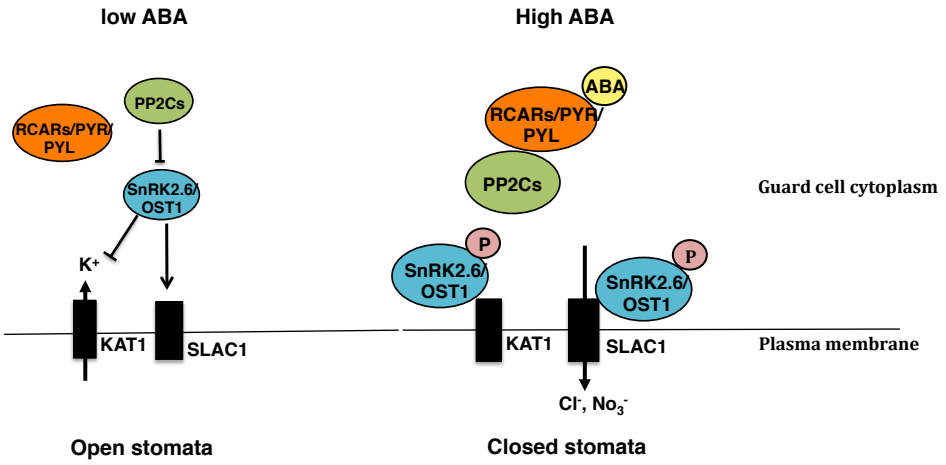

Figure 1.7 ABA signalling in guard cells
When ABA concentrations are minimal, PP2Cs dephosphorylate SnRK2.6/OST1, thereby K^+ inward channel KAT1 becomes active. In high ABA concentrations, ABA binds to the RCAR/PYR/PYL receptor-PP2C complex, releasing SnRK2. Phosphorylated SnRK2.6/OST1 blocks KAT1 and activates anion channel SLAC1, resulting in stomatal closure.

1.2.3.2.2 GTG1/GTG2 (G PROTEIN COUPLED RECEPTOR TYPE G PROTEIN 1 AND 2)

G protein coupled ABA receptors are plasma membrane localized cell surface receptors that are widely expressed in plants. Both GTG1 and GTG2 showed specific and saturable ABA binding activity in direct ABA binding assays. GTGs have GTPase activity and GDP bound GTGs have enhanced ABA binding ability which in turn initiates ABA signalling. GTG1/2 bind with GPA1 (G-PROTEIN α SUBUNIT 1) which abolishes its GTPase activity and represses ABA binding. GTP bound GPA1 represses ABA signalling. However, the downstream components of this pathway are not characterized yet (Pandey et al 2009, Wang and Zhang 2014).

1.2.3.3 Recent evidence on ABA perception and signalling mechanisms

Recent findings that several ABA receptors exist in different parts of the cell provide evidence that ABA is produced in different subcellular compartments. ABA synthesis enzymes

present in different compartments suggests that ABA synthesis occurs in different parts of the cell and that ABA levels contribute to the overall ABA homeostasis in the cells. For example ABA biosynthesis enzyme AtABA1 is localized in the chloroplast where as AtABA2 is in the cytosol (Cheng et al 2002, Rock and Zeevaart 1991). It has also been proposed that ABA produced in cytoplasm, plastids, vacuole and other subcellular organelles may have different physiological roles initiated by signalling networks via different ABA receptors in each specific compartment (Xu et al 2013b).

Takeuchi et al (2014) identified a potential ABA analogue AS6 that can inhibit the activity of PYLs. X-ray crystallography studies showed the structure of ABA facilitates the binding of ABA into PYR/PYL/RCAR receptors and thereby inhibits interaction with PP2Cs. AS6 ABA analog was able to block PYL-PP2C interaction indicating that binding of ABA to PYL receptors initiate ABA responses by repressing PP2Cs.

Inhibition of PP2Cs results in autoactivation of SnRK2 kinases and thereby positive regulation of ABA signalling. Recently the crystal structures of SnRK2.3 and SnRK2.6 were elucidated providing evidence that kinase activation is a two-step mechanism and details of how the ABA signal is transmitted to downstream components (Ng et al 2011). These studies also showed that autophosphorylation of SnRK2.6 is more efficient than that of SnRK2.3.

Lumba et al (2014) did a comprehensive transcriptomic data analysis in order to generate a mesoscale ABA signalling network. They showed that there are 3 main kinase hubs, MAP3K∂4, SnRK3.15, and SnRK3.22, that interact with PP2Cs and these kinases act as negative regulators of ABA response, in contrast to the SnRK2s involved in positive regulation of ABA signalling. SnRK3.15 and SnRK3.22 also interact with a large number of transcription factors and may have a role in overall ABA responses in the plant (Lumba et al 2014).

1.2.4 ABA in stress signalling

In plants and other organisms, such as algae, cyanobacteria, and fungi, ABA levels tend to increase with exposure to stress, suggesting a potential role of ABA in stress signal transduction (Lumba et al 2014). Exogenous ABA application mimics stress conditions in plants and provides a useful means to study the effect of ABA on stress signalling and tolerance (Bartels and Souer 2004). ABA distributes throughout the plant as an inactive glucose sugar conjugate and is converted to the active form by β-glucosidase (Wasilewska et al 2008). ABA acts as an endogenous messenger. Salt and drought stress signal transmission to initiate downstream gene expression occurs mainly through ABA signalling. In contrast, cold stress signal transduction can be ABA dependent or independent. Cold stress signalling mainly occurs in an ABA independent manner via the C-REPEAT/DEHYDRATION RESPONSIVE ELEMENT BINDING FACTORS (CBFs/DREBs) signalling pathway (Shinozaki et al 2003). However, freezing also results in reduced turgor pressure in cells, which induces ABA. ABA regulated genes also have been shown to confer tolerance to cold stress in Arabidopsis (Solanke and Sharma 2008). In addition, cold stress and drought stress signalling mechanisms converge, as CBF/DREBs act as coupling elements and function, together with ABREs, in ABA inducible gene expression in response to drought stress (Shinozaki et al 2003, Xin et al 2012).

A large number of ABA responsive genes have a common *cis*-element called the ABRE element in their promoter regions. An ABRE together with a Coupling Element (CE) results in ABA induction of gene expression (Busk and Pages 1998). The ABA RESPONSIVE ELEMENT BINDING FACTOR (AREB/ABF) family of transcription factors are the major transcription factors that regulate ABA induced gene expression. AREB/ABFs are bZIP transcription factors and their expression is induced by ABA and other potential stress conditions

(Choi et al 2000). Different ABFs are induced by ABA at different rates. For instance *ABF2, ABF3* and *ABF4* are induced faster than *ABF1*. Moreover, *ABF1* is induced only by cold stress whereas *ABF2* and *ABF3* are induced by salt stress. *ABF4* levels are induced by salt, drought and cold stress suggesting that distinct ABFs have roles in various ABA-dependent stress responsive pathways (Choi et al 2000).

There are nine group A bZIP transcription factors implicated in ABA signalling and they are subdivided into two groups based on where they are mainly expressed. The *ABI5/AtDPBF* family of genes includes *ABSCISIC ACID INSENSITIVE 5 (ABI5), ENHANCED EM LEVEL (EEL),* and *AREB3* and are expressed during seed maturation (Bensmihen et al 2002). Other AREB/ABF transcription factors are mainly expressed in vegetative tissues (Choi et al 2000).

SnRK2 is the major subfamily of SnRKs involved in abiotic stress responses. SnRK2 protein kinases phosphorylate AREB/ABFs and regulate their function in ABA regulated gene expression under stress conditions (Fujii and Zhu 2009). SnRK2.6/OST1 is an important regulator of stomatal closure in drought stress. The role of SnRK2.2 and 2.3 are mainly to transmit the ABA signal to inhibit seed germination and seedling growth in response to stress. A decuple *snrk2* mutant in Arabidopsis that carries mutations for all ten members of SnRK2 was hypersensitive to osmotic stress and also defective in ABA accumulation and ABA induced gene expression under osmotic stress indicating the critical role of SnRK2 kinases in osmotic stress signalling and tolerance. Moreover, *snrk2.2/3/6* triple mutant had impaired accumulation of proline, which is a compatible osmolite (Fujita et al 2011).

1.2.4.1 ABA and abiotic stress tolerance

In *Arabidopsis thaliana*, about 10% of the genome consists of ABA regulated genes (Nemhauser et al 2006). Approximately half of these genes are ABA induced genes and the rest are ABA repressed. ABA induced genes code for proteins that confer stress tolerance such as dehydrins, detoxifying enzymes of reactive oxygen species, regulatory proteins (transcription factors, protein kinases, phosphatases) and enzymes required for phospholipid signalling. Genes that are repressed by ABA are mostly related to growth (Cutler et al 2010). ABA biosynthesis mutants that are deficient in ABA identified in Arabidopsis (Koornneef et al 1998) and other crop plants (Liotenberg et al 1999) wilt and die under prolonged salt and drought stress, suggesting ABA plays an important role in osmotic stress tolerance.

Drought and high salinity generates osmotic stress in plant cells. Endogenous ABA levels are elevated in response to osmotic stress, which in turn coordinates the plant's responses to reduced water availability. Also seed maturation and post germinative growth creates cellular dehydration stress, which again results in accumulation of ABA in cells (Fujita et al 2011). The role of ABA in drought and salt stress is two fold: water balance and cellular dehydration tolerance. Water balance is achieved through guard cell regulation and the latter role by induction of genes that encode dehydration tolerance proteins in nearly all cells. ABA accumulation is induced by osmotic stress and this is as a result of activation of ABA biosynthesis as well as inhibition of ABA degradation (Zhu 2002). Thus, ABA mediated adaptive stress responses of plants to environmental stimuli occur via ABA responsive gene expression and regulation of the stomatal pore size. ABA responsive gene expression involves various transcription factors, ABA receptors, secondary messengers, protein kinase/phosphatase cascades and chromatin remodelling factors (Fujita et al 2011).

Both drought stress and salinity stress upregulates osmotic stress responsive genes that are ABA inducible. Most of the high salinity induced genes are also induced by drought suggesting there is overlap between salt and drought stress tolerance mechanisms (Roychoudhury et al 2013). A large number of transcription factors are induced by multiple stress conditions. AREB1/ABF2, AREB2/ABF4, ABF3 and MYB41 are some of the main transcription factors that are induced by both stresses: salt and drought in vegetative tissues (Fujita et al 2011).

Drought and salt stress results in osmotic imbalance and thus salt and drought stress tolerance mechanisms aim at restoring cellular homeostasis. These mechanisms are adaptive responses that either creates stress tolerance or avoidance of stress conditions. Modifications in metabolic pathways, synthesis of new proteins, changes in ion uptake and free radical scavenging are some of the stress responses at the cellular level immediately followed by stress signal transduction (Bhattacharjee and Saha 2014). High ABA levels in cells results in synthesis of storage proteins, desiccation tolerance and promoting dormancy through inhibition of seed germination (Finkelstein et al 2002). In the plant as a whole, the key adaptive responses include induction of stomatal closure as well as control of seedling growth and lateral root formation. While the balance between ABA and auxin levels slightly affects primary root growth, ABA represses lateral root formation while auxin promotes it (Wasilewska et al 2008, Zhao et al 2014).

Inhibition of seed germination under abiotic stress is another function of ABA. Seed germination occurs when levels of germination promoting Gibberellin are high and levels of dormancy promoting ABA are low. During late stages of maturation seeds accumulate ABI5 which in turn activates transcription of LATE EMBRYOGENESIS ABUNDANT (LEA)

proteins. LEA proteins confer osmotolerance to the embryo. ABA is necessary for activation of ABI5 and SnRK2.2 and SnRK2.3 phosphorylate ABI5 (Finkelstein and Lynch 2000, Lopez-Molina et al 2001). When seeds are in unfavourable environmental conditions, elevated endogenous ABA levels results in ABI5 accumulation preventing seeds from germinating.

1.2.4.1.1 ABA and salt tolerance

Salt stress severely impacts plant growth by affecting metabolic processes and photosynthetic efficiency. NaCl initially induces osmotic stress and eventually accumulation of both Na^+ and Cl^- ions generates ionic stress (Tester and Davenport 2003). However plants have evolved Na+ and osmotic stress sensors that identify the stress and response accordingly. Some responses are salt specific and distinct from responses to osmotic stress (reviewed in (Deinlein et al 2014)). High salinity in the soil is first sensed by the plant roots and salt and drought stress induce a rapid increase in cytosolic Ca^{2+} levels in the root cells. Ca^{2+}acts a second messenger inducing salt and drought responsive gene expression (Knight et al 1997, Tracy et al 2008). Hyperosmotic stress is coupled with Ca^{2+} signalling and Reactive Oxygen Species (ROS) signalling, thereby inducing a cascade of signalling events which results in downstream gene expression (Deinlein et al 2014).

Biochemical and molecular mechanisms of salt tolerance in plants include exclusion of salt ions, production of suitable osmolytes, changing the structure of the membranes to control ion uptake, and induction of enzymes that produce antioxidants and phytohormones. To manage salt or drought stress, cellular ABA levels increase dramatically. Plant cuticle has been shown to mediate stress signalling as well as ABA biosynthesis and signalling. In addition to its primary function providing mechanical support to the cell wall and plasma membrane, cuticle has been implicated in osmotic stress regulation. CED1 (9-CIS EPOXYCAROTENOID DIOXYGENASE

DEFECTIVE 1) is an essential protein in cuticle biogenesis and *ced1* mutants were sensitive to osmotic stress as cuticle mutants are unable to induce ABA biosynthesis in response to osmotic stress (Wang et al 2011).

ABA regulates root growth and architecture in plants under stress. Duan et al (2013) showed that salt has a strong inhibitory effect on lateral root growth while primary roots are less sensitive to salt stress. They also showed that endogenous ABA signalling affects root system architecture under stress conditions using ABA biosynthesis mutants (*aba1, aba2*) as well as signal transduction mutants such as *abi1*. Salt stress results in elevated levels of ABA exclusively in lateral root cells and induces a quiescent period in post-emergence lateral roots. Lateral roots in a quiescent stage forms a thick well developed Casparian strip which acts as a barrier to reduce diffusion of Na^+ ions through the endodermis. In the presence of Na^+ ions endodermal cells activate ABA signalling and arrest growth so that lateral roots do not elongate into high saline environments. Therefore ABA is an important signalling molecule in suppressing lateral root growth during salt stress (Duan et al 2013).

ABA regulates expression of many salt stress responsive genes via transcription factors that are elevated in response to salt. For instance ABF2/AREB1, ABF3, ABF4/AREB2, ABP9 and MYC/MYB, WRKY, AP2/ERF are some of the salt stress responsive transcription factors that enhance stress tolerance (Golldack et al 2014). A recent study shows that the PYL8/RCAR3 ABA receptor has a role in ABA mediated inhibition of primary root growth and also recovery of lateral root growth following exposure to ABA. PYL8/RCAR3 combines the action of ABA and auxin through direct interaction with MYB transcription factors, in growth recovery of post-emergence lateral roots (Antoni et al 2013, Zhao et al 2014).

There are proteins in the cell that are produced in an ABA dependent manner that have a role in osmotic tolerance. LEA proteins are highly hydrophilic small proteins identified to have an osmoprotectant role against cellular dehydration during late embryogenesis. LEA proteins have a role in salt stress tolerance (Bhardwaj et al 2013). Due to the hydrophilic nature, LEA proteins can sequester ions accumulating in the cell as well as act as chaperones and retain water molecules to prevent protein aggregation and inactivation of cellular enzymes (Marco et al 2015). In Arabidopsis 51 LEA proteins have been identified that belongs to nine different groups (Hundertmark and Hincha 2008). Jia et al (2014) showed overexpression of AtLEA14, which belongs to the LEA group 2 proteins, overactivates salt stress inducible genes such as *RD29B*, which are dehydration protective proteins, and subsequently confers salt tolerance in Arabidopsis.

In addition, ABA has been implicated in affecting histone H3 acetylation and methylation and thereby regulating stress inducible gene expression. Chen et al (2010) showed that histone modifications by HISTONE DEACETYLASE 6 (HDA6) in Arabidopsis are involved in inhibition of seed germination, salt stress responses and ABA and salt mediated gene expression in Arabidopsis.

1.2.4.1.2 ABA and drought tolerance

Drought is lack of water in the soil. Drought stress in plants arises due to water deficit conditions and results in removal of water from the cell membranes disrupting the lipid bilayer structure. In addition, protein denaturation and accumulation of cellular electrolytes results in disruption of cellular metabolism (Mahajan and Tuteja 2005). Therefore, drought causes osmotic stress, and osmotic stress causes dehydration and inhibition of water uptake in plants. ABA accumulates under osmotic stress conditions and plays an important role in the stress response

and tolerance of plants. In addition to autoactivation of SnRK2s by inhibition of PP2Cs in the ABA signalling cascade, hyperosmotic stress activates SnRKs (Monks et al 2001). SnRK2 kinases are a major component of the osmotic stress signalling pathway. The Arabidopsis triple mutant *snrk2.2 snrk2.3 snrk2.6* shows severe drought intolerance and ABA insensitivity (Fujii et al 2011). Also ABF2, ABF3 and ABF4 act as transcriptional activators in mediating ABRE dependent ABA signalling which, confers drought tolerance in vegetative tissues (Choi et al 2000).

ABA induces expression of many transcription factors and genes that encode products that act as enzymes in the synthesis of osmoprotectants (Daszkowska-Golec and Szarejko 2013). Osmolytes are compatible solutes such as amino acids (proline), sugar alcohols (mannitol, pinitol) and other sugars that accumulate without disrupting the function of proteins. Enzymes that produce osmolytes makes an osmotic adjustment facilitating a favourable water potential gradient and promote stress tolerance (Bray 1997).

Dehydrins and LEA-like proteins act as cellular chaperones that protect cellular membranes and macromolecules in the cell (Shinozaki and Yamaguchi-Shinozaki 2007). During seed maturation seeds undergo dehydration stress. LEA proteins accumulate in the embryo as a result of osmotic stress and their functions include protection of enzymes, lipids and mRNAs from dehydration. LEA proteins have been found to protect mitochondrial membranes from damage. LEA proteins are produced in an ABA dependent and ABA independent manner under osmotic stress (Bhardwaj et al 2013, Bhattacharjee and Saha 2014).

Under moderate water stress conditions plant root growth has to be maintained in order to keep the plants alive. ABA accumulates under moderate water stress and mediates auxin transport in the root tip, which enhances the proton pumps in the plasma membrane. Proton

secretions in the root tip play an important role in primary root growth and root hair development under moderate drought stress (Xu et al 2013a).

Based on the critical water level, drought tolerance is considered as mechanisms that confer tolerance to moderate dehydration. Further dehydration requires desiccation tolerance mechanisms in order to restore the ability of cells to rehydrate successfully (Hoekstra et al 2001).

1.2.4.1.3 ABA and desiccation tolerance

Water loss results in a change in turgor pressure that affects the cell walls. Desiccation tolerance is defined as evolution of cell walls that can withstand extensive water loss without damaging its structure or polymer organization. Desiccation tolerance mechanisms aim to restructure the cells walls and maintain normal growth under water stress conditions (Moore et al 2008).

Regulation of the stomatal pore is crucial in adapting plants to abiotic stress by reducing extensive water loss. Stomatal opening and closing occurs as a result of turgor pressure differences in the surrounding guard cells (Bhattacharjee and Saha 2014). In response to water stress, ABA concentration is increased in the guard cell cytoplasm and in the apoplast which results in a decrease in the turgor pressure due to activation of the K^+ outward rectifying channel and inhibition of the K^+ inward rectifying channel (KAT1 and 2). ABA also induces the anion channel SLAC1 resulting in release of anionic organic acids from the vacuole to the cytoplasm (Sirichandra et al 2009b). Reduced turgor pressure initiates closure of stomata as a mechanism of minimizing water loss from the plant. ABA levels rise in leaves immediately following water stress. CHLH/ABAR has been proposed as the chloroplast ABA receptor that links ABA

signalling within the chloroplast with ABA signalling in the nucleus. Overexpression of CHLH promotes stomatal closure and thereby dessication tolerance (Tsuzuki et al 2013).

SnRK2 OPEN STOMATA 1 (OST1) is a key SnRK2 protein kinase involved in regulation of the stomatal aperture by movement of guard cells during ABA signalling (Sirichandra et al 2009a). OST1 is activated by ABA, low humidity and osmotic stress and is an important kinase found in guard cells preventing rapid water loss. Loss of function mutants of SnRK2 do not exhibit ABA mediated stomatal closure activity and showed a wilty phenotype under dehydration stress conditions (Yoshida et al 2002, Sirichandra et al 2009a). Also SnRK2.6/OST1 physically interacts with ABI1 and 2 and ABI1 is required for ABA dependent activation of OST1 and both ABI and 2 are required for osmotic stress induced activation of OST1 (Yoshida et al 2006). Thus, SnRK2.6/OST1 acts as a positive regulator in the ABA induced stomatal closure. Moreover Yoshida et al (2002) showed that OST1 also positively regulates stress responsive genes such as RD29B and RD22.

Reactive Oxygen Species (ROS) has been identified as secondary messengers in ABA signalling in guard cells. In Arabidopsis two partially redundant guard cell expressed NADPH oxidase catalytic subunit genes, *AtRbohD* and *AtRbohF,* were found to be involved in ABA signalling in guard cells, ABA induced stomatal closure and ROS production, ABA activation of Ca^{2+} permeable channels in the plasma membrane of guard cells and increasing cytosolic Ca^{2+} levels in response to ABA. Thus, these two genes act as positive regulators of ABA signal transduction (Kwak et al 2003). Sirichandra et al (2009a) provided biochemical evidence that OST1 protein kinase physically interacts with AtRbohF NADPH oxidase and phosphorylates it.

1.2.5 Conclusions

ABA has a wide range of functions from plant development to biotic and abiotic stress signalling and tolerance. This review summarises the primary functions of ABA in salt, drought and desiccation tolerance through inhibition of seed germination, altering the root architecture, inducing stress responsive genes and gene products that act as osmoprotectants. ABA signalling cascades and stress tolerance mechanisms studied in Arabidopsis provide insight into application of stress tolerance strategies to commercial crops. While ABA is not the only plant hormone involved in stress responses, many of these responses occur in an ABA dependent manner, indicating the importance of ABA in plant stress response and tolerance.

1.3 Light and abscisic acid in plant development

Light and ABA both play critical roles in plant development. In several instances they both act on the same process. For example, both light and ABA inhibit hypocotyl elongation (Chen et al 2008, Lau and Deng 2010). Light, in general, promotes flowering while ABA delays flowering under normal conditions but promotes flowering in unfavourable conditions. Light promotes stomatal opening while ABA induces stomatal closure. Finally, light promotes germination while ABA inhibits germination via promoting dormancy (Finkelstein 2013). Seed germination is the main focus of this thesis and is discussed in more detail below.

1.3.1 Seed germination and dormancy

Seed germination is defined as testa (seed coat) rupture, followed by endosperm rupture, and eventually the emergence of the radicle (Finch-Savage and Leubner-Metzger 2006). Seed germination is a critical step for plant survival and the seed possesses the potential to determine whether or not to respond to the light and other germination initiation signals. Light and hormonal signals provide important cues to control the timing of seed germination.

Seed dormancy is a phenomenon that hinders germination of a viable seed. A dormant seed does not have the potential to germinate for a certain period of time, even if the environmental conditions are favourable. Both genetic and environmental factors contribute to seed dormancy. The plant hormones GA and ABA play important roles in regulating dormancy. Light, temperature and imbibition are some factors that release dormancy and promote seed germination (Finch-Savage and Leubner-Metzger 2006).

1.3.1.1 Role of hormones in seed dormancy and germination

Certain genes activated by plant hormones either promote or suppress seed germination. In general, the role of phytohormones in regulating seed germination is regarded as a balance between the two antagonistic hormones, namely the germination promoting hormone GA and the inhibiting hormone ABA (Miransari and Smith 2014). The roles of these hormones are tightly linked to environmental conditions where favourable conditions induce seed germination whereas unfavourable stress conditions repress it.

ABA levels are high in mature seeds and ABA is essential for maintaining dormancy and seed osmotolerance during embryogenesis (Finkelstein and Lynch 2000). ABA signalling component ABA INSENSITIVE 3 (ABI3) is an important regulator of seed dormancy and ABI3 positively regulate *ABI5* transcription. Thus, ABI5 acts downstream of ABI3 and is an important positive regulator of ABA signalling. In response to ABA, ABI5 represses seed germination in non-dormant seeds. Upon imbibition, ABA is broken down and gradually decreased in seeds, breaking dormancy (Lopez‐Molina et al 2002, Penfield and King 2009).

On the other hand, GA plays an important role in breaking seed dormancy and initiation of seed germination. DELLA proteins, which are negative regulators of GA signalling, repress seed germination and plant growth (Penfield and King 2009). GA levels in dry seeds gradually increase with imbibition and inhibit DELLA proteins. GA biosynthesis, as well as activation of GA signalling genes by light, promotes seed germination as well as maintains the ABA levels low during seed germination. Increased GA levels may result in cell elongation during radical emergence and testa rupture (Penfield and King 2009, Seo et al 2009).

There is a tight interaction between light, GA and ABA signalling pathways in seeds at the molecular level. Light regulates the concentrations of GA and ABA, and also GA

responsiveness by phytochromes. Phytochromes control seed germination by inducing the degradation of PIF1/PIL5. PIF1/PIL5 is involved in regulation of both ABA and GA metabolism. The amount of GA required for seed germination is less when endogenous ABA levels are low. The balance between ABA catabolism and GA biosynthesis in response to environmental conditions determines whether the seeds will germinate or not (Seo et al 2009).

1.3.1.2 Role of light in seed germination

Seed germination is very light sensitive and is induced by red (R) light. Variations in the R:FR light ratio affect the initiation of seed germination (Shinomura et al 1994). Seed germination is generally induced by R and inhibited by FR, termed the R/FR photoreversible low fluence response (LFR). In Arabidopsis, phyB is the predominant regulator of initiation of seed germination in LFR. phyA promotes germination via the FR high irradiance response (longer FR irradiation) and the very low fluence response (very short light pulse) to a wide range of wavelengths in seeds imbibed for longer periods in the dark in the absence of phyB. In addition, phyE plays a secondary role in seed germination (Mathews 2006, Seo et al 2009).

Phytochromes control seed germination by inducing the degradation of PIF1/PIL5. PIF1/PIL5 is negative regulator of germination involved in regulation of both ABA and GA metabolism and signalling. *pil5* mutants germinate well in all light conditions whereas PIF1/PIL5 overexpression results in inhibition of germination. In the dark (Figure 1.8),

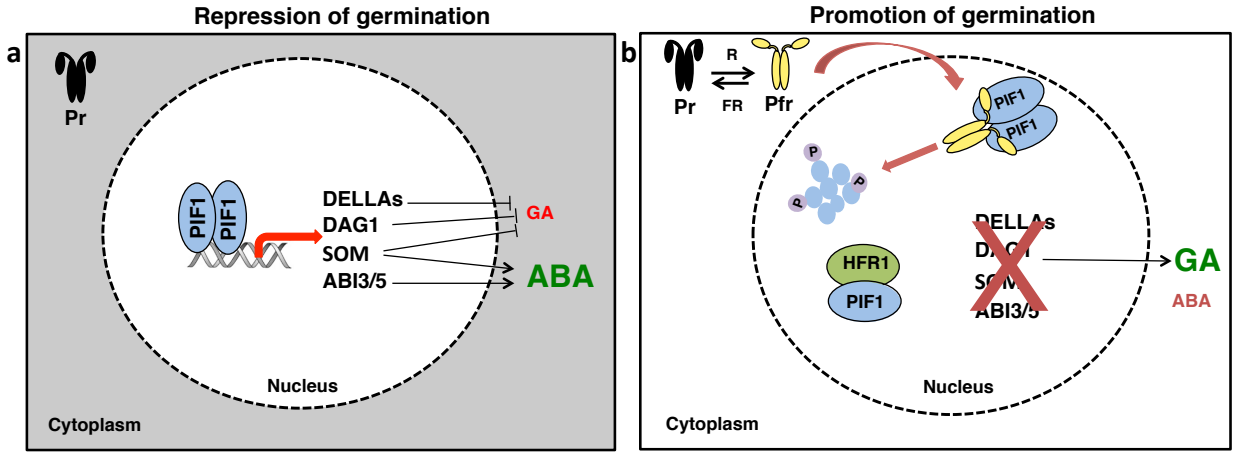

Figure 1.8 Regulation of seed germination by PIF1

(a) In the dark PIF1/PIL5 upregulates transcription of GA-inhibiting and ABA-promoting genes, resulting in repression of seed germination. (b) In the light, the activated Pfr form of phytochrome translocates to the nucleus and mediates degradation of PIF1. Also PIF1 is bound by HFR1, inhibiting its function. In the absence of PIF1, ABA synthesis is not stimulated and GA-inhibiting gene expression is not enhanced. Thus, GA accumulates while ABA levels decline, resulting in promotion of seed germination.

PIF1/PIL5 represses seed germination via activating *DELLA* genes, thus inhibiting GA signalling. In addition, PIF1/PIL5 activates transcription of the transcription factor DOF AFFECTING GERMINATION 1 (DAG1), which represses transcription of GA biosynthesis genes. PIF1/PIL5 also promotes the transcription of *SOMNUS* (*SOM*), which represses GAbiosynthesis and promotes ABA biosynthesis. In addition, PIF1/PIL5 activates transcription of *ABI3* and *ABI5*, thus promoting ABA signalling. Thus, in the dark PIF1 represses GA biosynthesis and signalling as well as activates ABA biosynthesis and signalling, inhibiting germination. In the presence of light, phytochromes interact with PIF1/PIL5, leading to its degradation. In the absence of PIF1/PIL5, repression of GA biosynthesis and signalling is relieved, ABA response is not promoted, and germination occurs (Oh et al 2004, Seo et al 2009, de Wit et al 2016).

Phytochromes also positively regulate HFR1, a positive regulator of seed germination. HFR1 directly interacts with PIF1 and prevents it from binding into its target genes, thereby downregulating transcription of PIF1 dependent genes (Shi et al 2013). The COP1-SPA complex as well as the CDD-CUL4 complex, of which DET1 is a component, targets HFR1 for degradation in the dark. In the light, phytochrome mediated inhibition of the COP1 and CDD complexes allows HFR1 to accumulate and inhibit PIF1 (Figure 1.8). In addition, DET1 and COP10 directly interact with PIF1, preventing its degradation and promoting PIF1 stability (Shi et al 2015). Thus DET1 directly regulates both positive (HFR1) and negative (PIF1) regulators of seed germination. In the dark, DET1 degrades HFR1 and stabilizes PIF1, repressing seed germination. In the light, DET1 is somehow inactivated, resulting in increased HFR1 and decreased PIF1, inducing seed germination. Thus DET1 is not only a central repressor of photomorphogenesis but also a central repressor of seed germination (Shi et al 2015).

In addition to light and hormones, all seeds need sufficient amounts of water/moisture for initiation of seed germination. Seeds require water in order to expand and elongate the seed embryo. Thus a seed first imbibes water and allow the radicle to grow inside the seed coat (Miransari and Smith 2014). Initiation of seed germination requires a minimum temperature that triggers the germination signal. Also negative temperatures are very harmful to seeds resulting in frost injuries in the embryo. Imbibition can still occur under low temperatures but damaged embryos prevent completion of seed germination. However the temperature range that is optimum for seed germination differs greatly with the species (Bradbeer 2013, Miransari and Smith 2014).

1.3.2 Role of HY5 in ABA signalling

In addition to its role in light signalling HY5 is also implicated in hormone signalling. HY5 exhibits important roles in ABA signalling during seed germination, seedling growth and root development. *hy5* and *abi5* seeds have reduced dormancy relative to their wild type Col and Ws respectively, indicating that HY5 and ABI5 are involved in regulating seed dormancy (Chen and Xiong 2008). HY5 binds to the promoter of *ABSCISIC ACID INSENSITIVE 5 (ABI5)* and enhances its expression. *hy5* mutants show defects in ABA responses and have very low *ABI5* mRNA levels. ABI5 regulated genes were also downregulated in *hy5*. Thus, HY5 regulates seed germination and seedling growth in response to ABA via ABI5 and positively regulates ABA signalling. In addition, ABI5 overexpression results in suppression of hypocotyl elongation in light suggesting that HY5-ABI5 is involved not only in ABA signalling but also in light mediated photomorphogenesis (Lau and Deng 2010, Chen et al 2008).

1.3.3 CUL4 E3 ligases in ABA signalling

Ubiquitination regulates ABA signalling at a variety of stages, from ABA perception to expression of downstream ABA responsive genes, targeting proteins for degradation. Several CUL4-DDB1 complexes have been implicated in ABA signalling (Stone 2014). DDB1 acts as an adapter in CUL4-DDB1 E3 ligases, allowing CUL4 to interact with a number of substrate receptors (DWDs or DCAFs). Different DCAFs form CUL4 E3 ligase complexes targeting substrates for degradation, thereby regulating ABA mediated functions in various developmental pathways (Table 1.1).

ABI5 interacts with the DWD proteins, DWA1 and DWA2 (DWD proteins hypersensitive to ABA, 1 and 2, respectively). DWA1 and DWA2 act as substrate receptors for a

CUL4 E3 ubiquitin ligase that targets ABI5 for degradation. DWA1 and DWA2 directly interact with each other and their double mutants show enhanced hypersensitivity to ABA and NaCl (Lee et al 2010). Thus, DWA1 and DWA2 act together as negative regulators in ABA signal transduction.

Another DWD protein, DWA3, also interacts with CUL4-DDB1 and has a negative role in ABA signalling. However, DWA3 does not interact with either DWA1 or DWA2. Although ABI5 and other ABA responsive transcription factors accumulate in *dwa3* mutants, no direct interaction was detected between DWA3 and these proteins. Hence, it was suggested that DWA3 acts upstream of DWA1 and 2, possibly repressing a negative regulator of DWA1 and 2, thereby negatively regulating ABA signalling (Lee et al 2011).

Another DDB1-CUL4 substrate receptor associated with negative regulation of ABA signalling is ABA HYPERSENSITIVE DCAF 1 (ABD1). ABD1 also directly interacts with ABI5 in the nucleus, leading to proteasome mediated degradation. ABD1 is involved in the regulation of a number of ABA mediated responses, including seed germination, seedling growth, stomatal closure, drought tolerance and also modulates ABA responsive gene expression (Seo et al 2014).

DET1-DDB1 ASSOCIATED 1 (DDA1) interacts with the CDD complex to target the ABA receptor PYL8 for degradation. DDA1 interacts directly with DDB1a in Arabidopsis and associates with the CDD complex via DDB1, as DDA1 does not show direct interaction with COP10 or DET1. Upon interaction of DDA1 with PYL8, the CUL4-CDD-DDA1 E3 ligase ubiquitinates PYL8 and it is degraded via the 26S proteasome pathway. Thus, by modulating the stability of the ABA receptor this CUL4 complex negatively regulate ABA signalling (Irigoyen et al 2014).

Thus seed germination is a complex process regulated by light, hormones and a number of other factors. In this study I investigated the role of light signalling component DET1 during seed germination and the effect of ABA, salt, and osmotic stress on these responses.

Chapter 2: Genetic interactions between *DET1* and intermediate genes in Arabidopsis ABA signalling

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2. Genetic interactions between *DET1* and intermediate genes in Arabidopsis ABA signalling

2.1 Abstract

Seed germination is regulated positively by light and negatively by the dormancy-promoting phytohormone abscisic acid (ABA). DE-ETIOLATED 1 (DET1) is a negative regulator of light signalling in *Arabidopsis thaliana*. In contrast, the bZIP transcription factor LONG HYPOCOTYL 5 (HY5) is a positive regulator of light signalling. HY5 also positively regulates ABA signalling by promoting the expression of *ABA INSENSITIVE 5 (ABI5),* a germination inhibiting transcription factor. Here we show that germination in *det1* mutants is sensitive to ABA. Double mutant analysis indicates that *det1* ABA sensitive germination requires *HY5* and *ABI5*. DET1 forms a complex with DAMAGED DNA BINDING protein 1A/B (DDB1A/B). Another DDB1 complex containing DWA1 and 2 (DWD hypersensitive to ABA 1/2) has also been shown to negatively regulate ABA response. Double mutant analysis indicates that *DWA1, DWA2, DDB1A,* and *DDB1B* are also required for the *det1* ABA sensitive germination phenotype. We also examined water loss in adult plants and found that the *det1* rapid water loss phenotype is independent of *HY5, ABI5, DWA1, DWA2,* and *DDB1B*. These findings provide insight into interactions between ABA and light signalling in Arabidopsis.

2.2 Introduction

Plant development is sensitive to various external factors such as light, temperature, water and gravity. These environmental signals interact with endogenous hormonal cues resulting in physiological changes during plant development. Among external stimuli, light has an especially important role in photosynthesis, chloroplast biogenesis, differentiation of the leaf meristem, floral induction and coordination of light regulated genes. Seedlings grown in the dark have a developmentally arrested phenotype with elongated hypocotyls, small folded cotyledons and undeveloped chloroplasts and are referred to as etiolated. In contrast, light grown seedlings exhibit short hypocotyls, open cotyledons and chloroplast development (Chen and Chory 2011, Lau and Deng 2012).

Light dependent seedling growth or photomorphogenesis is controlled by several photoreceptor systems. In plants, light signals are perceived by photoreceptors and passed on to a complex network of downstream components and signalling intermediates. Genetic screens have identified two types of photomorphogenetic mutants, namely light insensitive mutants in photoreceptors and transcription factors promoting photomorphogenesis (e.g. LONG HYPOCOTYL 5 - HY5) and constitutive photomorphogenic mutants (*constitutive photomorphogenic (cop) / de-etiolated (det) / fusca (fus))* that resemble light grown plants when grown in complete darkness. The COP/DET/FUS group of proteins are central repressors of photomorphogenesis involved in degradation of positive regulators. COP1 is a WD40 RING finger protein and E3 Ubiquitin ligase whose nuclear localization is regulated by light. In the dark, COP1 targets HY5 and other transcription factors for degradation which in turn promotes dark growth (Osterlund et al 2000, Huang et al 2014).

DET1 encodes a 62 kDa nuclear protein (Pepper et al 1994). DET1 interacts with

DAMAGED DNA BINDING PROTEIN 1A/B (DDB1A/B) and COP10 to form the CDD complex (Schroeder et al 2002, Yanagawa et al 2004, Ganpudi and Schroeder 2013). In addition, DET1 is involved in transcription regulation (Lau et al 2011) and binds histone 2B (H2B) (Benvenuto et al 2002). *det1* mutants exhibit a number of pleiotropic defects and *hy5* is able to rescue most of these phenotypes either partially or completely indicating that *hy5* is epistatic to *det1* (Chory 1992, Pepper and Chory 1997). *det1* has increased levels of HY5 protein indicating that DET1 is a negative regulator of HY5 level (Desai et al 2014, Osterlund et al 2000). DET1 regulation of HY5 level in the dark may be indirect since DET1 is required for nuclear localization of the COP1 E3 ligase (von Arnim et al 1997).

DDB1 is a conserved protein which forms CULLIN4 (CUL4) based E3 ligase complexes. CUL4-DDB1 complexes have diverse functions in plants in regulation of development and physiology, with target specificity determined by which DWD (DDB1 binding WD40) or DCAF (DDB1-CUL4-associated factor) is a component of the complex (Biedermann and Hellmann 2011, Lee et al 2008). Arabidopsis has two DDB1 homologues, DDB1A and DDB1B. Single mutants of *ddb1a* and viable weak *ddb1b* alleles lack obvious developmental phenotypes, while *ddb1a ddb1b* double mutants are embryo lethal, verifying the developmental importance of DDB1 (Schroeder et al 2002, Bernhardt et al 2010, Ganpudi and Schroeder 2013). In addition to DET1, CUL4/DDB1 also interacts with COP1 (Chen et al 2010).

The phytohormone abscisic acid (ABA), initially known as a leaf abscission and seed dormancy promoting sesquiterpenoid, plays an essential role in plant growth and development. In addition, ABA acts as an endogenous messenger in stress signal transduction pathways (Raghavendra et al 2010). In general, the role of phytohormones in regulating seed germination is regarded mainly as a balance between the ratios of two antagonistic hormones, namely

germination promoting gibberellin (GA) and germination inhibiting ABA (Seo et al 2009). Genetic studies of ABA regulation of gene expression and seed germination have identified a number of Arabidopsis mutants with altered ABA sensitivities (Hauser et al 2011, Lau and Deng 2010). Of particular relevance to this study are *ABSCISIC ACID INSENSITIVE 5 (ABI5)*, *DDB1 BINDING WD40 PROTEIN 1* and *2 (DWA1* and *DWA2)*, and *HY5*.

ABI5 is a ABA responsive basic leucine zipper transcription factor involved in ABA signalling during seed germination and seedling development. *abi5* mutants have decreased sensitivity to ABA inhibition of germination and altered expression of ABA-regulated genes. ABI5 regulates a subset of late embryogenesis-abundant genes during both seed and vegetative developmental stages (Finkelstein and Lynch 2000).

ABI5 interacts with the DWD proteins (DDB1-binding WD40 proteins) DWA1 and DWA2. DWA1 and DWA2 are substrate receptors for a DDB1-CUL4 E3 ubiquitin ligase that targets ABI5 for degradation. DWA1 and DWA2 directly interact with each other and their double mutants show enhanced hypersensitivity to ABA (Lee et al 2010).

HY5 encodes a basic leucine zipper transcription factor that positively regulates light signalling. Chen et al (2008) demonstrated that binding of HY5 to the promoter of *ABI5* upregulates the transcription of *ABI5*. Thus, HY5 integrates both ABA and light signal transduction pathways through direct activation of *ABI5* transcription (Chen et al 2008).

Studies in Arabidopsis have shown that HY5 is a positive regulator of the ABA signal transduction pathway (Chen et al 2008), while DWA1 and 2 act as negative regulators (Lee et al 2010). In this study, we investigated the potential role of DET1 in ABA signalling and the genetic interactions between *DET1* and genes (*HY5*, *DWA1*, *DWA2* and *ABI5*) that play important roles in the integration of light and ABA signalling.

2.3 Materials and Methods

2.3.1 Plant materials and growth conditions

All *Arabidopsis thaliana* mutants in this study are in the *Columbia-0* ecotype except for *abi5-1,* which is in the Ws-2 background (Finkelstein and Lynch 2000). *det1-1, cop1-4, ddb1a, ddb1b, ddb2* and their respective *det1* double mutants are as previously described (Chory et al 1989, Schroeder et al 2002, McNellis et al 1994, Al Khateeb and Schroeder 2007, Ganpudi and Schroeder 2013, Ly et al 2015). The *hy5* (SALK_096651C) (Chen et al 2008), *dwa1-1* (SALK_051022C) and *dwa2-1* (SALK_034658C) (Lee et al 2010) T-DNA insertion mutants and *abi5-1* (CS8105) point mutant 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 (except for germination assays, which used 0% sucrose) and 0.86% Phytoblend (Caisson) and were stratified at 4^0C for 2 days. The plates were then transferred to a growth chamber at 20^0C and 50% relative humidity for 14 days. Long day conditions (16 hrs of light and 8 hrs of dark) were provided by fluorescent bulbs (100 μM photons m^{-2} sec^{-1}). 14-day-old seedlings were transplanted to Sunshine mix number 1 (SunGro, Bellevue,WA).

2.3.2 Construction of double mutants in the *det1* background

All double mutants (*det1 hy5, det1 dwa1, det1 dwa2,* and *det1 abi5)* were generated using standard protocols (Weigel and Glazebrook 2002). Homozygous double mutants were identified based on their *det1* mutant phenotypes and PCR genotyping (Figure S2.1) using the oligonucleotide primers described below. HY5.1 (5'-ATTCCTTCCCAAAATGTCTCG-3') and HY5.2 (5'-ATGCGAGTGAATGACCATTTC-3') (Chen et al 2008) were used to detect the wild

type *HY5* allele while the *DWA1* wild type allele was detected using DWA1.1 (5'-GCTCTTGCACAGGAAACTTG-3') and DWA1.2 (5'-AATGTGTTGCTTCCCTTGATG-3') (Lee et al 2010). The following primer pair was used for the *DWA2* wild type allele DWA2.1 (5'-GCTCTTGCACAGGAAACTTG-3') and DWA2.2 (5'-AATGTGTTGCTTCCCTTGATG-3') (Lee et al 2010). T-DNA insertions were detected using LBb1.3 (5'-ATTTTGCCGATTTCGGAAC-3') in combination with one of the above primers for each genotype. ABI5.1 (5'-GGTTATTGTTGTGTATATGATGCAGTTG-3') and ABI5.2 (5'-CCACTACTCTTTTCCTTCCCC-3') were used to amplify the *ABI5* gene followed by digestion with AvaII to distinguish the *abi5-1* and wild type alleles (Bensmihen et al 2002). A Cleaved Amplified Polymorphic Sequence (CAPS) marker for *det1-1* (Pepper and Chory 1997) was used to confirm *det1* homozygotes in double mutants where the characteristic *det1* phenotype was not evident (for example *det1 hy5* double mutants).

2.3.3 Adult growth parameter measurements

For growth analysis of adult plants, the parameters measured were: flowering time, both in 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; as well as total number of inflorescences, silique length, and height at 6 weeks. Each adult growth assay was performed at least twice.

2.3.4 Seedling analysis

For hypocotyl analysis, seedlings were grown under either long day or dark conditions (after exposure to light for 6 hours to initiate germination) then scanned on a flat bed scanner

after 7 days. Hypocotyl lengths and cotyledon widths were measured using NIH Image J software (Schneider et al 2012). Chlorophyll measurements were done with 7 day old seedlings, 2 replicates per line with 20 seedlings each. Chlorophyll was extracted with 80% acetone overnight and A_{645} and A_{663} were measured using a Spectrophotometer (model 2100 pro, Ultrospec). Chlorophyll content was calculated according to the MacKinney method (Mackinney 1941). Anthocyanin was extracted using the standard method described in Fankhauser and Casal (2004). Seedling experiments were repeated at least twice for each double mutant.

2.3.5 Seed germination assays

In preparation for each germination experiment, plants were grown and seeds collected and stored in parallel. Sterilized seeds from each genotype were then sown on LS media, 0.86% Phytoagar and 0% sucrose supplemented with 0, 0.5, 2.5, or 5 μM ABA (Sigma). Plates were stratified at 4^0C for 2 days then transferred to 20^0C under long day conditions as described above. Seed germination was scored every 24 hours as a percentage of seeds with radical emergence (Bolle 2009) up to 5 days. In addition, cotyledon emergence and emergence of the first pair of true leaves were scored on day 10 and day 14, respectively. Germination assays for *cop1 det1* and *ddb1a det1* were done by identifying double mutants in a population of segregating heterozygotes because the double mutants are lethal and infertile, respectively. In addition, lower ABA concentrations (0.25 and 0.5 μM) were used for these assays so that doubles could be identified after cotyledon emergence.

For experiments involving transfer from control to ABA containing media, seeds were plated on filter paper on control media, stratified as above, then exposed to standard long day conditions. When all seeds of both wild type Col-0 and *det1* had germinated, but before any

cotyledon emergence was observed (36 h), filter paper with seeds was transferred from control to 5 μM ABA plates. Cotyledon emergence was scored daily following transfer.

2.3.6 Transpirational water loss assays

Water loss was measured using a method adapted 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 at room temperature on a weigh boat. The fresh weights were measured at 0, 1.5, 3, 4.5, and 6 hours. Water loss is expressed as percentage of weight loss versus initial fresh weight. Two replicates were used in each experiment per genotype and the experiments were repeated at least twice.

2.3.7 RNA extraction and Real time qPCR

RNA was extracted from approximately 50 dry seeds (experiment 1) or approximately 200 seeds imbibed in LS liquid media, 0% sucrose with or without 2.5 μM ABA for 2 days at 4^{o}C (experiment 2) 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 the RNA and cDNA samples were quantified using a Nano-drop spectrophotometer (Thermo Scientific). Real time qPCR was performed to detect *ABI5* transcript levels using ABI5.3 (5'-GCATATACAGTGGAATTGGA-3') and ABI5.4 (5'-CGGGTTCCTCATCAATGTCC-3') as the qPCR forward and reverse primers, respectively (Arroyo et al 2003). In addition, total RNA was extracted from 7-day-old seedlings (~50) using the same RNeasy plant mini kit. *DWA1* transcript levels were detected in wild type, *dwa2* and three different segregating *det1 dwa2* double mutant lines using DWA1.5 (5'-

ATTGACGGGTTCAGAGGATG-3') and DWA1.6 (5'-GTCCACAAACCAACCAGCTT-3') as forward and reverse primers, respectively. Both *ABI5* and *DWA1* transcript levels were calculated relative to housekeeping gene At5g60390 *EF1α* (5'-CTGGAGGTTTTGAGGCTGGTAT-3', 5'-CCAAGGGTGAAAGCAAGAAGA-3') (Jain et al 2006, Hossain et al 2012). Real time PCR was performed using a 40-fold dilution of cDNA in a 96-well plate using iQ SYBR Green Supermix (Bio-Rad). An iCycler equipped with iQ5 detection system (Bio-Rad) was used for the analysis.

2.3.8 Statistical analysis

A student's t-test was performed to statistically compare the single mutants with the appropriate wildtype control and the double mutants with *det1*. $P \leq 0.05$ was considered to be statistically significant. Each experiment was repeated at least three times for consistency and the results of a single representative experiment are shown.

2.4 Results

2.4.1 *det1* mutants are sensitive to ABA inhibition of germination

Due to evidence of interactions between light signalling and hormone response, we were interested in the role of DET1, a negative regulator of light signalling, in ABA mediated responses. Germination in *det1* has been previously shown to be light-independent (Chory et al 1989) but still requires GA (Nambara et al 1991). Consistent with light-independent germination, we find that *det1* exhibits early germination in control conditions (Figure 2.1a). However, as also described by Irigoyen *et al* (2014) we find that germination in *det1* mutants is hypersensitive

to ABA (Figure 2.1a). In contrast, cotyledon emergence in *det1* is resistant to ABA (Figure 2.1b).

There are several genes that interact either genetically or biochemically with *DET1* and have been implicated in ABA response. Previous studies suggest that HY5 acts as a positive regulator in the ABA signal transduction pathway (Chen et al 2008) while DWA1 and 2 act as negative regulators (Lee et al 2010). We hypothesized that in *det1* mutants there is an excess of HY5, which in the presence of ABA upregulates transcription of *ABI5,* thereby activating ABA signalling, resulting in less germination (Figure 2.2). In addition, DWA1 and DWA2 target ABI5 for degradation by acting as substrate receptors in a DDB1/CUL4 E3 Ubiquitin ligase complex. DWA1/2 and DET1 both form DDB1/CUL4 complexes and there are examples of genetic interactions between distinct DDB1 complexes such as DET1 and DDB2-containing (Al Khateeb and Schroeder 2007, Castells et al 2011). Thus genetic interactions between DET1 and DWA1/2 may be affecting the level of ABI5 and therefore germination. Therefore, our initial hypothesis is that ABA sensitive germination in *det1* is regulated by the abundance of ABI5 due to indirect effects via HY5 and/or DWA1/2 (Figure 2.2) (Note that this model is specifically for the *det1* ABA sensitive germination phenotype. The *det1* early control germination and ABA resistant cotyledon emergence phenotypes behave in the opposite way, that is have increased rather than decreased germination, therefore can't be explained by the model). In order to test this model, we generated *det1* double mutants with *hy5, abi5*, *dwa1*, and *dwa2* to assess the role of these genes in *det1* ABA sensitive germination.

2.4.2 *det1 hy5* phenotypes

hy5 has previously been shown to partially suppress *det1* phenotypes using *hy5-1,* a point mutation in the Landsberg background (Chory 1992), as well as with another allele (*ted5*), a point mutation in Columbia background (Pepper and Chory 1997). In this study we use a SALK T-DNA insertion allele, SALK_096651, in the Columbia background (Chen et al 2008). Therefore, to confirm the effect of this allele on *det1* phenotypes, we analyzed seedling and adult phenotypes of *hy5* and *det1 hy5* double mutants.

Consistent with previous studies, the *hy5* T-DNA allele partially rescues *det1* phenotypes, including the *det1* short hypocotyl phenotype in both light and dark, as well as reduced chlorophyll content in the light (Figure S2.2b,d,e). However, we did not see a significant effect on *det1* anthocyanin content in either light (Figure S2.2f) or dark (data not shown). In adults *hy5* partially suppresses the *det1* early flowering (days and leaves), reduced stature (rosette diameter, height, and silique length) and reduced apical dominance phenotypes (Figure S2.3). Thus the *det1 hy5* double mutant has an intermediate phenotype between *det1* and *hy5* consistent with other *hy5* alleles.

We then analyzed the effect of ABA on the germination of *det1 hy5* double mutant seeds and found that the *det1* ABA sensitive germination phenotype was suppressed in the double mutants (Figure 2.4a). Therefore, *det1* ABA sensitive germination phenotype requires *HY5*. *det1* early germination on control media was also suppressed in *det1 hy5* double mutants (Figure S2.4a), suggesting that HY5 contributes to this phenotype. In contrast, ABA resistant emergence of cotyledons and true leaves in *det1* was enhanced in the *det1 hy5* double mutants (Figure 2.4b).

2.4.3 *det1 abi5* phenotypes

abi5-1 is in the Wassilewskija (Ws-2) background thus we analyzed *det1* homozygotes with both mutant and wildtype versions of *ABI5,* derived from segregating F2 populations, in order to control for differences between the Ws-2 and Col-0 ecotypes. We then performed developmental analysis in order to test for genetic interactions between *det1* and *abi5.* With respect to dark hypocotyl length, neither *abi5* nor the Ws-2 background affected the *det1* short hypocotyl phenotype (Figure S2.5a). However, for many other phenotypes, including dark cotyledon width as well as adult rosette diameter, height, and silique length, variable modification of *det1* phenotypes was observed in both the *abi5* wildtype and mutant lines (Figure S2.5b-e), suggesting that modifier(s) of *det1* are segregating in the Ws-2 background but are independent of *ABI5*. Segregating F2 also exhibited increased flowering time (days) and decreased apical dominance (Figure S2.5f,g) independent of *abi5,* again indicating modifier(s) of *det1* in the Ws-2 background.

Despite the variation in developmental phenotypes, the role of *ABI5* in *det1* ABA sensitive germination is clear. While the Ws-2 background alone (light blue bars) did not significantly affect *det1* ABA sensitive germination, *det1 abi5* double mutants (pink bars) exhibit a highly germinative phenotype compared to the *det1* and *abi5* single mutants (Figure 2.5a and S2.6). Thus, *ABI5* is epistatic to *DET1* with respect to the ABA germination phenotype and *det1* ABA sensitive germination requires *ABI5*. On control media *abi5* did not affect *det1* early germination, however, this phenotype was suppressed in the Ws-2 background (Figure S2.6). True leaf emergence in *det1* and *abi5* on ABA occurred significantly earlier than in wildtype Col-0 and Ws-2, respectively, but the double mutants did not differ significantly from either single mutant (Figure 2.6b,c). In contrast, leaf emergence was delayed in *det1* in the Ws-2

background (blue bars) indicating that an unknown factor in the Ws-2 background suppresses *det1* ABA resistant leaf emergence.

2.4.4 *det1 dwa1/2* phenotypes

Since both DET1 and DWA1/2 form DDB1-CUL4 complexes and there are precedents for genetic interaction between DWD complexes (Al Khateeb and Schroeder 2007, Castells et al 2011), we analyzed developmental phenotypes of *det1 dwa1* double mutants to determine if *DWA1* interacts genetically with *DET1*. With respect to dark phenotypes, we found that the *dwa1* single mutant has significantly shorter hypocotyls than wild type in the dark, but did not affect apical hook angle (Figure S2.7a,b). In the *det1* background, *dwa1* had no significant effect on dark hypocotyl length but cotyledon width was decreased (Figure S2.7a,c). In light grown seedlings, the *det1* hypocotyl length and cotyledon width phenotypes were rescued by *dwa1,* while chlorophyll and anthocyanin content were not significantly affected (Figure S2.8, data not shown). In adult plants, *dwa1* single mutants show an early flowering phenotype both in terms of number of days and number of leaves (Figure S2.9a,b). In addition, *dwa1* enhanced the *det1* early flowering time phenotype in terms of number of leaves (Figure S2.9b). However, in terms of number of days, flowering time is slightly but significantly delayed in the *det1 dwa1* double mutant (Figure S2.9a). *dwa1* single mutants exhibit increased height, but *dwa1* did not have a significant effect on *det1* rosette diameter or height (Figure S2.9c,d). The *dwa1* single mutant also had an increased number of stems compared to wild type and variably enhanced stem number in the *det1 dwa1* double mutant (Figure S2.9e). In addition, *dwa1* resulted in an increased silique length phenotype in both the wildtype and *det1* backgrounds (Figure S2.10a). In the *det1 dwa1* double mutant, increased number of seeds per silique correlates with the

increased silique length (Figure S2.10b). Overall, these data suggest genetic interaction between *det1* and *dwa1*, specifically, *dwa1* suppresses the *det1* dark cotyledon width, light hypocotyl length and cotyledon width, early flowering time (days), silique length and seed number phenotypes and enhances the *det1* early flowering time (leaves) and decreased apical dominance phenotypes. In addition the *dwa1* single mutant exhibits previously undescribed reduced dark hypocotyl length, early flowering time (days and leaves), increased height and silique length and decreased apical dominance phenotypes.

We also generated *det1 dwa2* double mutants using the same *dwa2-1* allele used by Lee et al (2010) in order to assess *det1 dwa2* genetic interaction. Double mutant analysis of *det1 dwa2* seedling and adult phenotypes showed either no effect (dark and light cotyledon width, flowering time (leaves), and rosette diameter) or variable effects (dark and light hypocotyl length, flowering time (days), height, silique length, stem number) on *det1* phenotypes (Figure S2.11,12). Quantitative PCR analysis revealed that *DWA1* mRNA levels are upregulated in *dwa2* mutants but vary in the *det1 dwa2* mutant lines (Figure S2.12g). Thus the phenotypic variation in the *det1 dwa2* mutant lines might be due to variation in *DWA1* level. Alternatively, variation may be due to a segregating second insertion in the *dwa2* lines. *dwa2* single mutants exhibit several phenotypes in adult plants, including late flowering time (days and leaves), decreased rosette diameter and height, and increased silique length (Figure S2.12). Although the *det1 dwa2* developmental phenotypes show variability, their germination phenotypes are clear (see below).

The DWA1/2-DDB1-CUL4 complex degrades ABI5, negatively regulating ABA responses (Lee et al 2010), and our *det1 abi5* germination data indicates that ABI5 is required for the *det1* ABA sensitive germination phenotype. Thus, we analyzed *det1 dwa1* and *det1 dwa2* double mutants to investigate the role of *DWA1* and *DWA2* in *det1* ABA phenotypes. We

observed partial rescue of *det1* ABA sensitive germination in *det1 dwa1* and *det1 dwa2* (Figure 2.5a,6a), indicating that *DWA1* and *DWA2* are required for this phenotype. This result is unexpected however since we would predict *dwa1* and *dwa2* mutants to have increased levels of ABI5 upon ABA treatment and therefore less germination. On control media *dwa1* did not affect the *det1* early germination phenotype while *dwa2* variably enhanced it (Figure S2.4b,c). With respect to *det1* ABA resistant cotyledon emergence, *dwa1* had no effect, however cotyledon emergence was slightly decreased in *det1 dwa2* (Figure 2.5b, 6b), suggesting a role for *DWA2* in this phenotype.

2.4.5 Role of DDB1A, DDB1B and COP1

Our analysis so far indicates that the mechanism of *det1* ABA sensitive germination appears to be via HY5 and ABI5 with some contribution from DWA1 and DWA2. DET1 regulation of HY5 and therefore germination may be via the DET1/DDB1A, DET1/DDB1B, and/or COP1 complex. Thus we analyzed previously described *det1* double mutants with these three genes (Schroeder et al 2002, Ganpudi and Schroeder 2013, Ly et al 2015).

ddb1a did not affect *det1* early germination in control conditions, indicating that *DDB1A* is not essential for this phenotype (Figure 2.7a). However, the *det1* ABA sensitive germination phenotype was rescued in the *det1 ddb1a* double mutant, indicating that *DDB1A* is required for this phenotype. *ddb1a det1* double mutants also exhibited reduced levels of cotyledon emergence compared to *det1*, thus *DDB1A* is also required for the *det1* ABA resistant cotyledon emergence phenotype (Figure 2.7b).

With *ddb1b-2* (a relatively weak allele (Bernhardt et al 2010, Ganpudi and Schroeder 2013)), we observed partial rescue of *det1* ABA sensitive germination in low ABA

concentrations (Figure 2.8a) as well as partial rescue of *det1* ABA resistant cotyledon emergence (Figure 2.8b), indicating that *DDB1B* is involved in both these phenotypes. *det1 ddb1b* double mutants enhanced *det1* early germination in control conditions (Figure S2.4d) indicating that DDB1B acts with DET1, rather than against it, to regulate this phenotype.

In *det1 cop1-4* double mutants, *det1* early germination in control conditions was not only rescued, germination was in fact delayed (Figure 2.9a), indicating that *COP1* is required for *det1* early germination in control conditions. *cop1-4* single mutants initially exhibit ABA sensitive germination (day 2-3) but later germinate similar to wild type. Germination in *cop1-4 det1* double mutants is even more sensitive to ABA than *det1* (Figure 2.9a), thus the *det1* ABA sensitive germination phenotype is enhanced by *cop1-4*. Like *det1*, *cop1-4* single mutants also exhibit ABA resistant cotyledon and leaf emergence, however the *cop1 det1* double mutants exhibit delayed cotyledon emergence, and leaf emergence was never observed on either ABA containing or control media due to seedling lethality (Figure 2.9b). Thus both *DET1* and *COP1* are required for ABA resistant cotyledon emergence and viability in the other.

To summarize this section, *COP1* is required for *det1* early germination in control conditions, but *DDB1A* and *DDB1B* are not. *ddb1a* completely rescues and *ddb1b* partially rescues *det1* ABA sensitive germination, while *cop1* enhances this phenotype. *DDB1A, DDB1B* and *COP1* are all required for *det1* ABA resistant cotyledon emergence.

2.4.6 *ABI5 mRNA* level in *det1*

det1 seeds show an early germination phenotype on control media, so we analyzed mRNA levels of the germination inhibiting transcription factor ABI5. We examined *ABI5* mRNA levels in dry seeds using Real time qPCR and found that *det1* dry seeds have lower *ABI5*

mRNA level than wild type, consistent with the early germination phenotype (Figure 2.10a). We also examined *ABI5* mRNA levels in seeds imbibed in the presence or absence of ABA (Figure 2.10b) and found that the ABA-induced fold change in *ABI5* mRNA levels was nearly ten times higher in *det1* than in wild type, consistent with reduced germination of *det1* seeds on ABA.

2.4.7 Desiccation tolerance assays

In addition to germination, ABA also regulates desiccation tolerance. *det1* mutants desiccate very quickly and lose approximately 90% of fresh weight within 1.5 hours of leaf detachment at room temperature (Figure 2.11). This rapid water loss phenotype indicates that *det1* mutants have reduced drought tolerance. We performed transpirational water loss experiments with the *det1 hy5*, *det1 abi5, det1 dwa1, det1 dwa2,* and *det1 ddb1b* double mutants (we were unable to perform water loss assays on *ddb1a det1* and *cop1 det1* doubles because they are very tiny and lethal respectively). As shown in Figure 2.11, *det1 hy5, det1 abi5, det1 dwa1, det1 dwa2,* and *det1 ddb1b* double mutants did not differ significantly from *det1* with respect to water loss. Thus, the *det1* rapid water loss phenotype does not require *HY5, ABI5, DWA1, DWA2* or *DDB1B*.

2.5 Discussion

In this study, we investigated genetic interactions between *DET1* and intermediate genes in the ABA signalling pathway with respect to development and stress response.

2.5.1 Role of *HY5* in *det1* phenotypes

HY5 point mutants have previously been shown to be epistatic to *det1* (Chory 1992, Pepper and Chory 1997). In our study, using a *HY5* T-DNA allele (Chen et al 2008), we observed similar developmental interactions. HY5 has been implicated in DET1 regulation of *CAB2* gene expression (Maxwell et al 2003), photolyase gene expression (Castells et al 2010), and heat response (Delker et al 2014). Here we find that HY5 is required for *det1* ABA sensitive germination. In contrast to some of the developmental phenotypes examined, where *hy5* only partially rescues the *det1* phenotype, *hy5* is able to completely rescue the *det1* ABA sensitive germination phenotype. This suggests that HY5 homolog HYH (Holm et al 2002) is not acting redundantly in this instance.

In contrast to all other *det1* phenotypes examined, where *hy5* either suppressed or had no effect, *hy5* enhanced *det1* ABA resistant cotyledon and leaf emergence. Thus while DET1 generally acts a negative regulator of HY5, in this case they seem to be acting together. Alternatively, since *det1 hy5* germinates before *det1* on ABA, if the time between germination and cotyledon/leaf emergence was similar in the two genotypes, the net effect would be enhanced cotyledon/leaf emergence as observed. Nonetheless, *HY5* is not required for *det1* ABA resistant cotyledon emergence.

2.5.2 Role of *COP1* in *det1* ABA phenotypes

Our genetic analysis indicates that HY5 is required for *det1* ABA sensitive germination, consistent with previous studies implicating DET1 in negative regulation of HY5 level (Osterlund et al 2000, Desai et al 2014). COP1 also negatively regulates HY5 level when nuclear localized in the dark (Osterlund et al 2000). DET1 is required for COP1 nuclear

localization in the dark (von Arnim et al 1997), thus we wondered whether in this instance DET1 regulation of HY5 level and germination was indirect via COP1. Consistent with its role as a negative regulator of HY5, *cop1-4* single mutants also exhibited ABA sensitive germination. The *cop1-4 det1* double mutants exhibited enhanced levels of ABA sensitive germination relative to either single mutant, suggesting that DET1 and COP1 are acting additively rather than COP1 acting epistatically. *cop1* has previously been shown to enhance *det1* developmental phenotypes (Ly et al 2015, Ang et al 1994). With respect to cotyledon emergence, *cop1*, like *det1*, exhibits ABA resistant cotyledon emergence, yet the double mutant exhibits ABA sensitive cotyledon emergence. Thus *COP1* is required for *det1* ABA resistant cotyledon emergence.

2.5.3 Germination of *det1* mutants under control conditions

det1 exhibits early germination on control media. This finding is consistent with previously published phenotypes of *det1*, in which *det1* germination is light and phytochrome independent but still requires gibberellin (Chory 1992, Chory et al 1989, Nambara et al 1991). Recently Shi *et al* (2015) found that DET1 inhibits seed germination in the dark by stabilizing PHYTOCHROME INTERACTING FACTOR 1 (PIF1), a negative regulator of seed germination, and by degrading LONG HYPOCOTYL IN FAR RED 1 (HFR1), a positive regulator of seed germination. Thus *det1* mutants exhibit increased levels of HFR1 and decreased levels of PIF1, resulting in increased germination. We find that *det1* early germination on control media requires both HY5 and COP1, again consistent with previous studies (Chory 1992, Ang et al 1994). *dwa1, dwa2* and *ddb1a* do not show a significant effect on *det1* early germination on control media, whereas *ddb1b* enhances this phenotype. While *abi5* does not alter *det1* early

germination under control conditions, this phenotype is suppressed in the Ws-2 background, suggesting that modifier(s) of *det1* phenotypes are present in the Ws-2 background.

Gene expression analysis shows that mRNA levels of germination inhibiting transcription factor *ABI5* are low relative to the wild type in dry *det1* seeds and also in imbibed *det1* seeds, consistent with the *det1* early germination phenotype. However our genetic analysis indicates that *ABI5* does not have a role in germination of *det1* seeds in control conditions. Perhaps *det1 abi5* doubles are not significantly different from *det1* singles because *ABI5* levels are already low in *det1* and therefore absence of *ABI5* in the *det1 abi5* double mutant does not make any further difference in the germination phenotype.

2.5.4 Role of *ABI5* in *det1* ABA phenotypes

On ABA containing media, we predicted that the increased levels of HY5 in *det1* mutants upregulate transcription of *ABI5* and thereby activate the ABA signalling pathway. This hypothesis is supported by gene expression data (Figure 2.10) which indicate that while ABA results in an approximately 5 fold increase in *ABI5* mRNA level in wild type, an approximately 50 fold increase was observed in *det1*. In addition, our genetic analysis shows rescue of *det1* ABA sensitive germination in the *det1 abi5* double mutants, indicating that *ABI5* is required for this phenotype. Moreover, *det1* ABA sensitive germination is not only rescued in the *det1 abi5* double mutants but is now in fact ABA resistant, even more so than the *abi5* single mutant. Thus, while DET1 acts as a negative regulator of ABA signalling in wild type, in the absence of *ABI5* it acts as a positive regulator of ABA signalling, suggesting that ABI5 is not the only ABA signalling component downstream of DET1.

With respect to true leaf emergence, a very different story unfolds. ABA resistant true leaf emergence in *det1* mutants is not significantly altered in the *det1 abi5* double mutants, nor do the double mutants differ significantly from the *abi5* single mutants. Thus, ABA resistant true leaf emergence in *det1* mutants does not require ABI5, nor does ABA resistant true leaf emergence in *abi5* does require DET1. In contrast, *det1* ABA resistant true leaf emergence is completely suppressed in the Ws-2 background, suggesting that there is some component which varies between Col-0 and Ws-2 that is essential for *det1* ABA resistant true leaf emergence.

2.5.5 Genetic interactions between *DET1* and *DWA1/2*

Distinct DDB1-CUL4 complexes have been shown to influence each other. For example, the effect of *det1* on CUL4-DDB1/2 complexes has been shown by Castells et al (2011). *det1 ddb2* double mutants showed enhanced UV-C sensitivity in the dark relative to either single mutant indicating an additive effect with respect to nucleotide excision repair. In addition, Al Khateeb and Schroeder (2007) showed that *ddb2* effects *det1* phenotypes. Here our double mutant analysis indicates that *DWA1* and *DET1* also interact genetically. Specifically, *dwa1* modifies a subset of *det1* light and dark-grown seedling as well as adult phenotypes. In addition, *dwa1* single mutants have short hypocotyls in the dark relative to wild type, indicating *dwa1* may be involved in seedling etiolation response. However, *dwa1* does not affect dark hypocotyl length in the *det1* background, suggesting that DET1 is required for the *dwa1* short hypocotyl phenotype, therefore DET1 is epistatic to DWA1 with respect to dark hypocotyl length. Similarly, *dwa1* results in increased adult height in the wild type but not in *det1* background, suggesting that DET1 is also epistatic with respect to height. In contrast, *dwa1* results in decreased flowering time (leaves), decreased apical dominance and increased silique length in

both the wildtype and *det1* backgrounds, suggesting these are additive and independent phenotypes. Both *dwa1* and *ddb2* partially suppress the *det1* short silique and reduced seed number phenotypes. In *det1 ddb2* mutants this increased fertility appears to be due to partial rescue of the *det1* short stamen phenotype (Al Khateeb and Schroeder 2007); however no obvious difference in stamen length was observed in *det1 dwa1* mutants (data not shown).

We also examined genetic interactions between *dwa2* and *det1*. While overall the segregating *det1 dwa2* F2 lines exhibited variables phenotypes, the majority of lines did suppress the *det1* light hypocotyl length, apical dominance and silique length phenotypes, consistent with the effect of *dwa1* on *det1* phenotypes. While *dwa2* single mutants did not exhibit significant seedling phenotypes, adults were late flowering (days and leaves) with reduced height and rosette diameter. Interestingly, these are opposite to the phenotypes observed in *dwa1* single mutants. Our gene expression analysis indicates that *dwa2* mutants have increased *DWA1* mRNA levels, thus perhaps the *dwa2* phenotypes observed are due to excess *DWA1*.

2.5.6 *det1* ABA sensitive germination requires *DWA1/2*

Our developmental analysis indicates that genetic interactions occur between *DET1* and *DWA1/2* and therefore it is possible that genetic interactions also occur during germination. We find that both *dwa1* and *dwa2* result in partial rescue of the *det1* ABA sensitive germination, thus *DWA1* and *DWA2* are required for this phenotype. However, DWA1 and DWA2 target ABI5 for degradation, thus *dwa1/2* mutants would be expected to have higher levels of ABI5 following ABA treatment, resulting in less germination (Lee et al 2010). In contrast, the *det1 dwa1/2* double mutants exhibit more germination than *det1* alone on ABA. Perhaps the decreased abundance of functional DWA1/2-DDB1-CUL4 complexes liberates DDB1A/B to increase the

abundance of other DDB1 complexes, which negatively regulate ABA signalling. For example, the *det1-1* allele is a splice site mutation which still retains ~1% of wildtype message levels (Pepper and Chory 1997), thus perhaps decreased DWA1/2 allows more DDB1A/B to complex with the residual DET1. The partial rescue of *det1* developmental phenotypes by *dwa1* is consistent with this hypothesis. This model predicts that loss of function alleles of other unrelated DDB1 complex components might also affect *det1* germination. Consistent with this hypothesis, *det1 ddb2* double mutants also rescue *det1* ABA sensitive germination (Figure S2.12). Alternatively, other DDB1 complexes also negatively regulate ABA signalling. ABA-hypersensitive DCAF1 (ABD1)–DDB1–CUL4 complexes target ABI5 for degradation (Seo et al 2014). DWA3 forms DDB1-CUL4 complexes which negatively regulate ABA signalling (Lee et al 2011) In addition, the DET1-DDB1-associated protein 1 (DDA1) DDB1–CUL4 complex has also been shown to negatively regulate ABA signalling by targeting ABA receptors (PYR/PYL/RCAR) for degradation (Irigoyen et al 2014). Note that DET1's role in the DDA1 complex could also explain the *det1* ABA sensitive germination phenotype, however this is not consistent with the rescue by *hy5* of this sensitivity nor with the fact that DET1 acts as a positive regulator of ABA signalling in the absence of ABI5.

2.5.7 *det1* ABA sensitive germination requires *DDB1A/B*

If the above models are correct, that is, if *dwa1/2* rescue of *det1* sensitivity is due to increased communal levels of DDB1A/B, then *det1 ddb1a/b* mutants should exhibit enhanced sensitivity. However, this is not what we observe. We find that *ddb1a* completely rescues *det1* ABA sensitive germination and a weak allele of *ddb1b* partially rescues this phenotype. The strength of these phenotypes is consistent with allele strength and with *DDB1A/DDB1B* level in

seeds (Figure S2.13). While this indicates that DDB1A and to some extent DDB1B are required for *det1* ABA sensitive germination, it does not fit the above model. In fact *ddb1a* mutants may have lower levels of all the CUL4-based E3 ligase complexes described above, including DWA1/2, DWA3, ABD1, and DDA1-type, all of which are negative regulators of ABA signalling. Thus *ddb1a* mutants would be predicted to have increased ABA signalling and decreased germination, but in the *det1* background we observe the opposite effect. What is the basis of this effect?

Suppression of a *det1* phenotype by *ddb1a* is unusual. For the majority of phenotypes reported, *ddb1a* enhances *det1* phenotypes, suggesting that DET1 and DDB1A are acting together at the molecular level (Schroeder et al 2002, Al Khateeb and Schroeder 2007, Ganpudi and Schroeder 2013). The fact that *ddb1a* suppresses *det1* ABA sensitive germination suggests that the two proteins are acting antagonistically at the molecular level with respect to this phenotype. DET1 and DDB1A have been shown to exhibit opposite activities in *in vitro* transcription assays, where DET1 acts as a repressor and DDB1A acts as an activator (Lau et al 2011) thus perhaps it is DET1's role as a transcriptional repressor, rather than its role in E3 ligase complexes that is driving the germination phenotype.

2.5.8 Germination and cotyledon emergence of *det1* on ABA are opposite phenotypes

Thus *det1* mutants exhibit ABA sensitive germination and this phenotype is suppressed by *hy5, abi5, dwa1/2* and *ddb1a/b*. In contrast, *det1* mutants exhibit ABA resistant cotyledon and true leaf emergence and this phenotype is suppressed by *dwa2, ddb1a/b* and *cop1* (note that in this case *dwa2, ddb1a,* and *ddb1b* are acting in the expected direction, that is, they result in more

ABA sensitivity, consistent with loss of negative regulators). Interestingly, *cop1-4* mutants exhibit the same pattern of ABA sensitive germination and resistant cotyledon emergence. What is the basis of these opposite phenotypes? Potentially tissue specific differences between the radical and cotyledons or over developmental time could result in these distinct phenotypes. ABA regulates germination both via inhibition of radical emergence and via post-germination inhibition of seedling growth (Lopez-Molina et al 2001), thus perhaps DET1 is acting differently at these two time points. In support of the role of time in these distinct phenotypes, Irigoyen *et al* (2014) reported that *det1* exhibited ABA sensitive true leaf emergence at seven days, and we do not observe the *det1* resistant phenotype before ten days. While *det1* ABA sensitive germination required HY5 and ABI5, *det1* ABA resistant cotyledon emergence did not. This result is consistent with studies indicating that while ABI5 is a key regulator of germination, its contribution decreases during seedling establishment (Finkelstein et al 2005).

To separate the effect of ABA on germination and cotyledon emergence in *det1*, wildtype and *det1* seed were germinated in control conditions then transferred to ABA containing media. In these experiments *det1* exhibited ABA resistant cotyledon emergence immediately (Figure S2.15). Thus *det1* germination and cotyledon emergence respond oppositely to ABA. The delay in observing ABA resistant cotyledon emergence in *det1* in continuous ABA conditions must be due to delayed *det1* germination.

2.5.9 Rapid water loss phenotype in *det1* is independent of *HY5, ABI5, DWA1/2* and *DDB1B*

Desiccation tolerance is one of the effects of ABA and can be assessed by transpirational water loss. Water loss assays showed that *det1* mutants lose significantly more water than wild

type. In contrast with the other phenotypes examined, *hy5*, *abi5, dwa1, dwa2* and *ddb1b* had no effect on the *det1* rapid water loss phenotype. This is consistent with the results of Chen *et al* (2008) who reported that while HY5 is involved in many ABA-mediated stress responses, HY5 may not be involved in ABA-induced stomata movement or ABA responses in adult plants. Other constitutive photomorphogenic mutants such as *cop1* and *cop10* exhibit defects in stomatal closure (Mao et al 2005, Delgado et al 2012). Recently, COP1 was shown to be required for cytoskeletal rearrangement and anion channel activity in guard cells (Khanna et al 2014).

In summary, we find that *det1* early germination in control conditions requires *HY5* and *COP1,* while *det1* ABA sensitive germination requires *HY5, ABI5, DDB1A/B* and *DWA1/2*, and *det1* ABA resistant cotyledon emergence requires *DDB1A/B, DWA2,* and *COP1*. In contrast, the *det1* rapid water loss phenotype is independent of all the above genes. These results illustrate the genetic complexity of *det1* phenotypes.

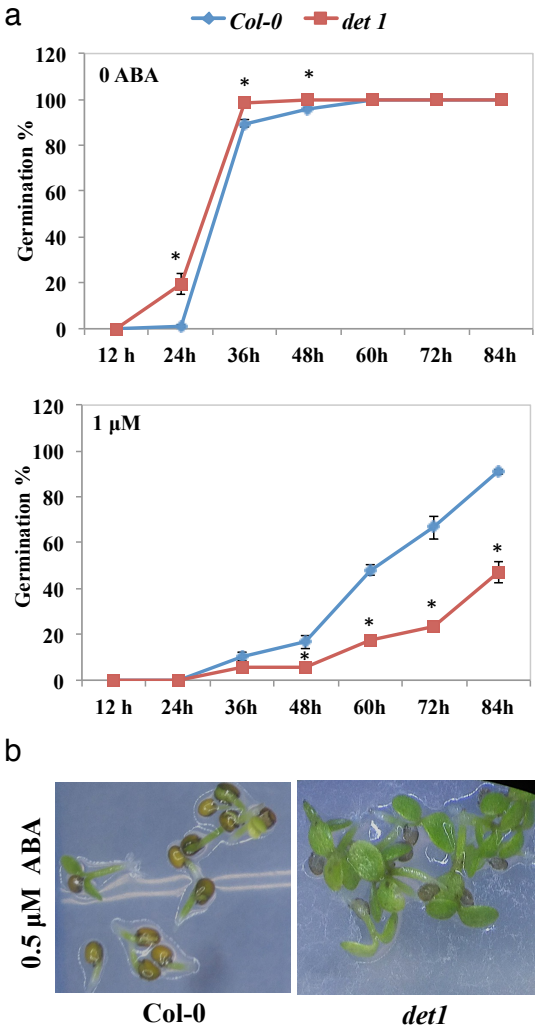

Figure 2.1 *det1* is sensitive to ABA inhibition of germination but resistant to ABA inhibition of cotyledon emergence

(a) Germination (%) on 0 or 1 µM ABA. Values are mean ± SE of 3 replicates of 50-100 seeds, *=P<0.05 of *det1* vs wild type. (b) Representative cotyledon development on 0.5 µM ABA after 10 days.

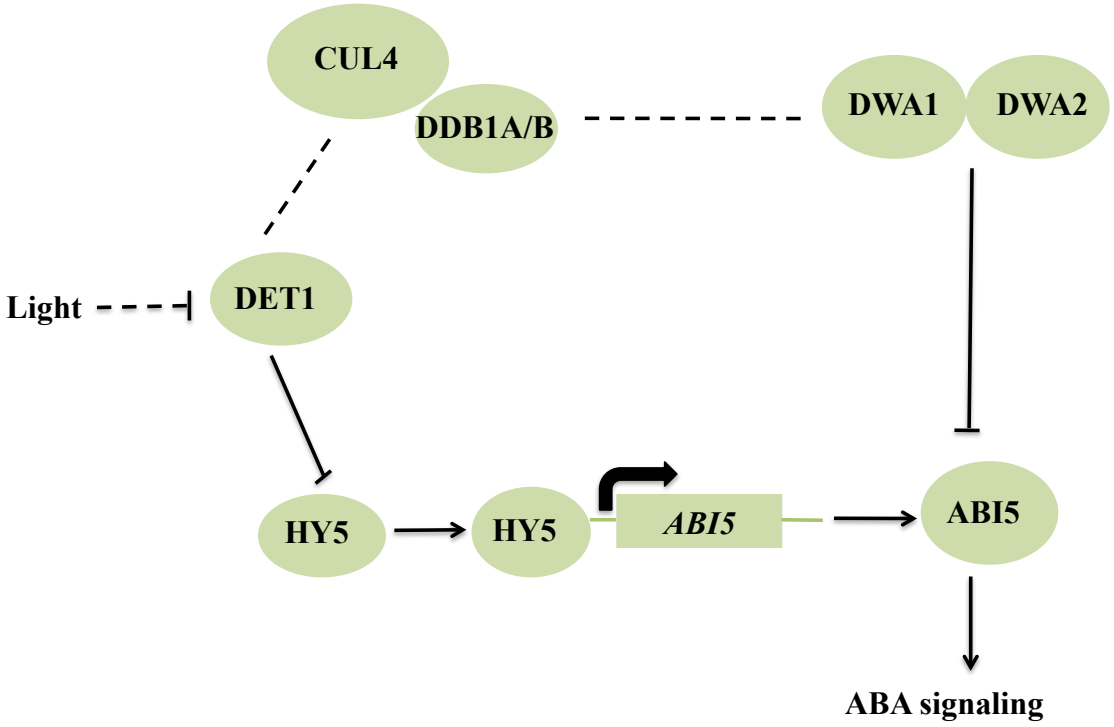

Figure 2.2 Hypothetical model showing possible interactions of genes involved in det1 ABA sensitive germination.

The model shows that *det1* ABA sensitive germination may be due to two pathways. Firstly, DET1 is a negative regulator of HY5. HY5 in turn positively regulates *ABI5* transcription in the presence of ABA, promoting ABA signalling and inhibiting germination. Secondly, DET1 forms a complex with DDB1A/B and CULLIN 4. Another DDB1-CUL4 complex containing DWA1&2 has been shown to regulate ABA response via degradation of ABI5. It is possible that these components of distinct DDB1 complexes, DET1 and DWA1/2, interact genetically during ABA response.

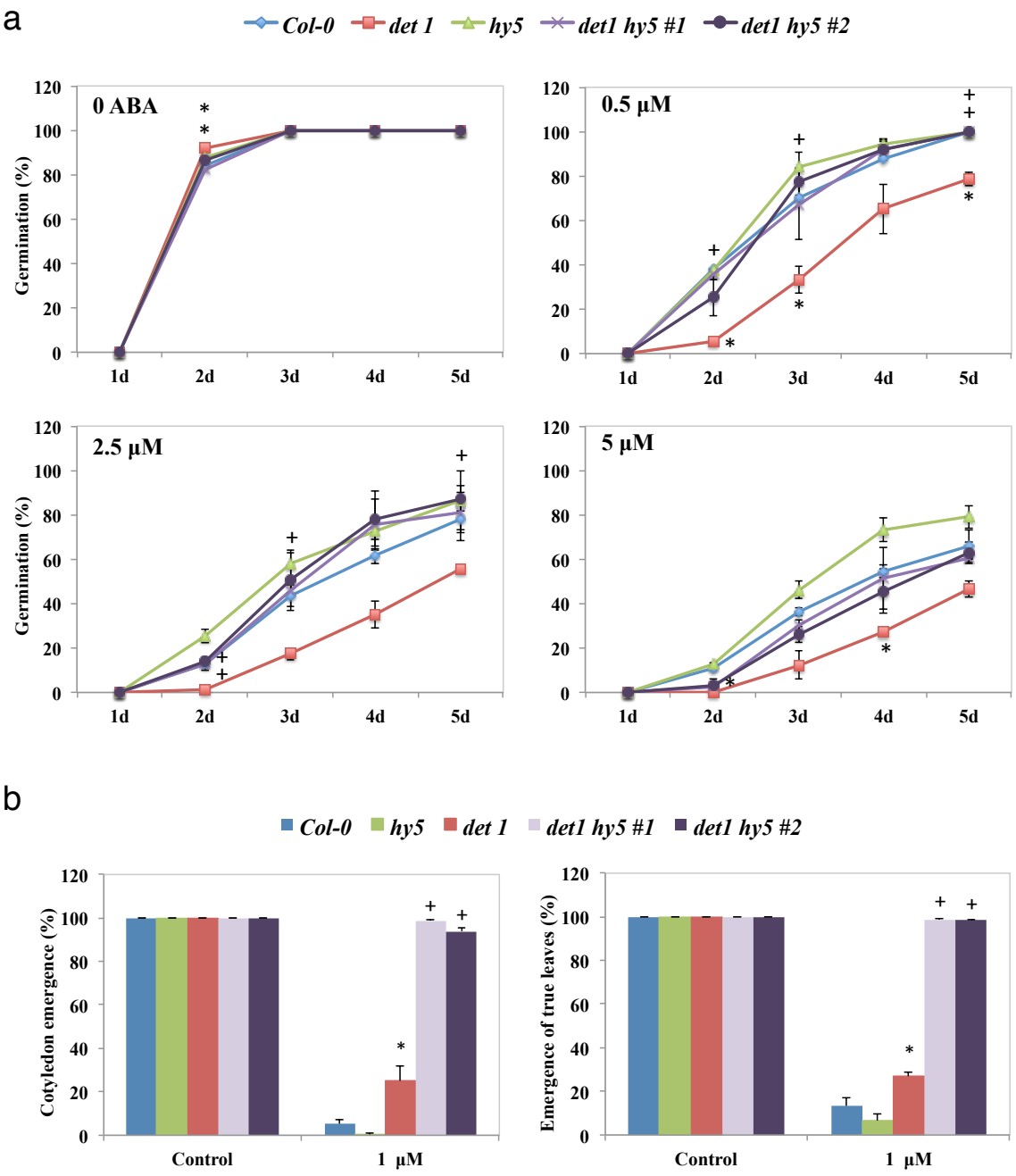

Figure 2.3 Germination in *det1 hy5* double mutants

(a) Germination (%) on 0, 0.5, 2.5 or 5 µM ABA. (b) Emergence of cotyledons and first pair of true leaves (%) on 0 or 1 µM ABA after 10 and 14 days respectively. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*. Values are means ± SE of (a) 2 replicates and (b) 3 replicates of 50-100 seeds.

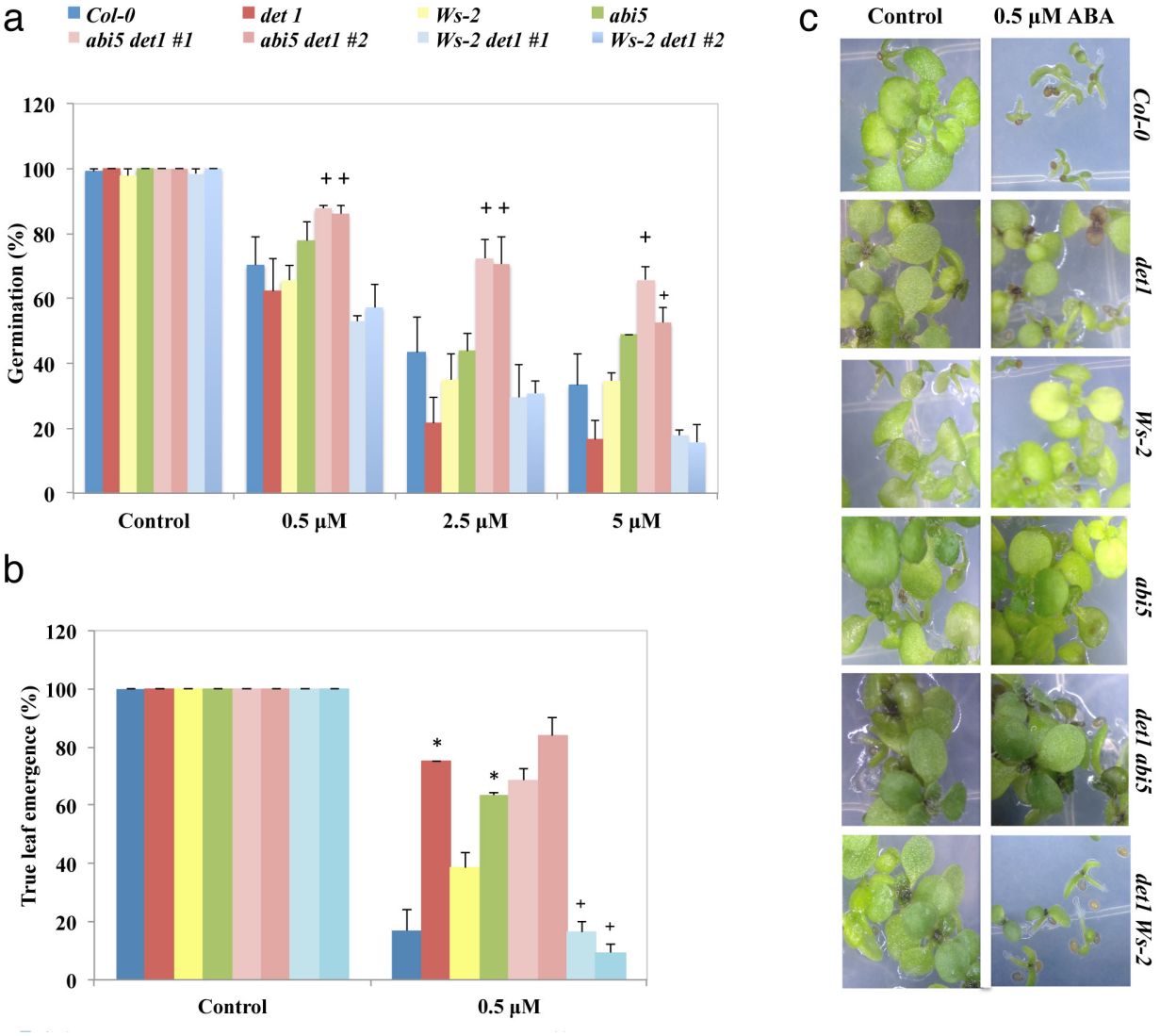

Figure 2.4 Germination in *det1 abi5* double mutants

(a) Germination (%) of *det1 abi5* on 0, 0.5, 2.5 or 5 µM ABA after 2 days. (b) True leaf emergence (%) on 0.5 µM ABA after 14 days. Values are means ± SE of (a) 3 replicates and (b) 2 replicates of 50-100 seeds, *=P< 0.05 of single mutants vs appropriate wild type and + =P ≤ 0.05 of doubles vs *det1*. (c) Representative seedlings after 14 days.

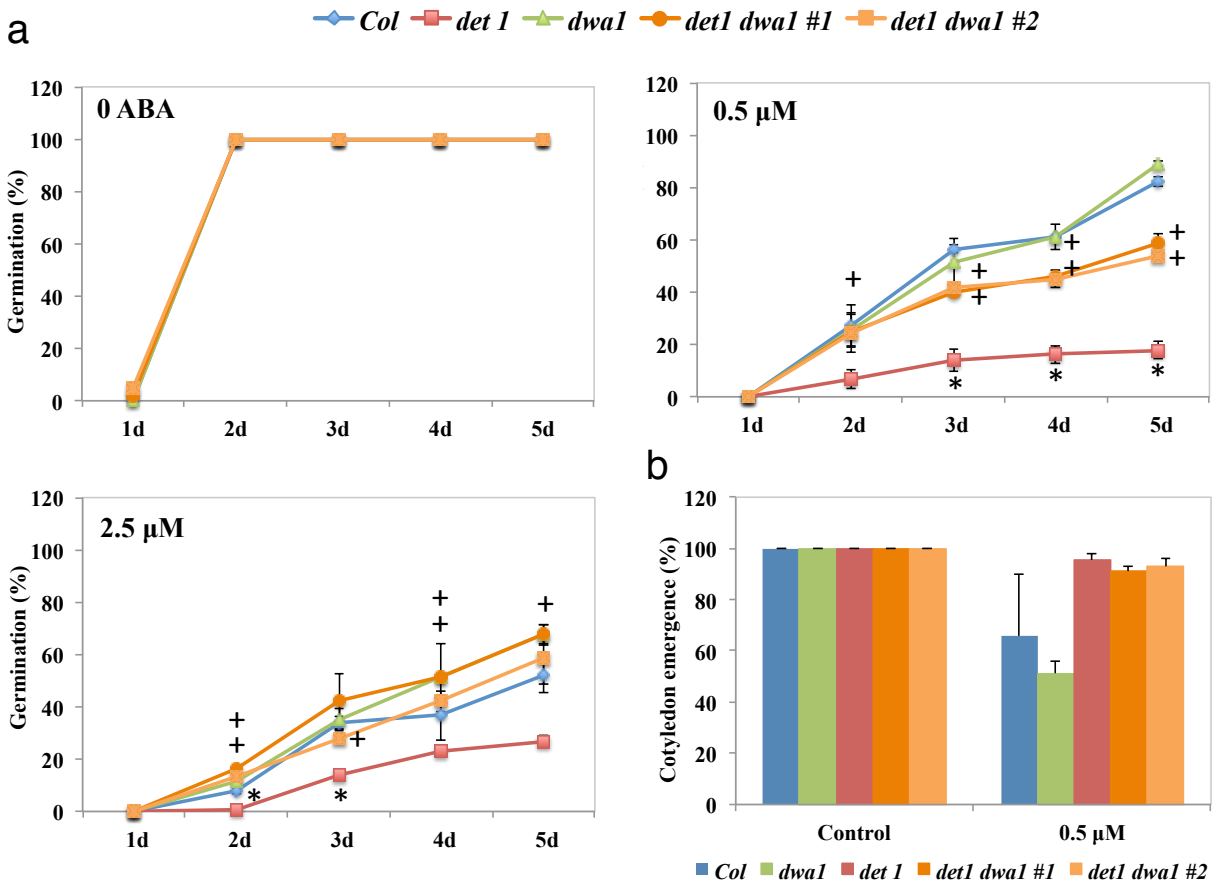

Figure 2.5 Germination in *det1 dwa1* double mutants

(a) Germination (%) of *det1 dwa1* on 0, 0.5, or 2.5 µM ABA. (b) Cotyledon emergence (%) on 0.5 µM ABA after 10 days. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

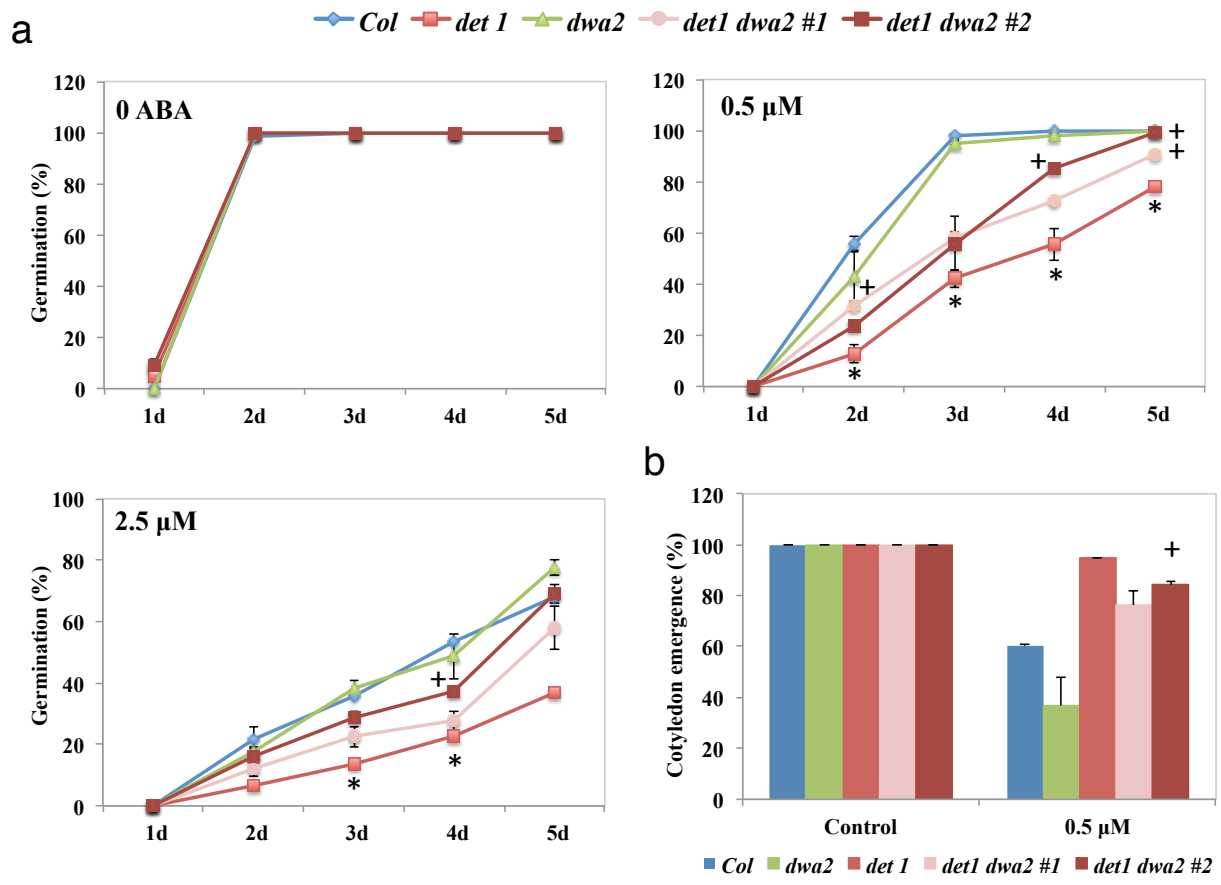

Figure 2.6 Germination in *det1 dwa2* double mutants

(a) Germination (%) of *det1 dwa2* on 0, 0.5 or 2.5 µM ABA. (b) Cotyledon emergence (%) on 0.5 µM ABA after 10 days. Values are means ± SE of 2 replicates of 50-100 seeds, *=P< 0.05 of single mutants vs wild type and doubles vs *det1*. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

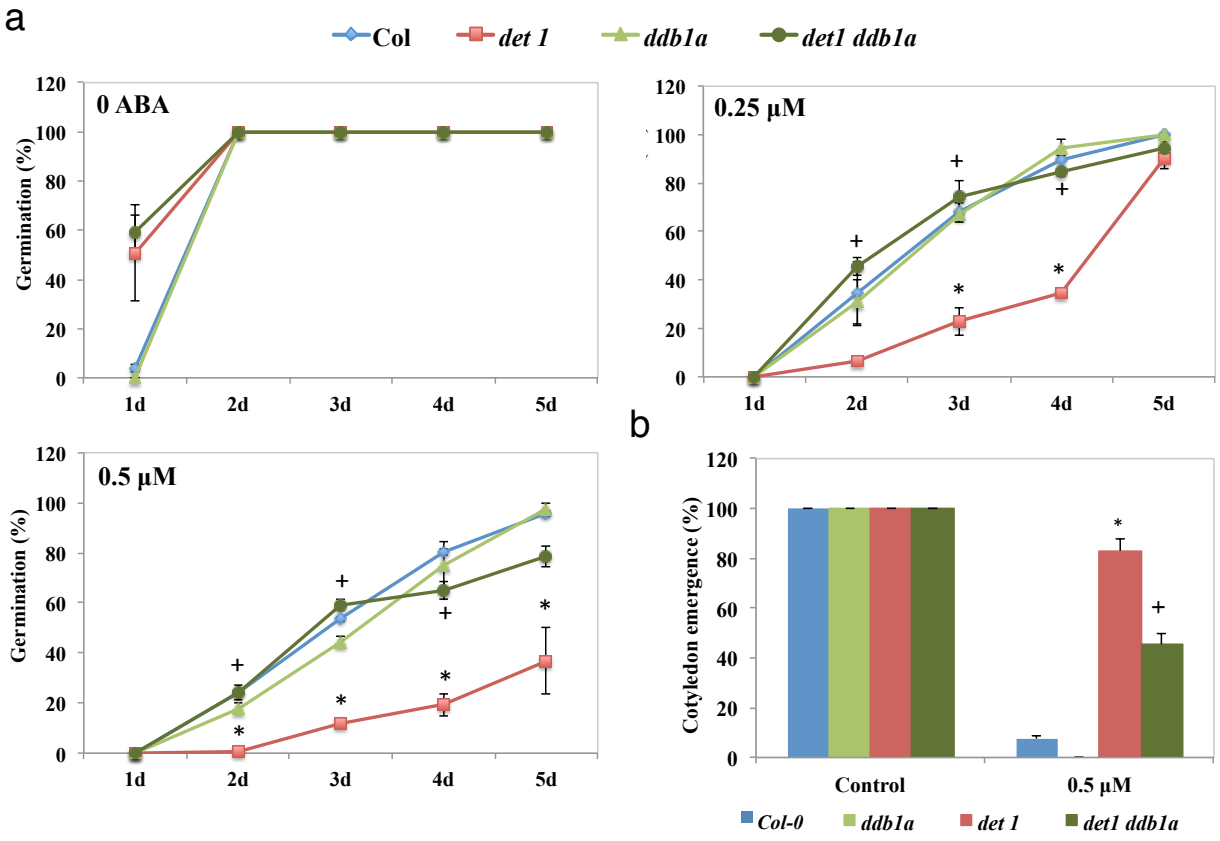

Figure 2.7 Germination in *det1 ddb1a* double mutants

(a) Germination (%) of *det1 ddb1a* on 0, 0.25 or 0.5 µM ABA. (b) Cotyledon emergence (%) on 0.5 µM ABA after 10 days. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

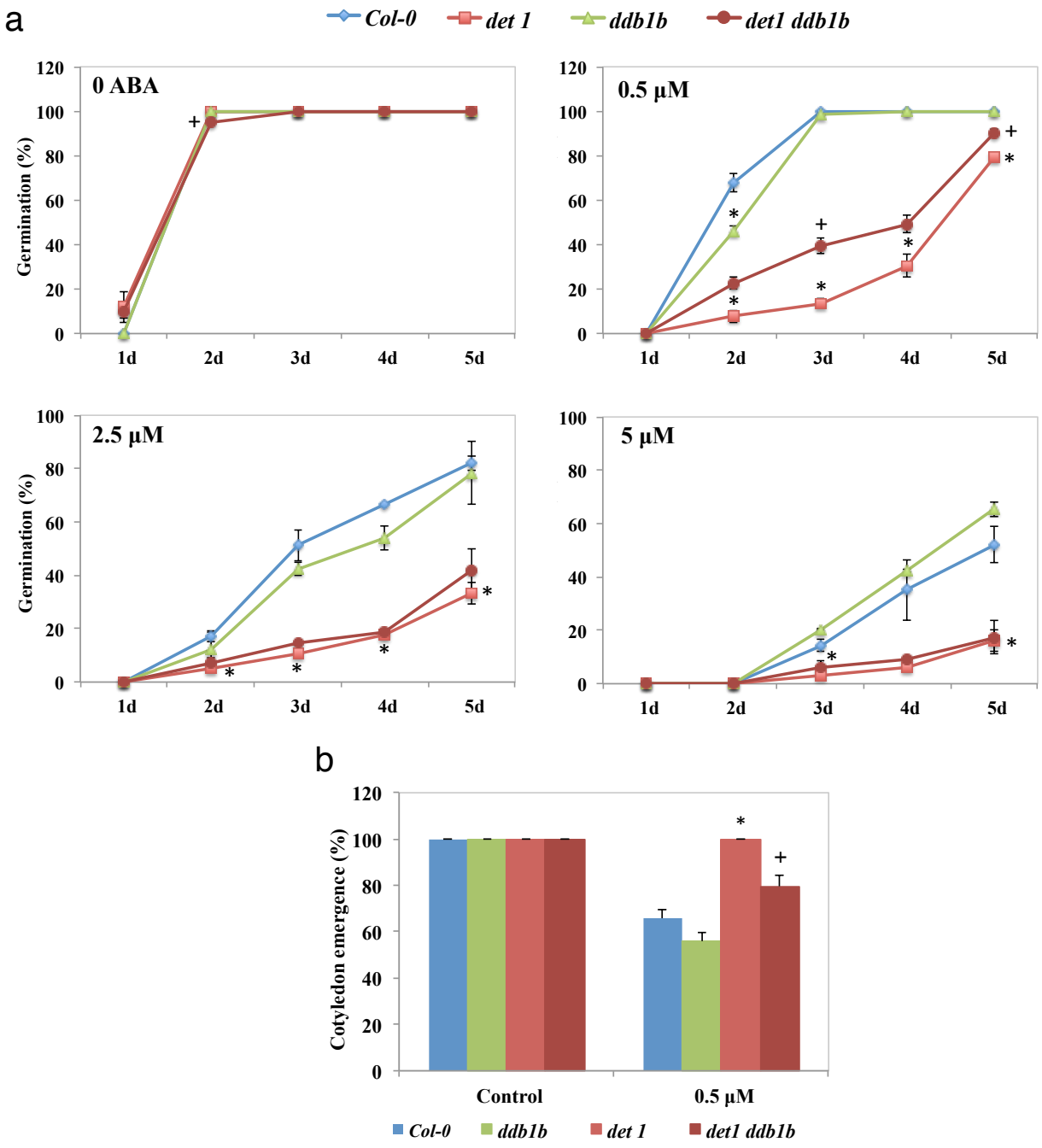

Figure 2.8 Germination in *det1 ddb1b* double mutants

(a) Germination (%) of *det1 ddb1b* on 0, 0.5, 2.5 or 5 µM ABA. (b) Cotyledon emergence (%) on 0.5 µM ABA after 10 days. Values are means ± SE of 2 replicates of 50-100 seeds, *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

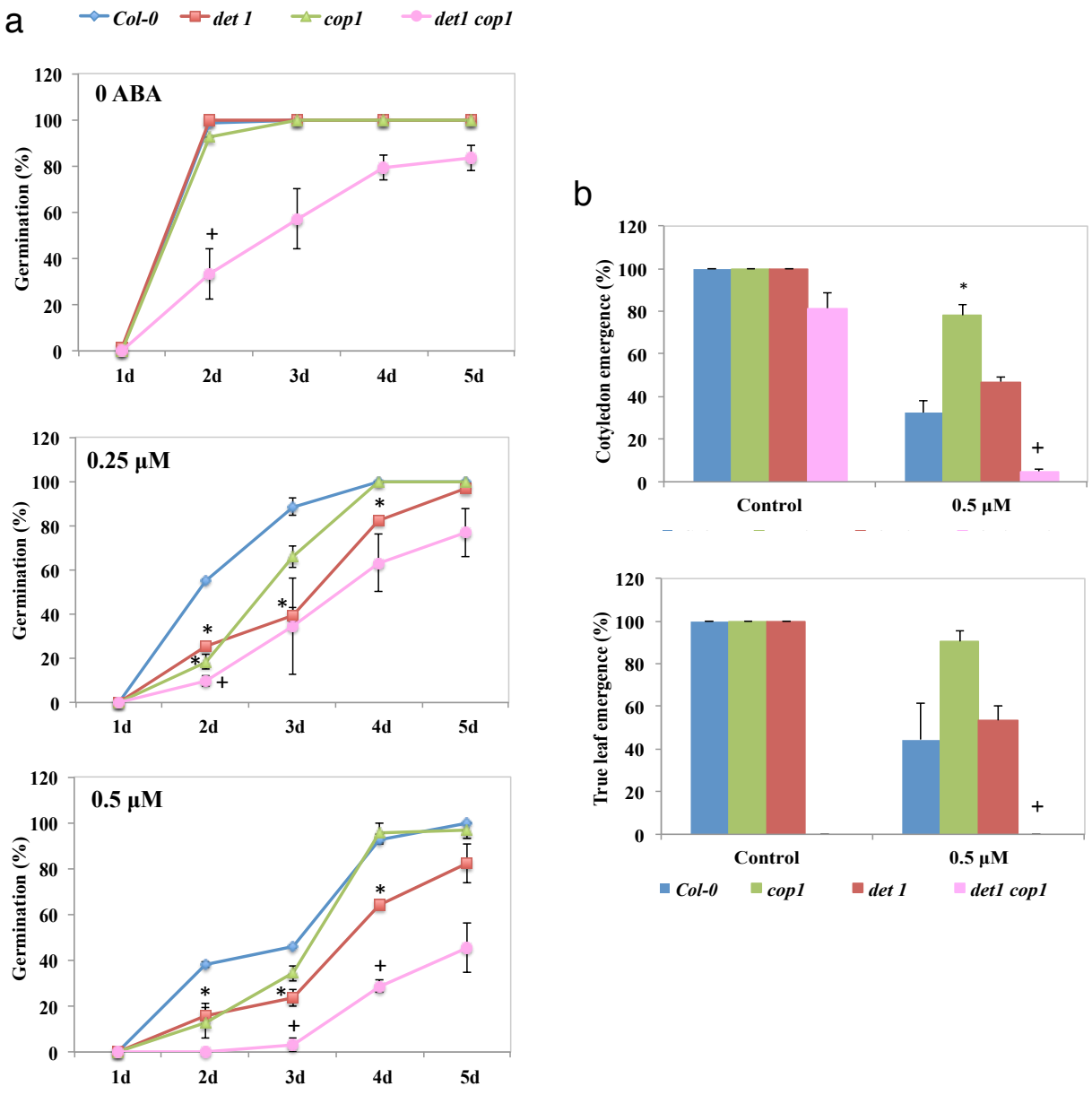

Figure 2.9 Germination in *det1 cop1-4* double mutants

(a) Germination (%) of *det1 cop1-4* on 0, 0.25 or 0.5 µM ABA. (b) Cotyledon emergence at 8 days and true leaf emergence at 10 days (%) on 0.5 µM ABA. Values are means ± SE of 2 replicates of 50-100 seeds, *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

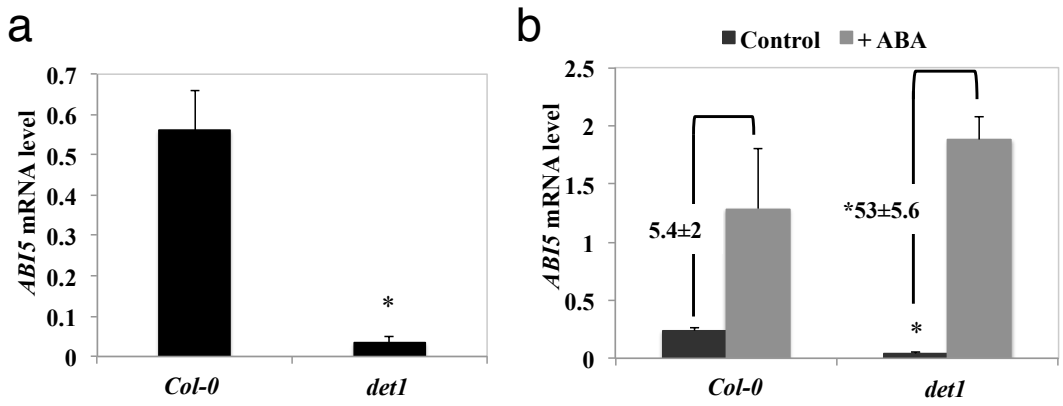

Figure 2.10 *ABI5* gene expression in *det1*

(a) Real-time PCR analysis of *ABI5* mRNA levels in Col-0 and *det1* dry seeds. (b) *ABI5* mRNA levels in Col-0 and *det1* seeds imbibed in liquid media in the presence or absence of 2.5 μM ABA for 48 hours during cold stratification at 4^0C. Brackets indicate fold change due to ABA treatment. Values are normalized relative to reference gene *EF1α*. Error bars indicate SE of 3 technical replicates. *=P< 0.05 of *det1* vs Col-0.

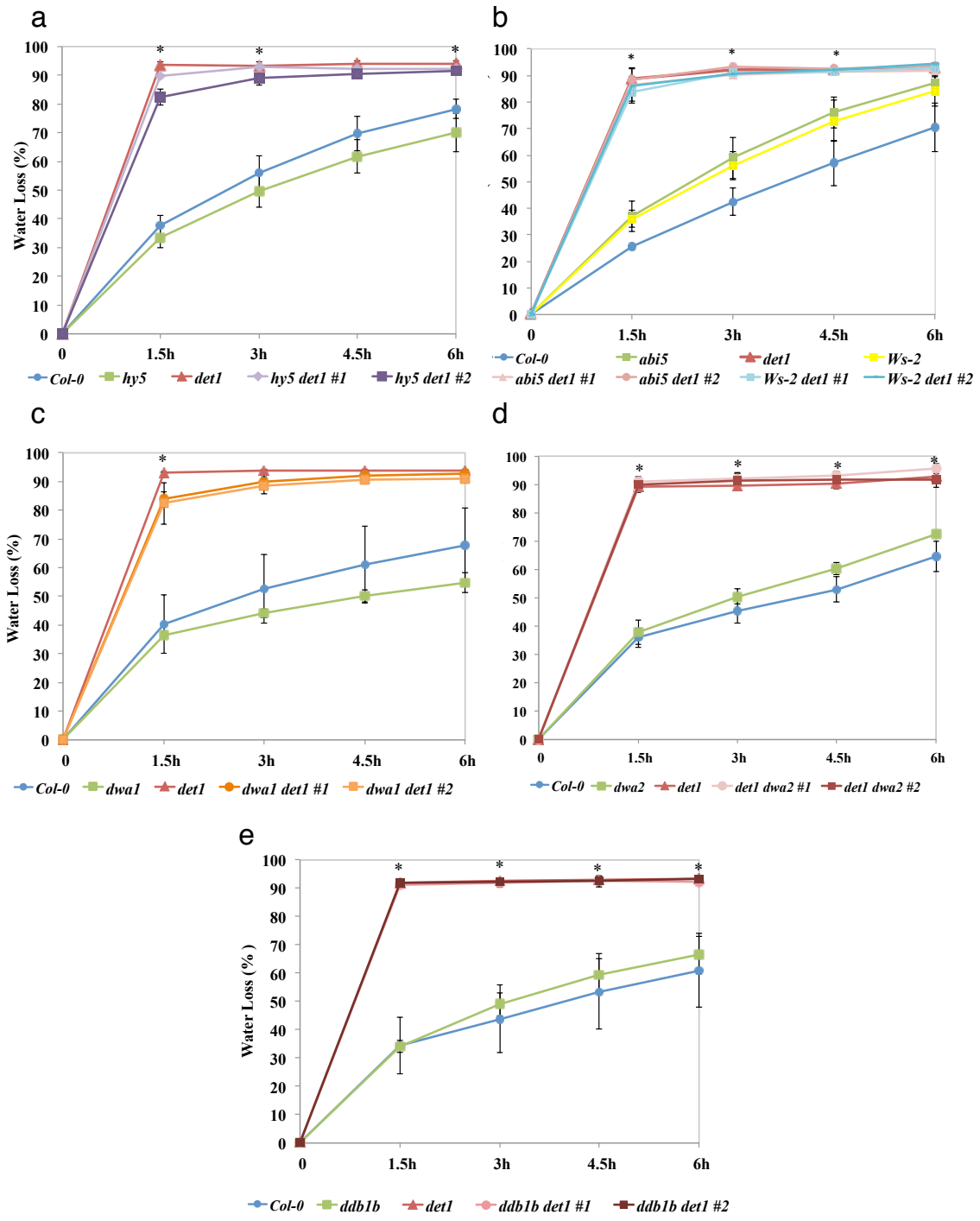

Figure 2.11. *det1* rapid water loss phenotype does not require HY5, ABI5, DWA1, DWA2 or DDB1B.

Water loss from detached leaves as % loss of fresh weight. Values are means ± SE of 2 samples of 3-6 leaves, *=P< 0.05 of single mutants vs Col-0, double mutants vs *det1*.

2.6 Supplementary data

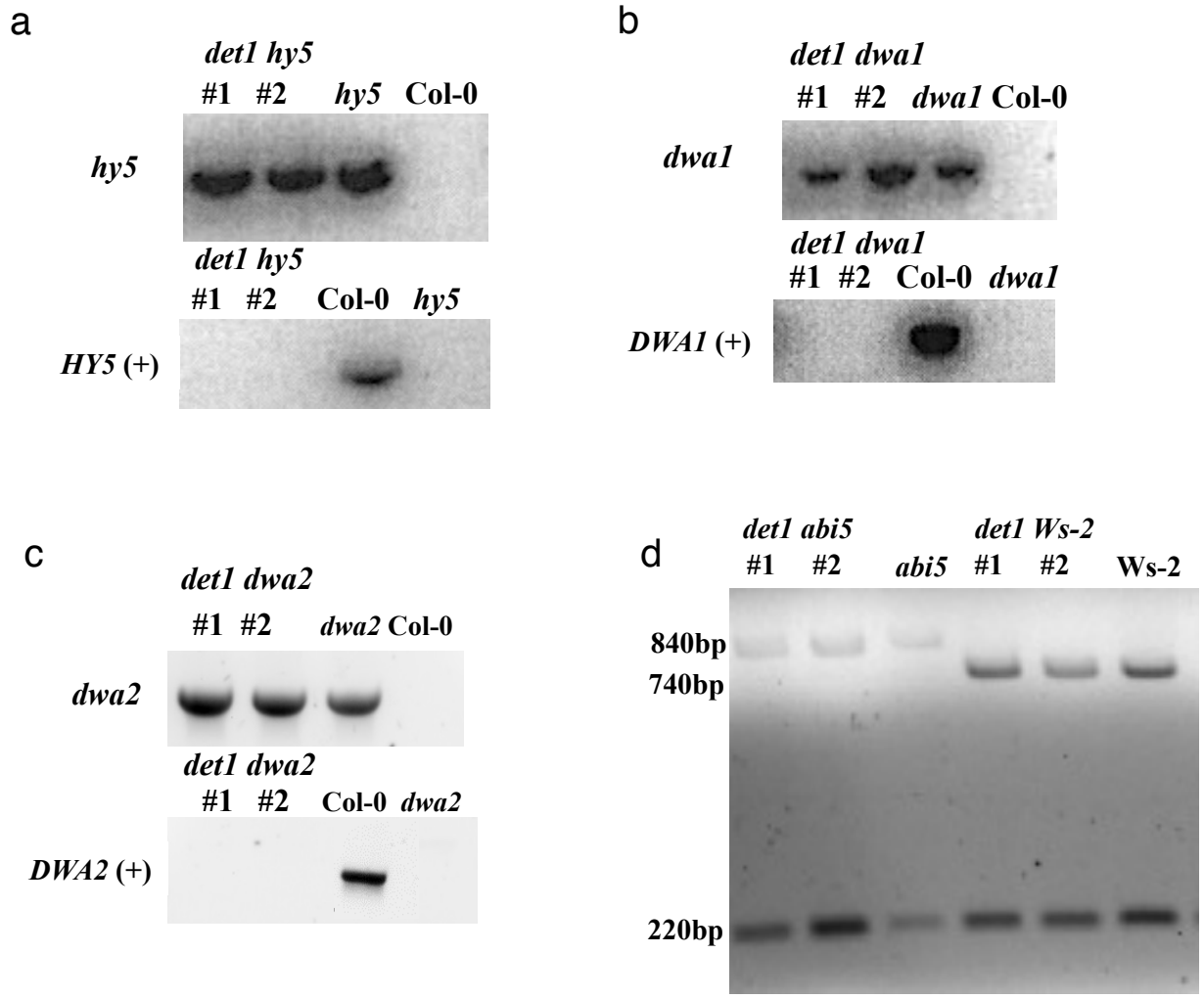

Figure S2.1 PCR genotyping of double mutants generated for this study

Mutant and wildtype PCR reactions of (a) *det1 hy5*, (b) *det1 dwa1*, (c) *det1 dwa2* and (d) *det1 abi5*. Genotyping for *abi5-1* mutant was followed by digestion with Ava II (Bensmihen et al 2002).

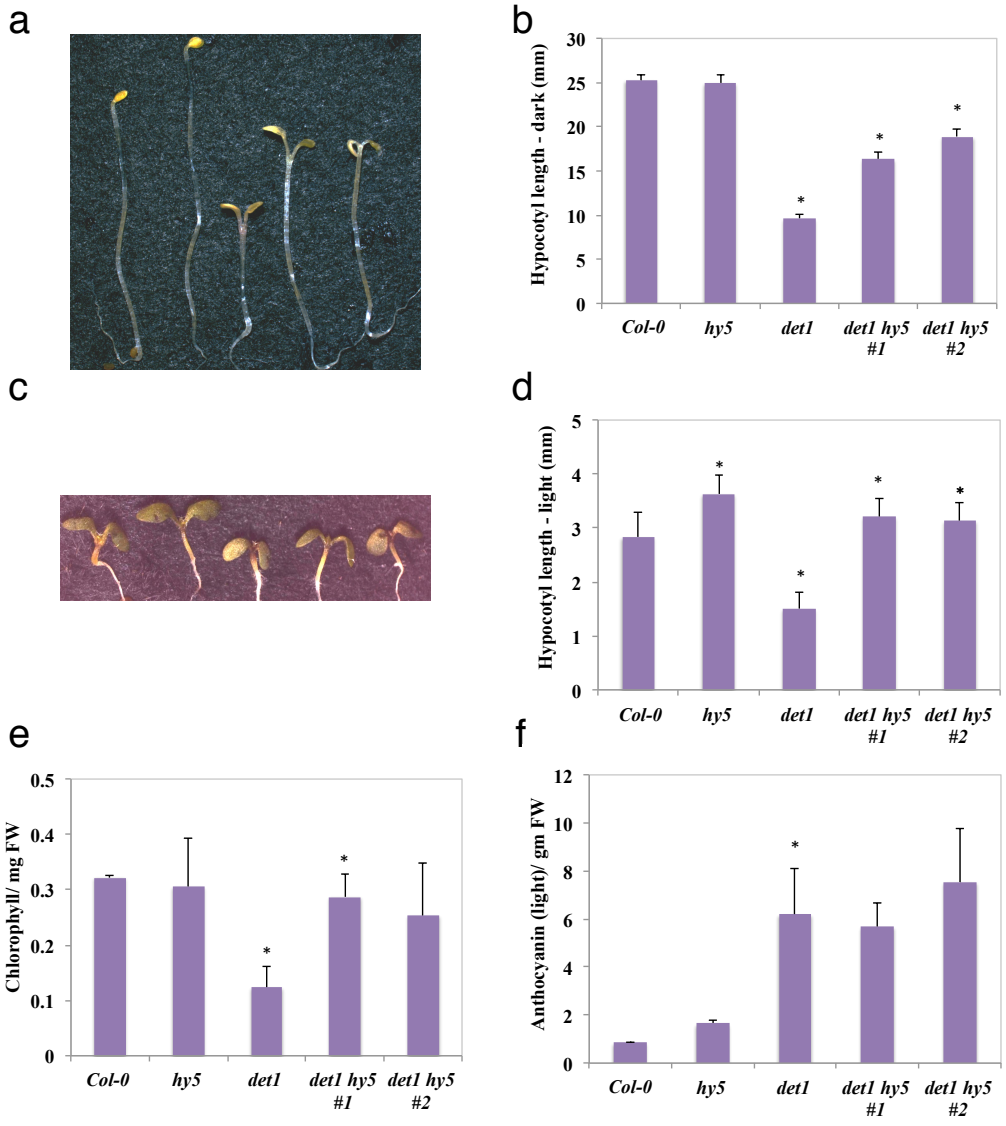

Figure S2.2 Phenotypic analysis of *det1 hy5* seedlings.

(a) Dark grown seedlings from left: Col-0, *hy5*, *det1*, *det1 hy5 #1* and *det1 hy5 #2*. (b) Dark hypocotyl length (n=10). (c) Light grown seedlings from left: Col-0, *hy5*, *det1*, *det1 hy5 #1* and *det1 hy5 #2*. (d) Light hypocotyl length (n=10). (e) Chlorophyll content (mg of chlorophyll/mg of fresh weight) (n = 2). (f) Anthocyanin content (A530- A657 / g fresh weight) (n = 2). Error bars indicate 95% CI. * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

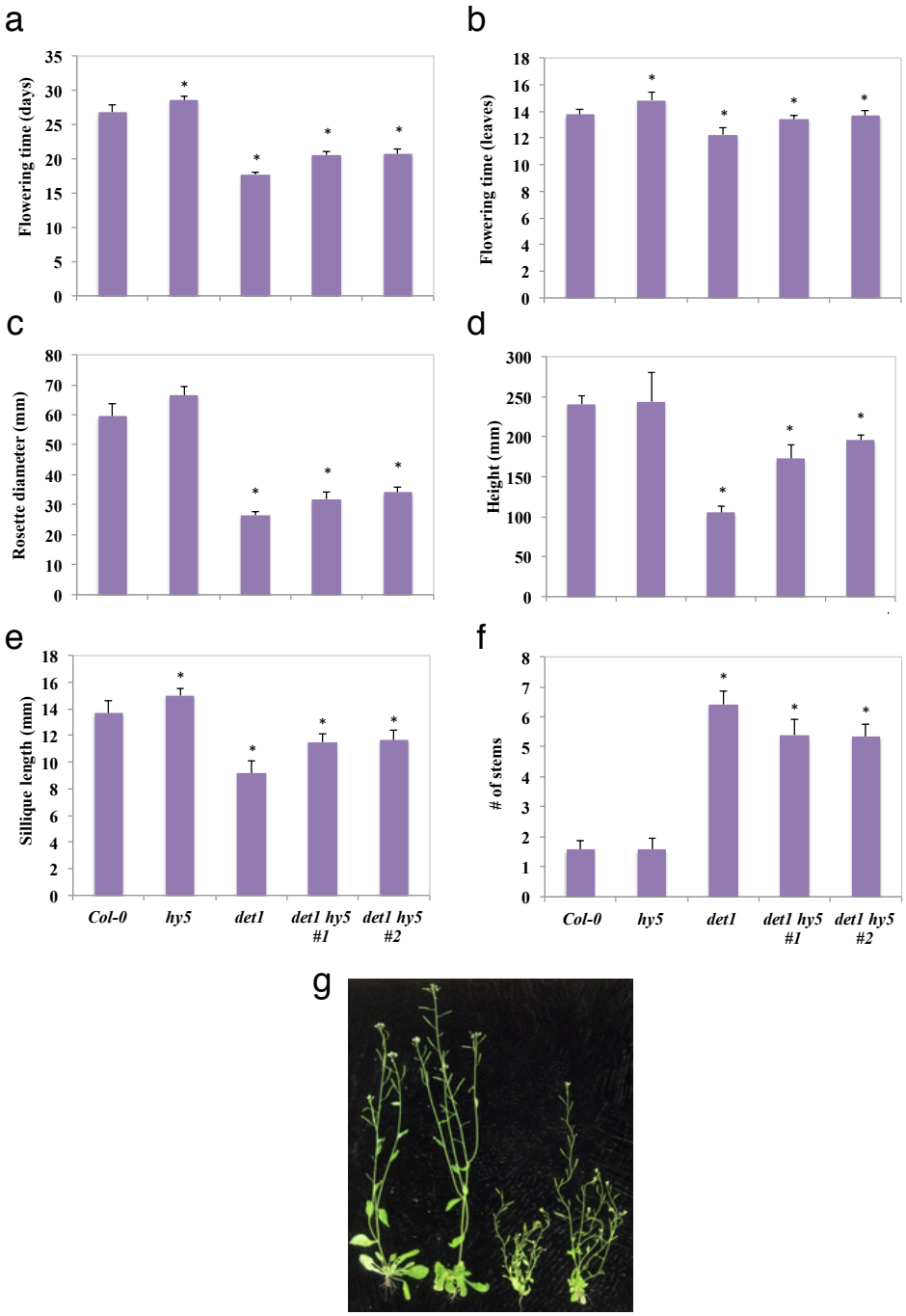

Figure S2.3 Phenotypic analysis of *det1 hy5* adults

(a) Flowering time (in days). (b) Flowering time (in # of leaves). (c) Rosette diameter. (d) Plant height. (e) Silique length. (f) Number of stems. (g) Adult plants from left: Col-0, *hy5*, *det1*, *det1 hy5 #1*. Error bars indicate 95% CI (n=12). * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

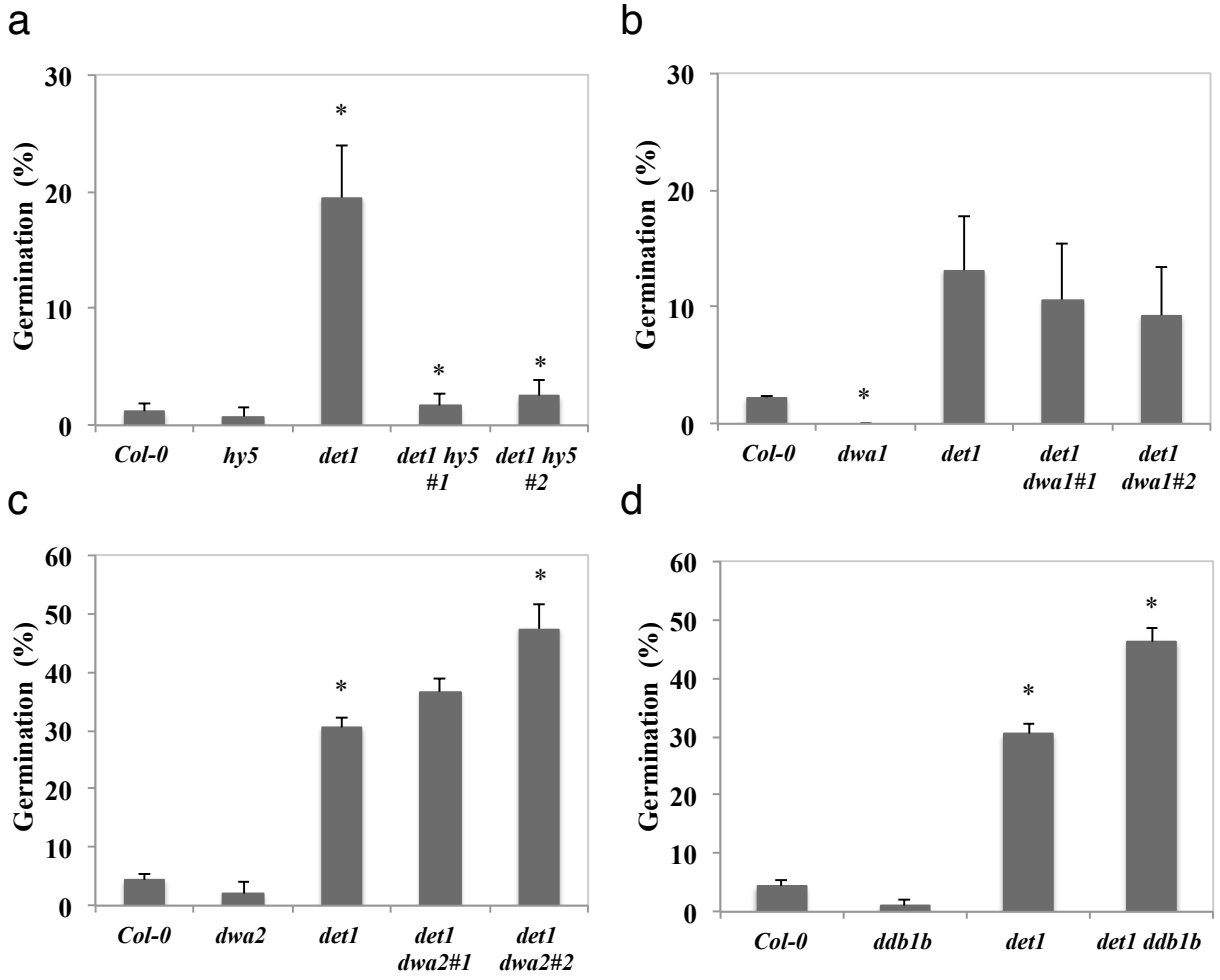

Figure S2.4 Germination phenotypes on control media

(a) *det1 hy5* after 24 hours. (b) *det1 dwa1* after 24 hours. (c) *det1 dwa2* after 36 hours. (d) *det1 ddb1b* after 36 hours. Values are mean ± SE of 2 replicates of 50-100 seeds, * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

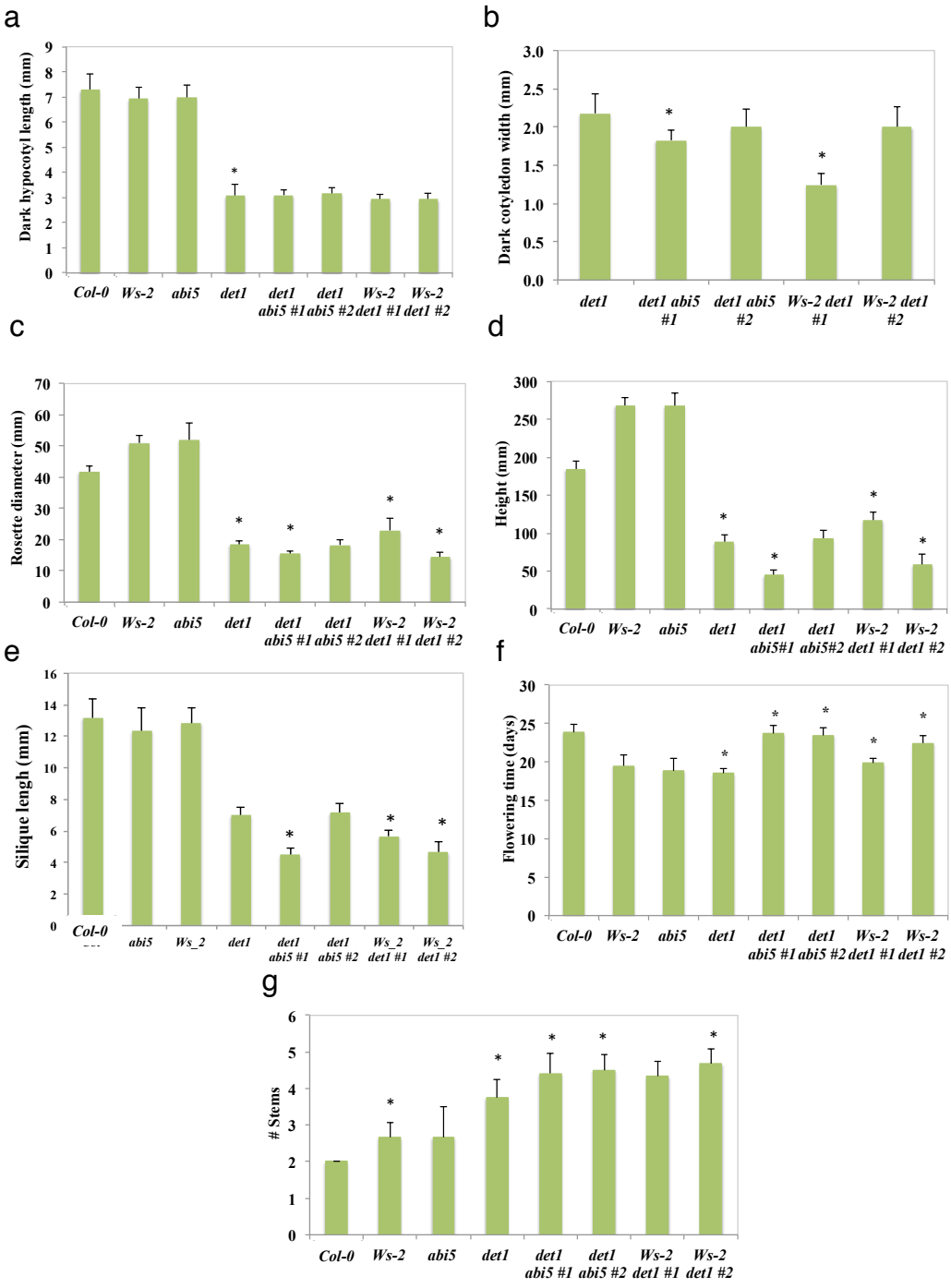

Figure S2.5 Phenotypic analysis of *det1 abi5* seedlings and adults

(a) Dark hypocotyl length. (b) Dark hypocotyl width. (c) Rosette diameter. (d) Plant height. (e) Silique length. (f) Flowering time (in days). (g) Number of stems. Error bars indicate 95% CI (n=12). * indicates $P \leq 0.05$ of *det1* relative to Col-0, *abi5* relative to Ws-2 and double mutants relative to *det1*.

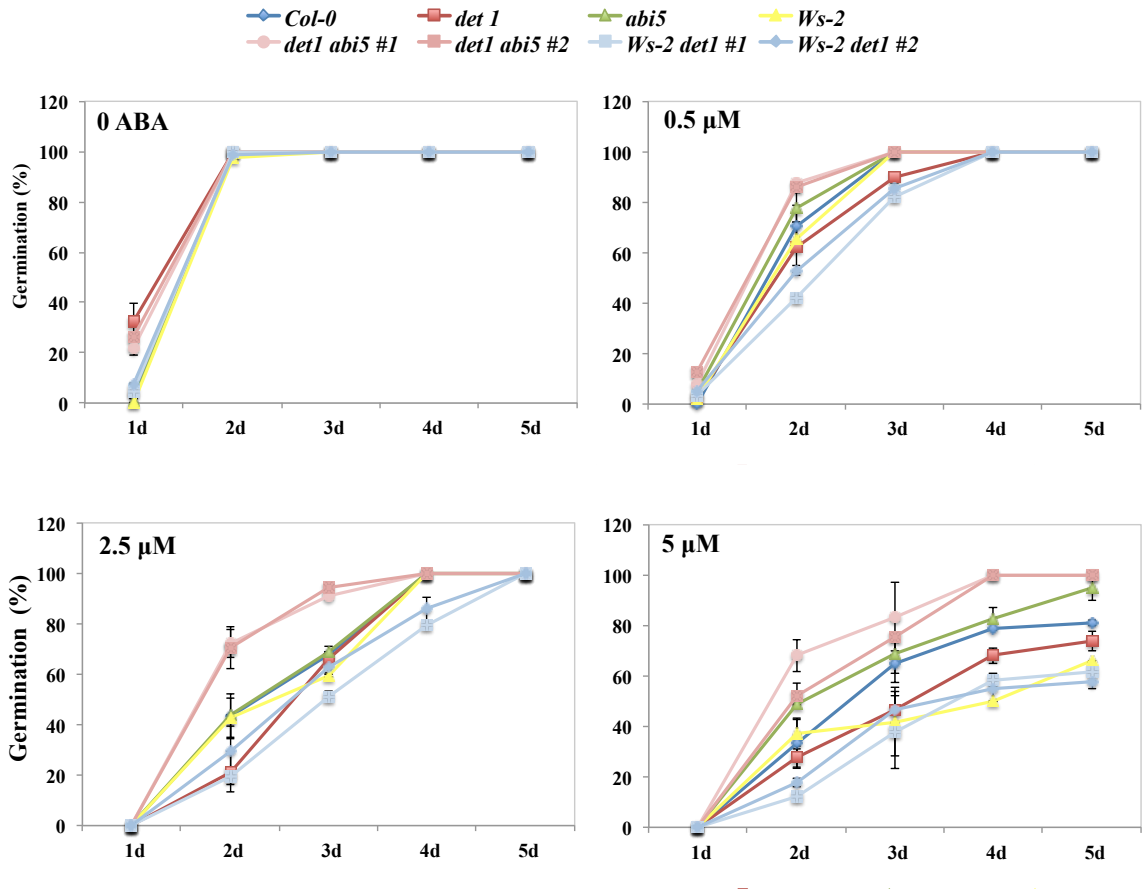

Figure S2.6 ABA Germination phenotypes of *det1 abi5*

Values are mean ± SE of 3 replicates of 50-100 seeds.

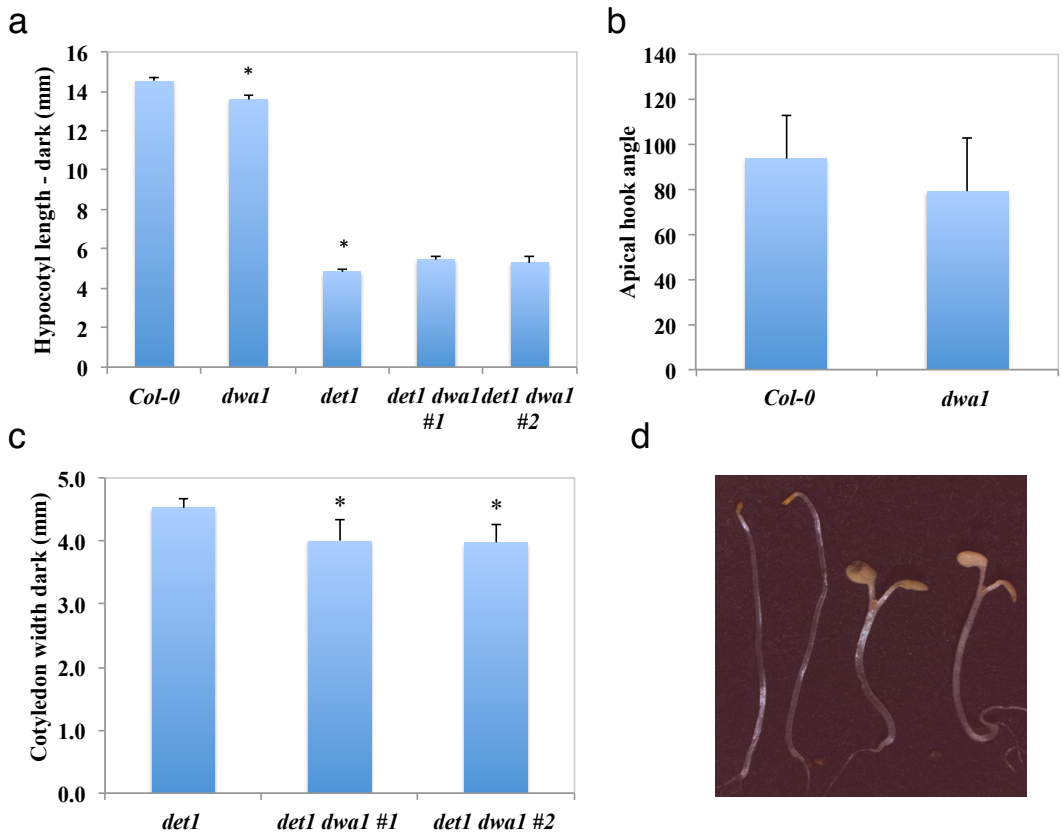

Figure S2.7 Phenotypic analysis of *det1 dwa1* dark grown seedlings

(a) Dark hypocotyl length. (b) Apical hook angle. (c) Dark cotyledon width. (d) Dark grown seedlings from left: Col-0, *dwa1*, *det1*, *det1 dwa1 #1*. Error bars indicate 95% CI (n=10). * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

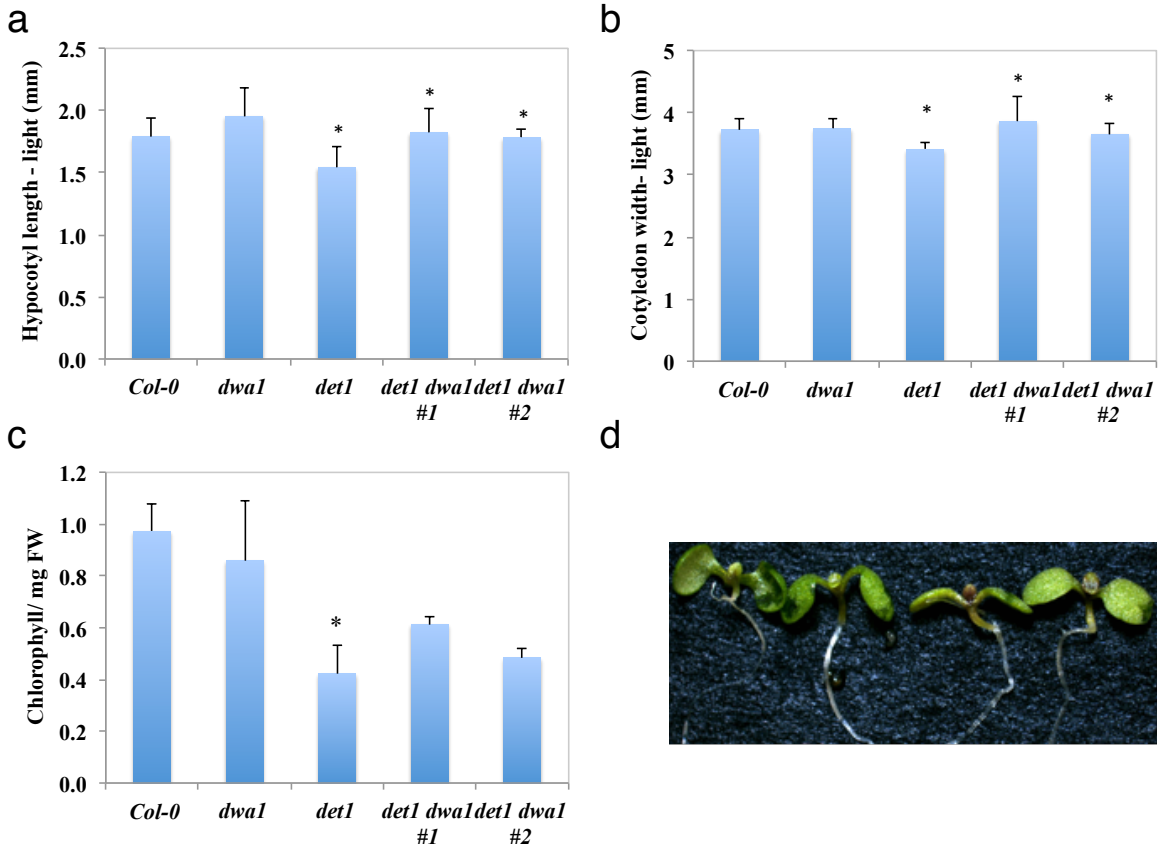

Figure S2.8 Phenotypic analysis of *det1 dwa1* light grown seedlings

(a) Light hypocotyl length (n=10). (b) Light cotyledon width (n=10). (c) Chlorophyll content (mg of chlorophyll/mg of fresh weight) (n = 2). (d) Light grown seedlings from left: Col-0, *dwa1*, *det1*, *det1 dwa1 #1*. Error bars indicate 95% CI. * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

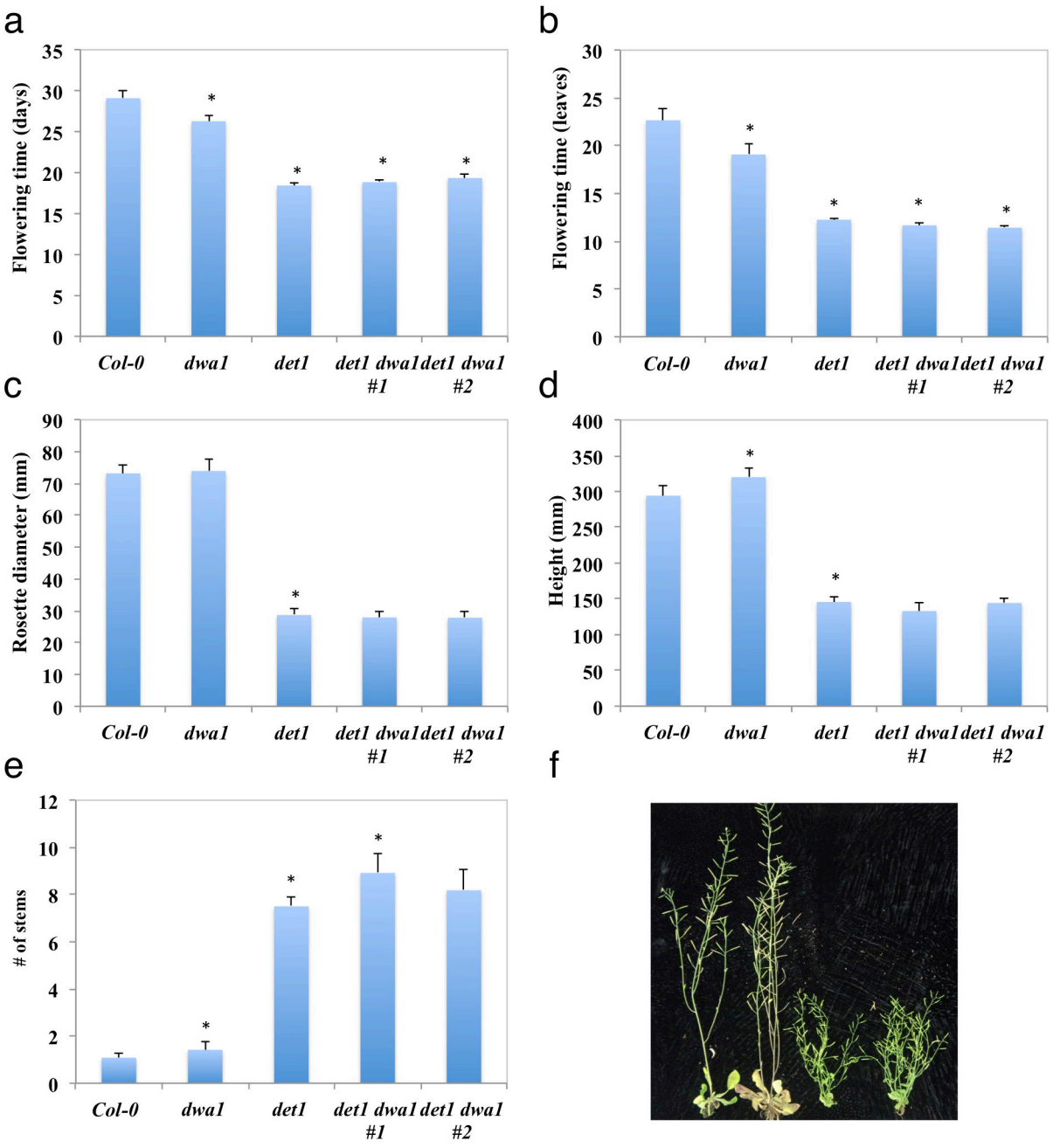

Figure S2.9 Phenotypic analysis of *det1 dwa1* adults

(a) Flowering time (in days). (b) Flowering time (in # of leaves). (c) Rosette diameter. (d) Plant height. (e) Number of stems. (f) Adult plants from left: Col-0, *dwa1*, *det1*, *det1 dwa1 #1*. Error bars indicate 95% CI (n=12). * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

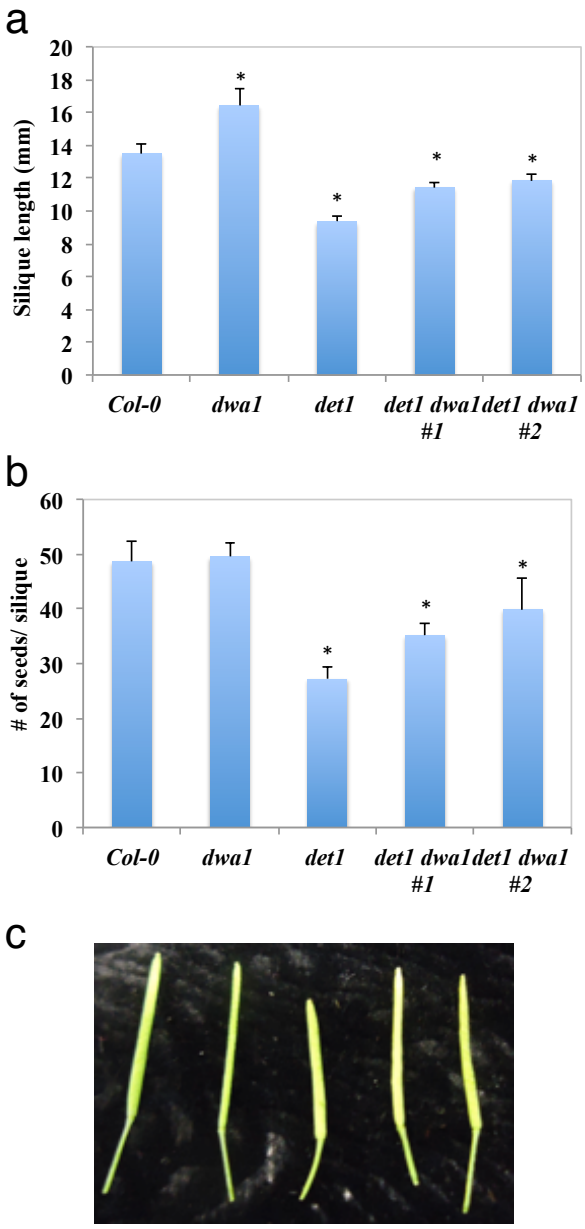

Figure S2.10 Phenotypic analysis of *det1 dwa1* siliques and seeds

(a) Silique length (n=12). (b) Number of seeds/silique (n=6). (c) Siliques from left Col-0, *dwa1*, *det1*, *det1 dwa1 #1* and *det1 dwa1 #2*. Error bars indicate 95% CI. * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

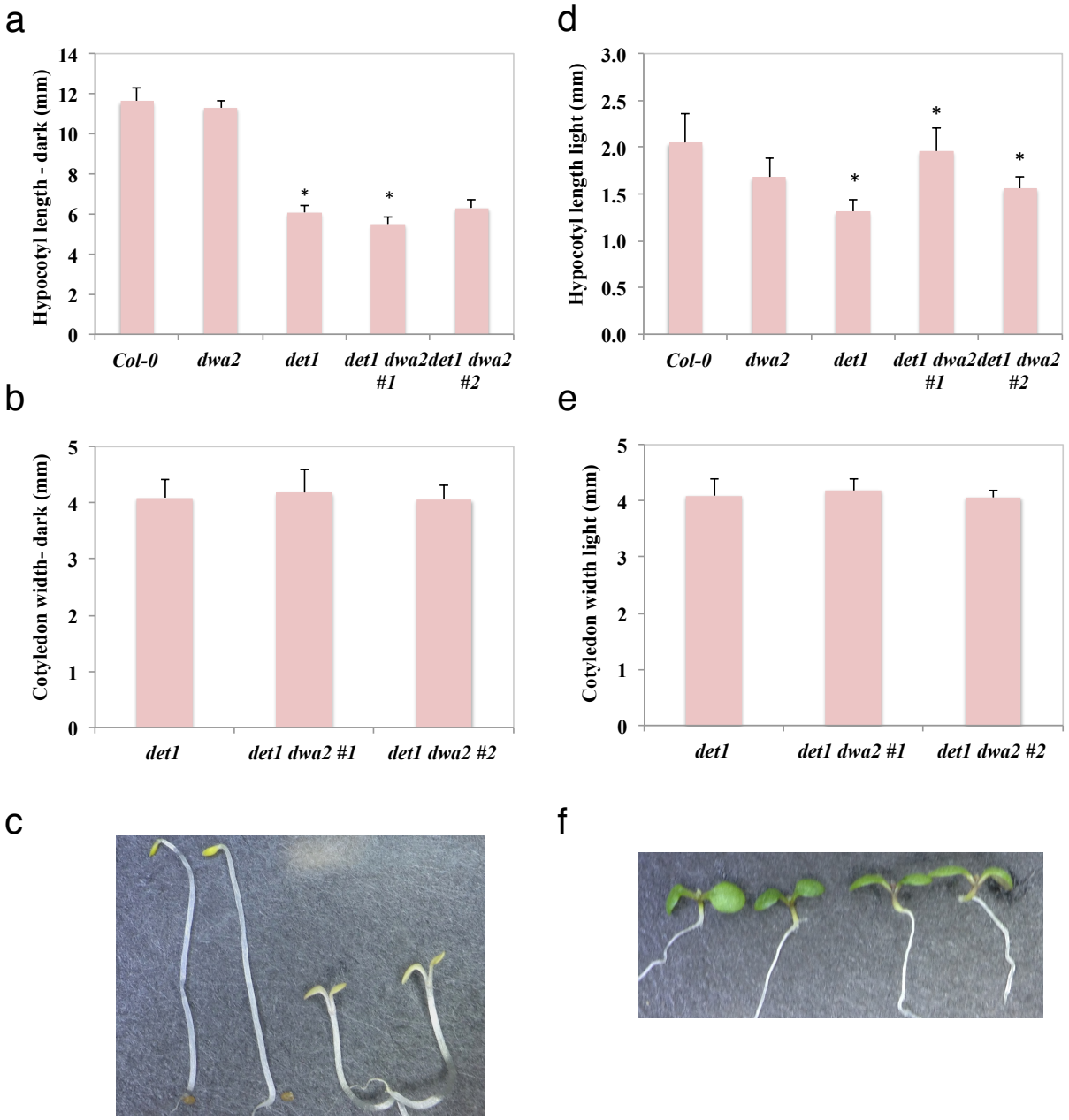

Figure S2.11 Phenotypic analysis of *det1 dwa2* seedlings

(a) Dark hypocotyl length. (b) Dark cotyledon width. (c) Dark grown seedlings from left: Col-0, *dwa2*, *det1*, *det1 dwa2 #1*. (d) Light hypocotyl length. (e) Light cotyledon width. (f) Light grown seedlings from left: Col, *dwa2*, *det1*, *det1 dwa2 #1*. Error bars indicate 95% CI (n=10). * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

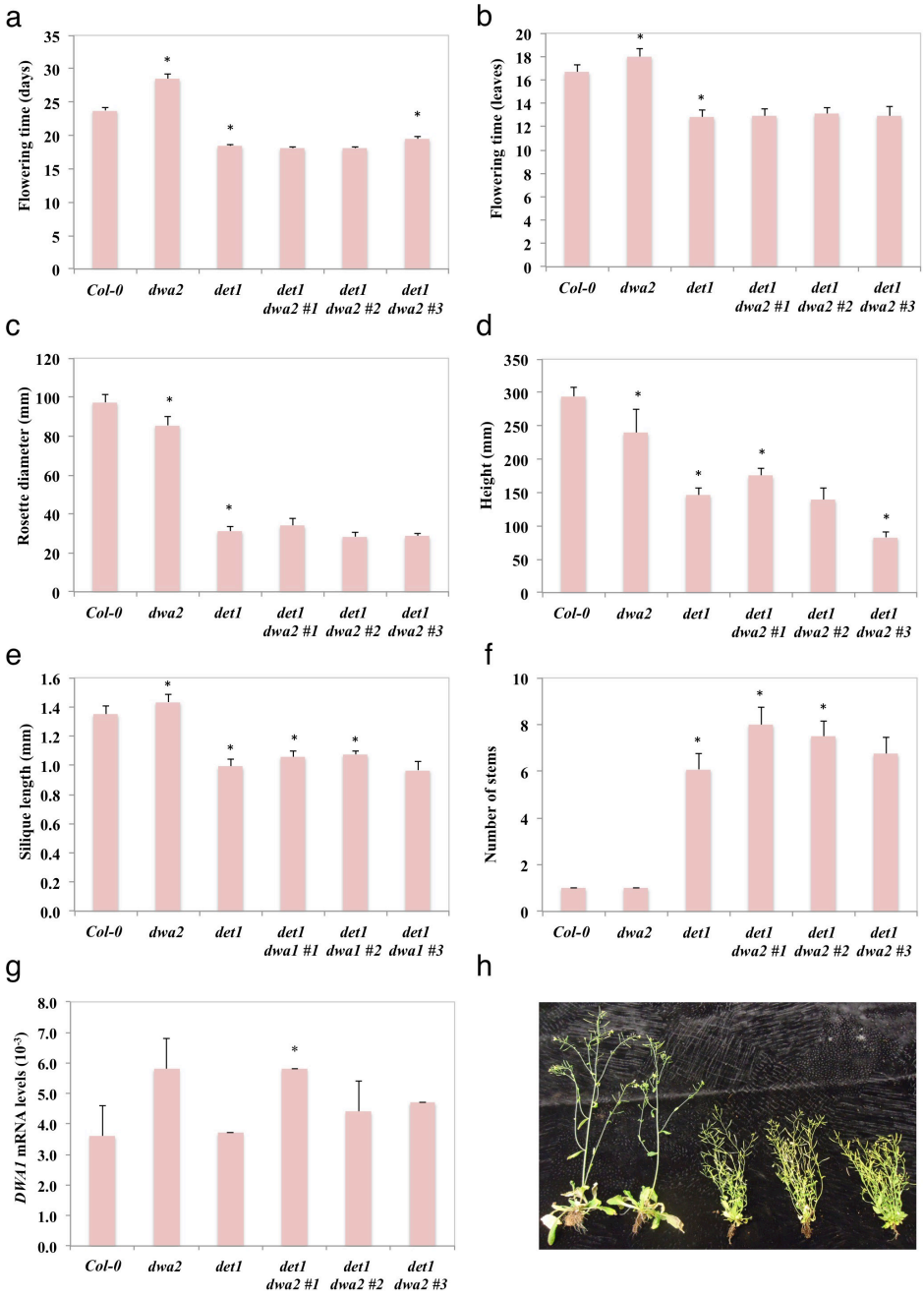

Figure S2.12 Phenotypic analysis of *det1 dwa2* adults

(a) Flowering time (days). (b) Flowering time (# of leaves). (c) Rosette diameter. (d) Plant height. (e) Silique length. (f) Number of stems. Error bars indicate 95% CI (n=12). (g) Real-time PCR analysis of *DWA1* (At2g19430) mRNA levels in 7 day old seedlings. Values normalized relative to reference gene *EF1α*. Error bars indicate SE of 3 technical replicates. * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*. (h) Adult plants from left: Col-0, *dwa2*, *det1*, *det1 dwa2 #1* and *det1 dwa2 #2*.

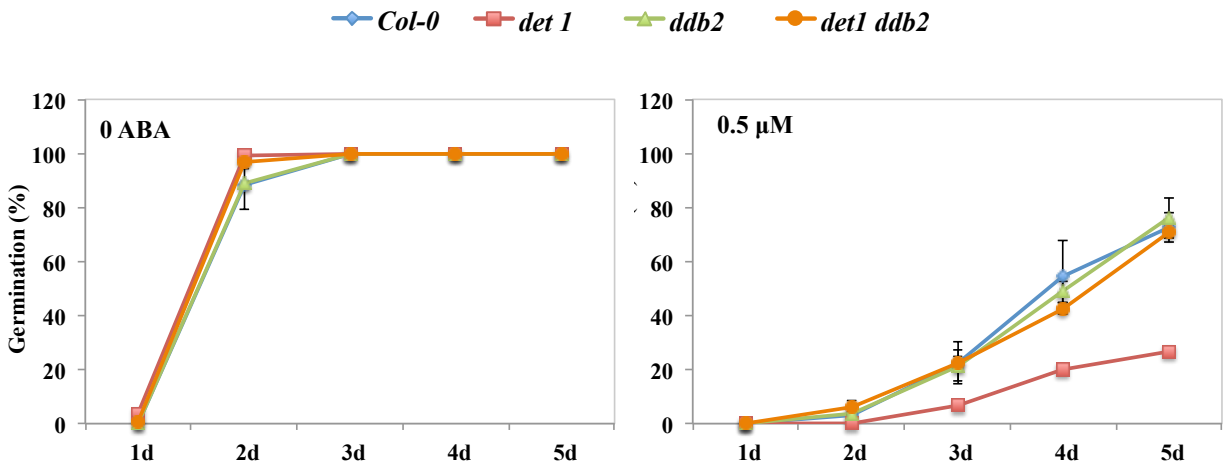

Figure S2.13 ABA Germination phenotypes of *det1 ddb2*

Values are mean ± SE of 2 replicates of 50-100 seeds.

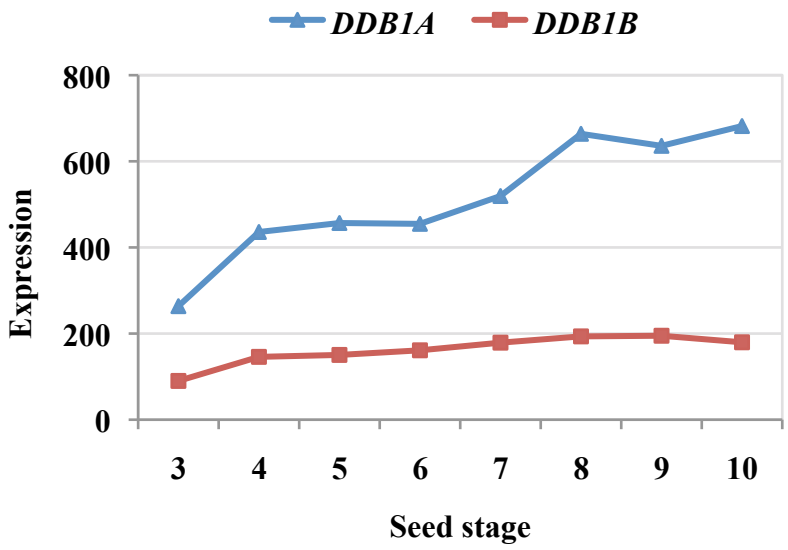

Figure S2.14 Expression levels of *DDB1A* and *DDB1B* during seed development.

Data from (Schmid et al 2005) accessed via AtGenExpress.

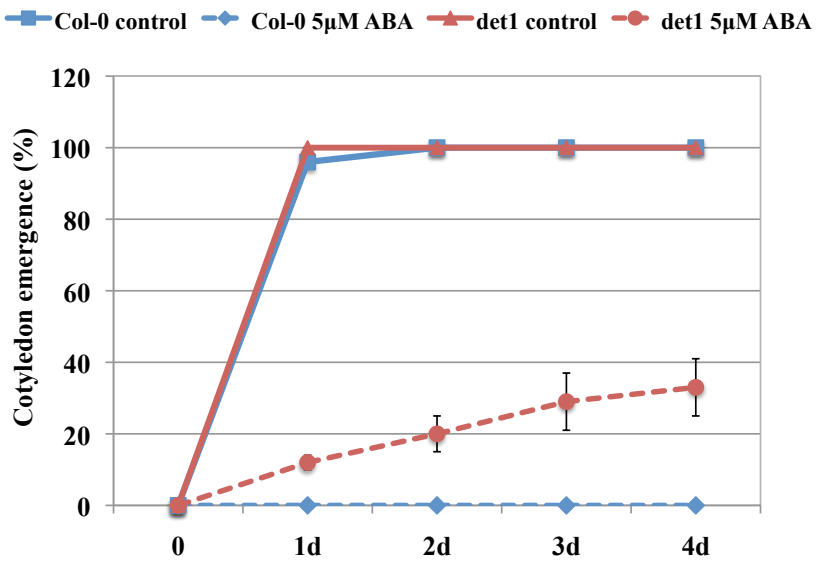

Figure S2.15 ABA resistant cotyledon emergence in *det1*

det1 and wildtype seeds were germinated on control media then transferred to 5 µM ABA at 36 h. Cotyledon emergence following transfer to ABA is indicated. Values are mean ± SE of 2 replicates of 50-100 seeds.

Chapter 3. Role of Arabidopsis *DE-ETIOLATED 1* (*DET1*) during seed germination in salt and osmotic stress conditions

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3. Role of Arabidopsis *DE-ETIOLATED 1* (*DET1*) during seed germination in salt and osmotic stress conditions

3.1 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 ABA signalling. We found that *hy5* suppressed the *det1* salt and mannitol resistant germination phenotype, thus, the *det1* stress resistant germination requires HY5. In order to identify genes downstream of HY5 that were differentially expressed in *det1* mutants, publicly available microarray data was used for enrichment analysis. The analysis revealed that ABA regulated genes, including *ABA RESPONSIVE ELEMENT BINDING FACTOR 3* (*ABF3*), are downregulated in *det1*. We investigated the role of *ABF3, ABF4,* and *ABF1* in *det1* phenotypes. Double mutant analysis showed that *abf4* and *abf1* suppress the *det1* salt/osmotic stress resistant germination phenotype. Molecular analysis revealed that while *ABF3* is induced by salt in wildtype seeds, *ABF4* and *ABF1* are repressed, and all three of these genes are underexpressed in *det1* seeds. In addition, *abf1* suppressed *det1* rapid water loss and stomata phenotypes. Thus interactions between *ABF* genes appear to regulate *det1* salt/osmotic stress response phenotypes.

3.2 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 agriculture and food production faces today (Deinlein et al 2014).

The plant hormone abscisic acid (ABA) controls a wide array of physiological processes in plants, including regulating water balance and conferring osmotic stress tolerance (Raghavendra et al 2010). ABA signals that the plant is under stress, thereby inducing a number of stress responses, including upregulation of stress responsive gene expression (Busk and Pages 1998). This results in the accumulation of osmoprotectant proteins, 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) in ABA signalling and 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) 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)* and upregulates transcription, thereby positively regulating ABA signalling (Chen et al 2008). We previously

showed that *det1* mutants are sensitive to ABA inhibition of seed germination and this sensitivity requires both HY5 and ABI5. Thus in *det1* mutants increased levels of HY5 appear to result in upregulation of the seed germination inhibiting transcription factor *ABI5,* resulting in less 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). Here we investigate the role of HY5 and ABI5 in ABA-mediated salt and osmotic stress phenotypes of *det1* mutants.

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 RESPONSE ELEMENT BINDING FACTORS/ABA 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, AREB1/ABF2, ABF3 and AREB2/ABF4 are the main downstream transcription factors involved in ABA-mediated osmotic stress signalling in vegetative tissues. *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

light downregulates *ABF3* expression. *ABF1* and *ABF4* are also targets of HY5 but *ABF2* is not (Lee et al 2007).

In this study we used genetic analysis to dissect the germination phenotypes of *det1* mutants in salt and osmotic stress conditions and investigate the role of HY5 and ABI5 in these phenotypes. We also investigated the role of *ABF* genes in *det1* germination phenotypes. In addition, we examined growth and developmental phenotypes of *det1 abf* double mutants in order to characterize genetic interactions between the *ABF* genes and *DET1* during development.

3.3 Materials and methods

3.3.1 Plant materials and growth conditions

Except for *abi5-1,* which is in the Ws-2 background, all *Arabidopsis thaliana* mutants in this study are in the *Columbia-0* ecotype. *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 4^0C 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 20^0C and 50% relative humidity for 14 days. Long day conditions (16 hrs of light and 8 hrs of dark) were provided by fluorescent bulbs (100 µM photons m^{-2} sec^{-1}). After 14 days seedlings were transplanted to Sunshine mix number 1 (SunGro, Bellevue,WA).

3.3.2 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 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.

3.3.3 Seedling analysis

For hypocotyl analysis, plates were grown under long day or dark conditions (after exposure to light for 6 hours 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, 2 replicates per line of 20 seedlings each. Chlorophyll was extracted with 80% acetone overnight and A_{645} and A_{663} were measured using a Spectrophotometer (model 2100 pro, Ultrospec). Chlorophyll content was calculated according to the MacKinney method (Mackinney 1941). Seedling experiments were repeated at least twice for each double mutant.

3.3.4 Adult growth parameter measurements

For adult growth analysis, the 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; in addition to total number of inflorescences, silique length, and height at 6 weeks. Each adult growth assay was performed at least twice.

3.3.5 Seed germination assays

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

3.3.6 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 1h exposure to light (100 μM photons m^{-2} sec^{-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 transcriptional 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).

3.3.7 RNA extraction and real time PCR

RNA was extracted from approximately 50 light or dark grown seedlings, or approximately 200 seeds imbibed in LS liquid media (0% sucrose with or without 150 mM NaCl) for 2 days at 4^{o}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′-GCAACCGAAGATGTAGTAGTCA-3′); *ABF3* (At4g34000) ABF3_c1276F (5′-

TACGATGGAACTGGAAGCAG-3′) and ABF3_c1385R (5′-GAGGCTCCAGAAGCTGATTT-3′); *ABF4* (At3g19290) ABF4_c1300F (5′-AACTGGAAGCCGAAATTGAAA-3′) and ABF4_c1404R (5′-ACGTTTCTTTCAGCTGCTCAT-3′). Amplified samples were normalized against *EF1α* (At5g60390) (CTGGAGGTTTTGAGGCTGGTAT, CCAAGGGTGAAAGCAAGAAGA) (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. Independent qPCR reactions were done at least 3 times and the mean values were calculated.

3.3.8 Transpirational water loss assays

In order 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 versus initial fresh weight. Two replicates were used in each experiment per genotype and the experiments were repeated at least twice.

3.3.9 Measurement of stomatal index

Whole leaves of similar developmental stage (5^{th} leaf of each plant) were detached, mounted in water, and observed immediately under an upright light microscope (Zeiss AxioVision). Five areas of 220 x 170 μm^2 per leaf from 3 different plants were imaged at 40X

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) x 100%. SI was calculated individually for each leaf and the mean was calculated per genotype (Kang et al 2009).

3.3.10 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 (4^{th} or 5^{th} 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 20^0C and 50% relative humidity, in the dark. After 2, 3 and 4 hours, 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 100X oil immersion. Pore width and length of at least 12 different stomata/genotype/treatment were measured using NIH Image J software.

3.3.11 Statistical analysis

Each experiment was repeated at least three times 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 as statistically significant.

3.4 Results

3.4.1 *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. Surprisingly, the opposite phenotype was observed. *det1* mutants exhibited resistant germination on media supplemented with salt, mannitol, or sorbitol (Figure 3.1, S3.1). 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 appear to exhibit osmotic stress resistant germination.

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

Previous 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 et al 1997). We previously found that HY5 is required for *det1* ABA sensitive germination. We proposed that on ABA *det1* mutants have excess HY5, and upregulation of germination inhibiting ABI5 via HY5 results in less 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 2016b), 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 (Figure 3.2a, S3.2). Hence this appears to be a sucrose dependent

phenotype, since on 0.6% sucrose *hy5* exhibited salt and mannitol resistant germination as reported (Figure S3.3). In *det1 hy5* double mutants, the *det1* resistant germination phenotype was suppressed on both salt and mannitol media. 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 (Figure 3.2b, S3.4). As expected, the *abi5* single 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 (Figure S3.4b). 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 dormancy-promoting 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. To sum up, *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 probably driving the early germination phenotype in *det1* mutants (Figure 3.3a).

3.4.3 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. All these phenotypes require HY5 (Fernando and Schroeder 2015). In *det1* mutants, the germination and seedling photomorphogenic response are light independent phenotypes and may be molecularly similar. In order to identify the gene/genes driving early germination in *det1* mutants, we examined public microarray data comparing *det1* and wildtype dark grown seedlings. We first hypothesized that Gibberellic Acid (GA) related genes might be upregulated in *det1,* promoting germination. However, genes promoting GA responses (for example GASA4 AT5g15230) and GA biosynthesis genes (e.g. GA-20 oxidase like protein AT4g21200) are underexpressed in *det1* dark grown seedlings (Hu et al 2002, Schroeder et al 2002). Therefore, we performed whole genome microarray data analysis using publicly available microarray data from CATdb (Gagnot et al 2008) in order to find differentially expressed genes in *det1* dark grown seedlings that might contribute to early germination. ChIP Enrich was used to identify the enriched Gene Ontology terms (GO terms) in dark grown *det1* seedlings. This analysis showed that, as expected, primarily photosynthesis related genes were upregulated in *det1* in the dark (Figure S3.5). Downregulated genes in dark grown *det1* seedlings included a number of growth related terms, consistent with its unelongated 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

(Figure 3.3b). Thus this analysis suggests 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 these GO terms. In the "response to ABA stimulus" GO term we found an ABF3 transcriptional module (Figure 3.3c). That is, the bZIP transcription factor ABF3 interacts with ABRE and DPBF binding site motifs within the 1 kb upstream region of genes that respond to ABA stimulus, and both *ABF3* and genes responding to ABA stimulus are underexpressed in *det1* mutants in the dark. Thus decreased levels of *ABF3* may result in 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 (Figure 3.3a). *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 (Figure S3.6) (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 more 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 (Yoshida et al 2010), and are somewhat induced by salt and osmotic stress (Figure S3.6) (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).

Firstly, we validated our dark grown seedling microarray analysis and examined the effect of light on *ABF3, ABF4,* and *ABF1* level using Real time PCR. As predicted, *ABF3* mRNA levels were lower in dark grown *det1* seedlings than in wild type. In light grown seedlings, *ABF3* levels were decreased in wild type relative to dark levels, and *det1* levels were not significantly different from wild type (Figure 3.4a). *ABF4* levels were also reduced in wild type in the light, but no significant difference was observed in *det1* in either condition (Figure 3.4b). In contrast, *ABF1* levels were upregulated in both dark and light in *det1* mutants (Figure 3.4c). In order to examine the role of *ABF3, ABF4,* and *ABF1* in *det1* phenotypes, we obtained T-DNA loss of function alleles of the three genes (Figure S3.7a), generated double mutants with *det1* (Figure S3.7b), then analyzed seedling and adult developmental phenotypes, as well as stress responses with respect to germination and water loss.

3.4.4 *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 (Figure S3.8a-c). In light grown seedlings, *abf3* mutants had short hypocotyls and enhanced the *det1* short hypocotyl phenotype (Figure S3.8d,e), suggesting an additive effect. *abf3* mutants also resulted in decreased cotyledon width in both the wildtype and *det1* backgrounds (Figure S3.8f). The *abf3* single mutant did not show a difference in chlorophyll content relative to the wildtype, but suppressed the *det1* pale phenotype (Figure S3.8g). In adults, *abf3* mutants exhibited delayed flowering time in terms of both days and

number of leaves, and suppressed the *det1* early flowering phenotype (Figure S3.9a,b), again suggesting an additive effect. *abf3* mutants exhibited decreased rosette diameter but did not affect rosette width or height in *det1* (Figure S3.9c,d). *abf3* did suppressed the *det1* short silique phenotype though (Figure S3.9e). *abf3* mutants also exhibited increased apical dominance, that is decreased stem number, in both the wildtype and *det1* backgrounds, thus suppressing the *det1* decreased apical dominance phenotype (Figure S3.9f). Thus, *abf3* suppressed the *det1* chlorophyll level, flowering time, silique length and apical dominance phenotypes and enhanced the *det1* light hypocotyl length phenotype.

3.4.5 *det1 abf3* salt/mannitol germination responses

det1 mutants show resistant germination on both salt and mannitol. *det1* mutants have low levels of *ABF3,* thus if *det1* phenotypes are due to the absence of ABF3, then *det1* and *abf3* should exhibit similar phenotypes. As previously reported (Finkelstein et al 2005), we found *abf3* germination was resistant to salt and mannitol (Figure 3.5). However *det1* exhibited a stronger resistance phenotype than *abf3* in both stress conditions, indicating that lack of ABF3 cannot be the sole basis of the *det1* phenotype. In the *det1 abf3* double mutants we observed some variation between lines. On salt, one of the double mutants (#1) resembled *abf3* while the other (#2) behaved like *det1*. On mannitol, double mutant #1 resembled *det1* while the other (#2) exhibited an enhanced phenotype. We also examined ABA inhibition of germination in these mutants and found that *abf3* did not have any significant effect on germination either in the wildtype, consistent with previous reports (Finkelstein et al 2005), or in the *det1* background (Figure 3.6).

3.4.6 *det1 abf4* seedling and adult phenotypes

Since *ABF1* and *ABF4* are close homologs of *ABF3* that exhibit some functional redundancy, we also examined growth and germination responses of *abf4* and *abf1* mutants. In contrast to *abf3*, *abf4* mutants had short hypocotyls in the dark and decreased both *det1* hypocotyl length and cotyledon width (Figure S3.10a-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 (Figure S3.10e,f). Similar to *abf3, abf4* suppressed the *det1* decreased chlorophyll phenotype (Figure S3.10g).

In adults, similar to *abf3*, *abf4* partially suppressed the *det1* early flowering phenotype (Figure S3.11a,b) and decreased apical dominance phenotype (Figure S3.11f). In addition, *abf4* enhanced the *det1* decreased rosette diameter and height phenotypes (Figure S3.11c,d) but had with no significant effect on silique length (Figure S3.11e).

3.4.7 *det1 abf4* salt/mannitol germination responses

In germination assays, the *abf4* single mutant exhibited resistance to salt and mannitol (Figure 3.7). 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 (Figure 3.7a). On the other hand, on mannitol *det1 abf4* mutants germinate slower than *det1* and similar to *abf4* single mutants, showing partial recue. This indicates that *abf4* suppresses *det1* and that ABF4 is required for the *det1* salt/mannitol resistant germination phenotype (Figure 3.7b). However, like *abi5*, the fact that the loss of dormancy-promoting ABF4 results in less germination in *det1* suggests this is an indirect effect. On ABA (Figure 3.8), *abf4*

exhibited resistant germination while the *det1 abf4* double mutants were nearly as resistant as the *abf4* single mutants, indicating that *ABF4* is required for *det1* ABA sensitive germination.

3.4.8 *det1 abf1* seedling and adult phenotypes

In the dark, *abf1* partially suppressed the *det1* hypocotyl length and cotyledon width phenotypes (Figure S3.12a-c). In the light, *abf1* single mutants had significantly longer hypocotyls than Col-0 and also suppressed the *det1* hypocotyl length phenotype, but did not affect cotyledon width or chlorophyll content (Figure S3.12d-g). In adults, *abf1* exhibited early flowering in terms of both days and number of leaves but suppressed the *det1* early flowering (days) phenotype (Figure S3.13a,b). Even though we did not observe a height phenotype in *abf3* and *abf4*, *abf1* mutants were significantly taller than the wild type (Figure S3.13d). In addition, *abf1* suppressed a number of *det1* growth phenotypes namely decreased rosette diameter, height, and silique length (Figure S3.13c-e). *abf1* single mutants exhibited decreased apical dominance but suppressed the *det1* reduced apical dominance phenotype (Figure S3.13f). Overall *abf1* suppressed eight of the eleven *det1* phenotypes examined and did not enhance any *det1* phenotypes.

3.4.9 *det1 abf1* salt/mannitol germination responses

Surprisingly, germination in the *abf1* single mutant was significantly delayed relative to wild type in control media, but did not exhibit a significant phenotype on stress media (Figure 3.9). This is consistent with reports that *abf1* was delayed in control but not on salt and sorbitol media (Finkelstein et al 2005). Similar to *abf1* suppression of *det1* adult growth and seedling phenotypes, germination of the *det1 abf1* double mutant was significantly delayed relative to

det1. In 100 mM salt, germination of the double mutant was even slower than wild type whereas in control conditions and on mannitol, the germination rate of the *det1 abf1* lines were more or less like the *abf1* single. Therefore, *abf1* completely suppresses the *det1* early germination phenotypes on control as well as salt and osmotic stress media. Thus, *ABF1* is epistatic to *DET1* with respect to both the control and stress germination phenotypes. On ABA, the *abf1* single mutant, like *det1*, exhibited sensitive germination (Figure 3.10). 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.

3.4.10 *ABF* mRNA levels in *det1* mutants

In order to examine the effect of salt on *ABF* gene expression in the *det1* mutant background we analyzed *ABF3, ABF4,* and *ABF1* RNA 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 (Figure 3.11), 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* (Figure 3.11a). Surprisingly, *ABF4* was repressed by salt in wildtype seeds (Figure 3.11b). Although *ABF4* mRNA levels were low in *det1, ABF4* was also repressed by salt in *det1* seeds. *ABF1* was also repressed by salt treatment in wild type but not in *det1* (Figure 3.11c).

3.4.11 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 (Figure 3.12). 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* (Figure 3.12c), suggesting that *ABF1* is required for the *det1* rapid water loss phenotype. Since *abf1* was able to partially suppress the *det1* rapid water loss phenotype, we examined stomata phenotypes in the *det1 abf1* double mutants in order to investigate the basis of this phenotype.

3.4.12 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* has a higher stomatal index than wild type and this phenotype was rescued in the *det1 abf1* double mutant (Figure 3.13a). 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, 3 and 4 hours (Figure 3.13b,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. In the double mutants, *abf1* rescued the *det1* stomatal aperture phenotype. Thus *abf1* was able to suppress both the *det1* increased stomatal index and increased stomatal aperature phenotypes, contributing to decreased water loss.

3.5 Discussion

Complex mechanisms regulate salt and osmotic stress tolerance in plants during seed germination and seedling growth. *det1* mutants exhibit a variety of phenotypes under stress conditions. The genetics behind these phenotypes and the role of DET1 in salt and osmotic stress response is the focus of this study. We found that *det1* exhibits salt resistant germination and that HY5 but not ABI5 is required for this phenotype. Analysis of public microarray data indicated that ABA response genes, including the transcription factor ABF3, are downregulated in *det1* mutants. Thus we examined the role of ABF3 and its homologues ABF4 and ABF1 in *det1* germination and developmental phenotypes.

3.5.1 Role of ABF3, ABF4, and ABF1 in *det1* salt/osmotic stress resistant germination

On salt and mannitol containing media, the *abf3* single mutant showed significantly more germination than the wild type, indicating that ABF3 plays an important role in salt/osmotic stress response. 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 ABA, salt, or sorbitol compared to the wildtype. However they reported that *abf3 abi5* double mutants show significantly enhanced germination on all three types of media, suggesting redundant roles

of ABF3 and ABI5 (Finkelstein et al 2005). In our *abf3 det1* double mutants, we observed variable response to salt and osmotic stress. Double mutant #2 resulted in more germination than line #1 in control, salt, and osmotic stress conditions. Interestingly we did not observe variation between these two lines in seedling and adult growth analysis or in ABA assays. The basis of this variation in germination is unknown. Although our *abf3* T-DNA allele has been previously shown to result in complete loss of function (Finkelstein et al 2005, Reeves et al 2011), the T-DNA insertion is located in an intron in the 5' UTR, so we tested whether it retains any residual function. However we did not detect any restoration of *ABF3* levels in the *det1 abf3* double mutants (Figure S3.14). Possibly a modifier of *det1* phenotypes is segregating in the background. Finally, loss of *ABF3* could result in variable upregulation of homologous genes. We are in the process of examining these possibilities.

ABA is a key player in stress signal transduction, triggering a cascade of events that eventually lead to induction of stress responsive genes. ABFs act downstream of SNF1-Related protein Kinase 2 proteins (SnRK2s) and are phosphorylated by SnRK2s, thereby positively mediating ABA signalling (Nakashima et al 2009, Yoshida et al 2010). Thus we examined the effect of ABA on *det1 abf3* germination. Yoshida et al (2010) showed that neither the *abf2,3*, or *4* single mutants nor the *abf2 abf3 abf4* triple mutant exhibit any difference from the wild type with respect to ABA sensitive germination. Similarly we saw that the *abf3* single mutant does not show a significant difference in germination relative to the wild type on ABA. In addition, *abf3* does not affect the *det1* ABA sensitive germination phenotype. Therefore, ABF3 is not required for *det1* ABA sensitive germination.

We found *abf4* single mutants germinated before Col-0 on both types of stress media, suggesting ABF4's role in inhibition of seed germination is a general osmotic effect and not just

an effect of ionic stress. Our results are consistent with Kim et al (2004), using the same SALK T-DNA allele we used, showed that *abf4* germinate earlier than wild type on control media and were resistant to salt stress. When we examined the effect of *abf4* on *det1*, we observed that ABF4 is required for *det1* precocious germination in control as well as salt and osmotic stress conditions. However, like ABI5, lack of ABF4, an inhibitor of germination, would result in more germination not less, therefore this may also be an indirect effect. Nonetheless *abf4* appears to be epistatic to *det1* under these conditions.

On ABA, we found that *abf4* also exhibited a resistant germination phenotype, again consistent with the results of Kim et al (2004), but in contrast to the absence of phenotype reported by Yoshida et al (2010). We found that *abf4* rescued the *det1* ABA sensitive germination phenotype. This time the effect was in the expected direction, that is lack of ABF4 resulted in more germination in the *det1* background.

We found that *abf1* did not exhibit salt or osmotic stress germination phenotypes, consistent with the results of Finkelstein et al (2005). However, *abf1* germination was delayed in control conditions. *abf1* completely rescued the *det1* precocious germination in control, salt and osmotic stress conditions, thus *abf1* is epistatic to *det1*. However, like ABF4, this rescue is in the unexpected (less germination) direction. On ABA, *abf1* mutants exhibited sensitive germination like *det1*, yet rescued the *det1* sensitive germination phenotype.

Therefore, ABF1 and ABF4 show similar functional properties with respect to *det1* germination phenotypes, rescuing control, salt, osmotic, and ABA germination phenotypes. Amino acid sequence alignment shows the degree of similarity between ABF1 and ABF4 is high, therefore they sub-group together on the ABF family phylogenetic tree, suggesting this pair of genes may have similar functional properties (Bensmihen et al 2002). Furthermore, our

molecular analysis of the *ABF* genes shows that while *ABF3* mRNA levels were induced by salt in wild type seeds, both *ABF4* and *ABF1* were repressed. Thus, *ABF3* appears to inhibit germination in the wild type seeds in salt conditions, resulting in reduced germination, while *ABF1* and *ABF4* seem to act antagonistically.

Thus, counterintuitively, reduced levels of *ABF1* and *ABF4* seem to result in less germination, both in salt conditions and in *det1* mutants. How could this be? Perhaps a homologous gene is acting redundantly. To test this we examined *ABF3* levels in our *det1 abf1* and *det1 abf4* double mutants and found that in fact *ABF3* was highly upregulated in the double mutants (Figure 3.14). The increased *ABF3* then provides a basis for the decreased germination observed in the *det1 abf1* and *det1 abf4* double mutants.

3.5.2 Effects of stress on *ABF* gene expression

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 three 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 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. In seven day old seedlings, *ABF1* and *ABF4* are induced twofold by ABA while *ABF3* was induced 8.5 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. We found that while *ABF3* was upregulated by salt in imbibed seeds as expected, *ABF1* and *ABF4* were unexpectedly downregulated. The basis of this conflicting pattern is unknown but is it consistent with the opposing effects we observe with respect to *det1* germination on medium containing salt. We also found that *ABF1/3/4* levels were very low in *det1* mutants in both in control and salt stress conditions. Thus low levels of the germination inhibiting ABF proteins in *det1* mutants may contribute to precocious germination in both control and stress conditions.

3.5.3 Role of *ABF* genes on *det1* seedling and adult phenotypes

With respect to growth and development, the *abf* mutants exhibited a number of different phenotypes. In dark grown seedlings, *abf4* has shorter hypocotyls whereas *abf1* and *abf3* did not have a significant phenotype. In the double mutants, *abf4* enhanced while *abf1* suppressed the *det1* short hypocotyl phenotype. In contrast, both *abf4* and *abf1* decreased *det1* dark cotyledon width. In light grown seedlings, *abf3* has shorter hypocotyls and decreased cotyledon width in both the wildtype and *det1* backgrounds suggesting ABF3 may have some contribution in promoting light growth and DET1 and ABF3 act in the same pathway with regards to this phenotypes. ABA and light have antagonistic effects on gene expression of some genes (Weatherwax et al 1996). *ABF3* is downregulated by light (Lee et al 2007). However, we see an effect of *abf3* mutation in light seedlings but not in dark grown seedlings. *ABF4* levels are also downregulated by light and *abf4* mutants exhibit phenotypes in both dark and light. For example, *abf4* has larger cotyledons in the light but enhances the *det1* small cotyledon phenotype, while both *abf3* and *abf4* suppress the *det1* decreased chlorophyll phenotype. *abf1* increases light

hypocotyl length in both the wildtype and *det1* backgrounds. *ABF1* is upregulated in *det1* in both light and dark, consistent with *abf1* rescue of both light and dark grown *det1* phenotypes.

In adults, both *abf3* and *abf4* exhibit delayed flowering as well as delay flowering in *det1*. In contrast, *abf1* shows early flowering but delays flowering in *det1*. A recent study done using triple and quadruple *abf* mutants found that flowering is delayed in all the *abf* multiple mutants relative to wild type in terms of both days and number of rosette leaves at bolting. However they found no significant difference between the triple and quadruple mutants indicating absence of one gene does not significantly affect flowering time (Yoshida et al 2015).

Overall in adults, *abf4* partially suppressed the *det1* flowering time and apical dominance phenotypes but enhanced the decreased rosette diameter and height phenotypes. *abf3* partially rescued the *det1* flowering time, apical dominance and silique length phenotypes while *abf1* suppressed the *det1* flowering time, apical dominance, rosette, height and silique length phenotypes. These results indicate that *ABF* genes are not always redundant. *ABF* genes are expressed mostly in vegetative tissues and are reported to have a role in stress tolerance in vegetative tissues (Fujita et al 2011)

3.5.4 *det1* rapid water loss phenotype is independent of *ABF3* and *ABF4*

abf3 and *abf4* single mutants did not show a significant difference relative to the wild type in our water loss assays. Kim et al (2004) found the transpiration rate in *abf3* mutants was slightly higher than in wild type, while *abf4* did not differ significantly from wild type, similar to our results. However, the *abf2 abf3 abf4* triple mutants show increased water loss rates, suggesting the *ABF* genes may act redundantly with respect to desiccation tolerance (Yoshida et al 2010). The authors suggest this may be linked to stomatal movements during water-deprived

conditions. However, *abf3* and *abf4* single mutants have significantly reduced tolerance to drought stress. In water-deprived conditions, the survival rates of *abf3* and *abf4* mutants were lower than those of wild type. In addition to alterations in transpiration rates, this phenotype can be partially attributed to the down-regulation of LEA class dehydrin protein coding genes in the *abf1 abf3 abf4* triple mutant, which play critical roles in cellular dehydration stress tolerance (Kim et al 2004, Yoshida et al 2010).

In the *det1* background neither *abf3* nor *abf4* rescue the rapid water loss phenotype. Thus ABF3 and ABF4 are not required for the *det1* water loss phenotype. We have previously shown that this *det1* phenotype is also independent of HY5 and ABI5 (Fernando and Schroeder 2015).

3.5.5 Role of *ABF1* in *det1* desiccation tolerance

Despite the fact that *ABF1* is transcriptionally less abundant compared to the other AREB/ABF genes, Yoshida et al (2015) showed recently that ABF1 does have a role in ABA signalling under drought stress. By comparing triple to quadruple *abf* mutants they showed that lack of *ABF1* reduces drought tolerance and ABA sensitivity through downregulation of downstream drought responsive transcription factors, yet does not alter transpiration rate or stomatal apertures (Yoshida et al 2015). In our experiments, although the *abf1* single mutant did not show a clear water loss phenotype, the *det1 abf1* double mutants exhibited significantly less water loss than *det1*. This suggests that *ABF1* plays a role in transpiration in the *det1* background. We hypothesized that this effect might be associated with stomata number or closure so we examined stomatal phenotypes of these mutants.

We determined stomatal index (SI), an indicator of stomatal density, and found that *det1* mutants have a higher SI. 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.

We also examined stomatal apertures and found that *det1* mutants exhibit delayed stomatal closure in response to ABA compared to wild type (Figure 3.13). 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 the case of COP1 (Khanna et al 2014). *det1 abf1* double mutants also rescued the *det1* delayed stomatal closure phenotype, 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/mannitol 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 *abf4* and *abf1* rescue the *det1* resistant

germination phenotype, but *abf3* does not. *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.

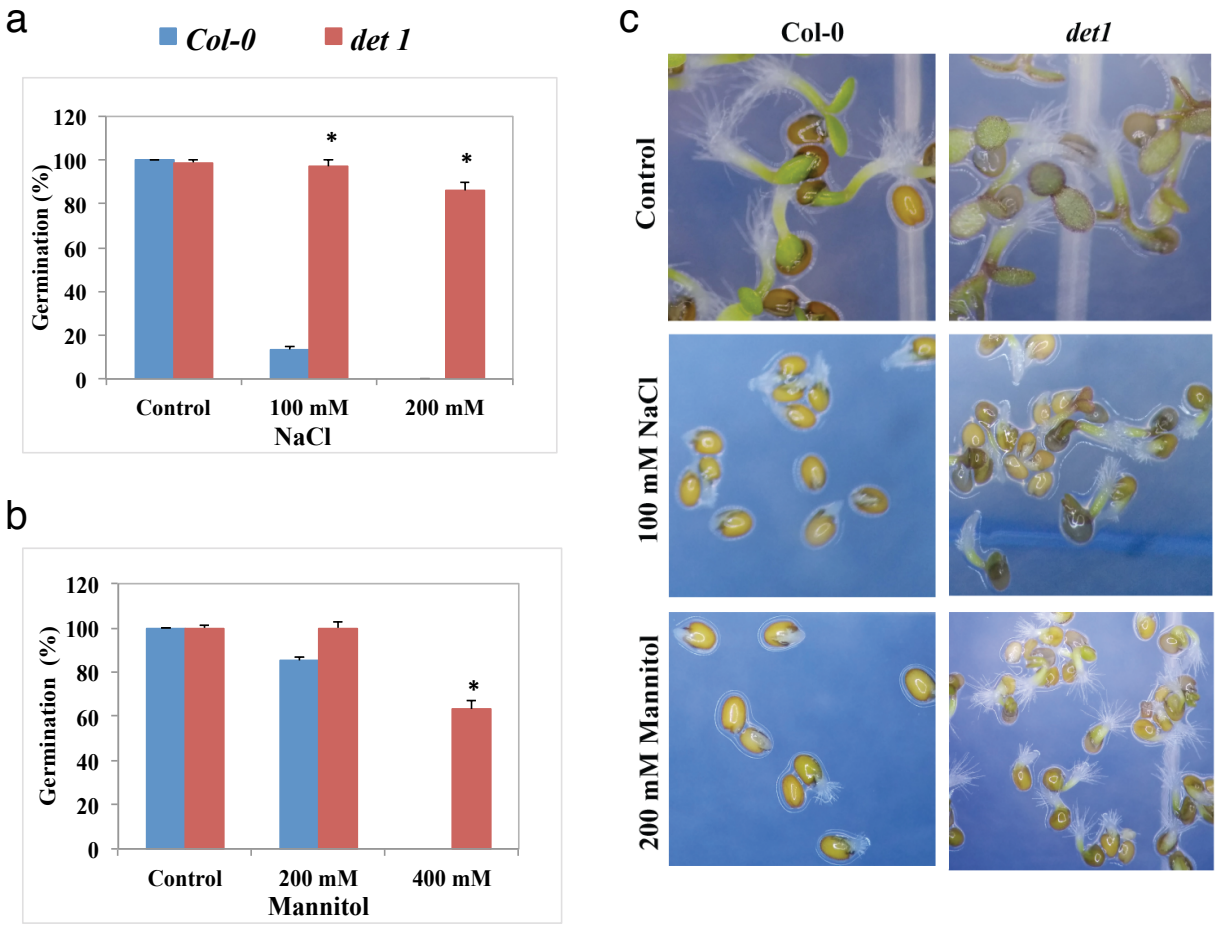

Figure 3.1 *det1* mutants exhibit salt and mannitol resistant germination.

Germination of *det1* mutants on (a) NaCl and (b) Mannitol after 3 days. Values are means ± SE of 2 replicates of 50-100 seeds. *=P< 0.05 of *det1* versus wild type. (c) Representative germination and cotyledon emergence of Col-0 and *det1* on control media, 100 mM NaCl and 200 mM Mannitol after 3 days.

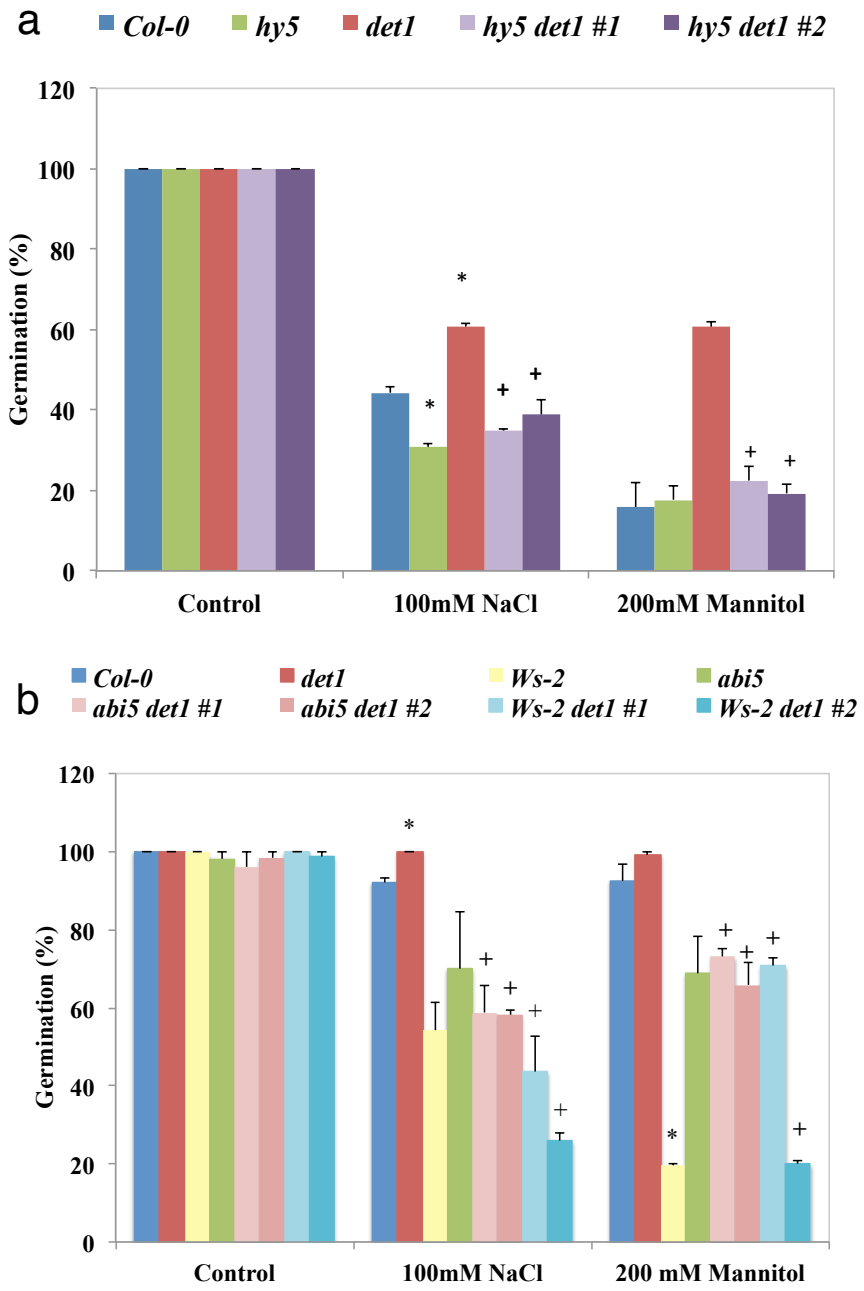

Figure 3.2 Germination in *det1 hy5* and *det1 abi5* double mutants

Germination (%) of (a) *det1 hy5* and (b) *det1 abi5* on control, 100 mM NaCl and 200 mM Mannitol after 2 days. Values are means ± SE of 2 replicates of 50-100 seeds.. *=P ≤ 0.05 of single mutants vs appropriate wildtype, + =P ≤ 0.05 of doubles vs *det1*.

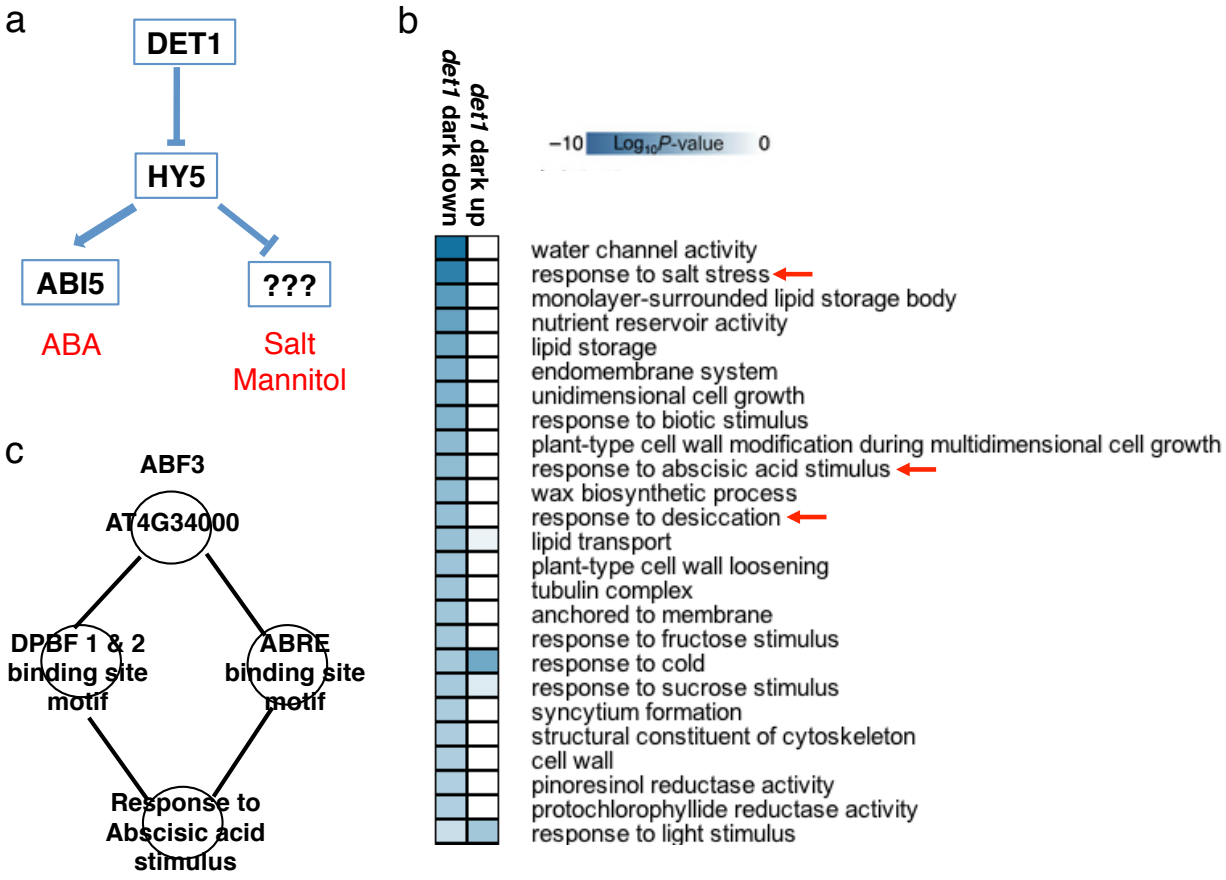

Figure 3.3 Identification of candidate gene(s) driving germination in *det1* mutants in salt/osmotic stress conditions.

(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.

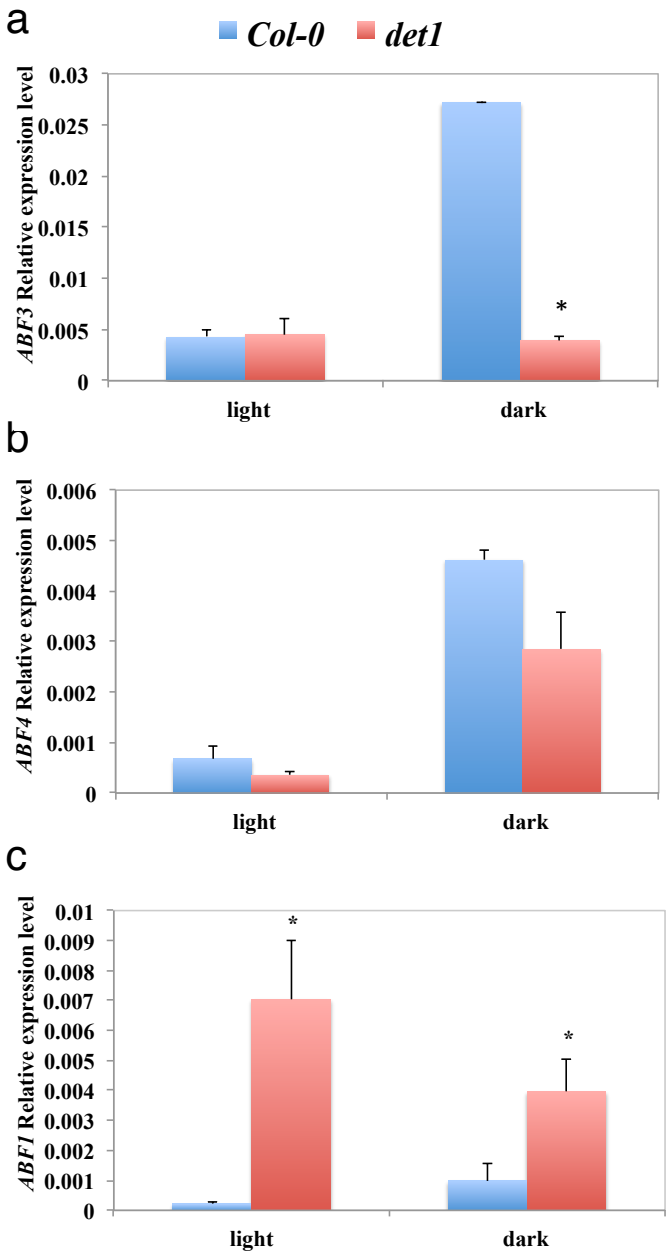

Figure 3.4 *ABF* gene expression in *det1* dark and light grown seedlings

(a) *ABF3* (b) *ABF4* and (c) *ABF1* mRNA transcript abundance in dark and light grown wildtype and *det1* seedlings. Values are normalized relative to the reference gene *EF1α*. Error bars indicate SE of 3 technical replicates. *=P≤ 0.05 of *det1* vs Col-0.

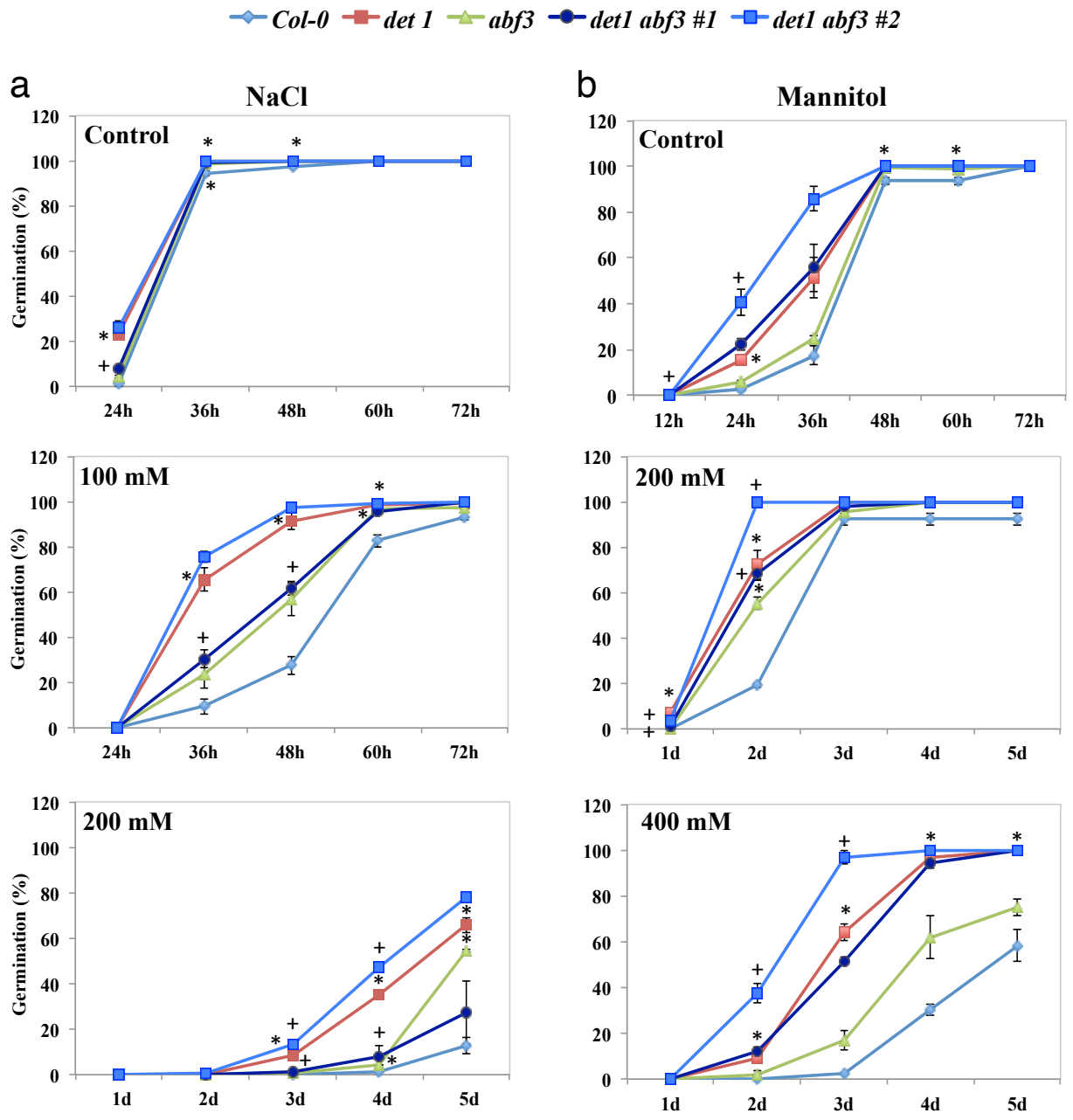

Figure 3.5 Germination in *det1 abf3* double mutants

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

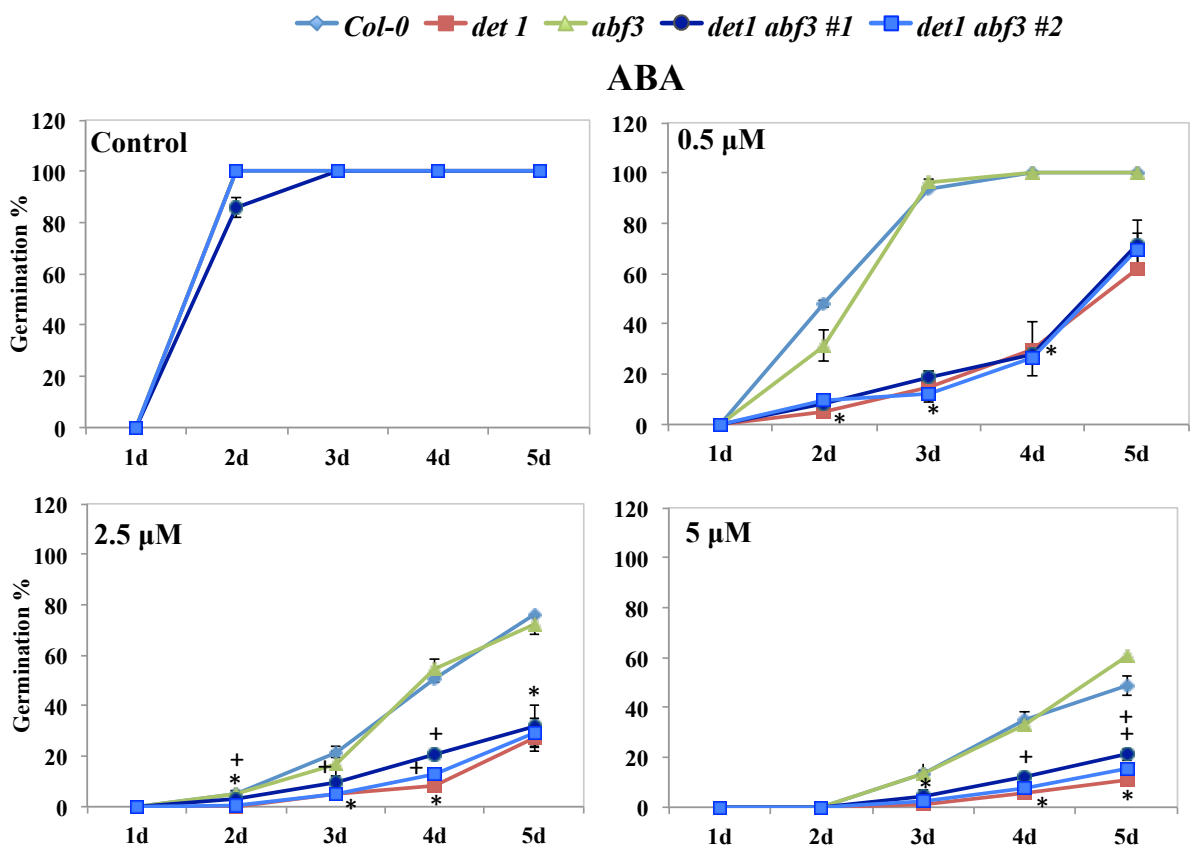

Figure 3.6 Germination in *det1 abf3* double mutants on ABA media.

Germination (%) on media supplemented with 0, 0.5 µM, 2.5 µM, or 5µM ABA. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

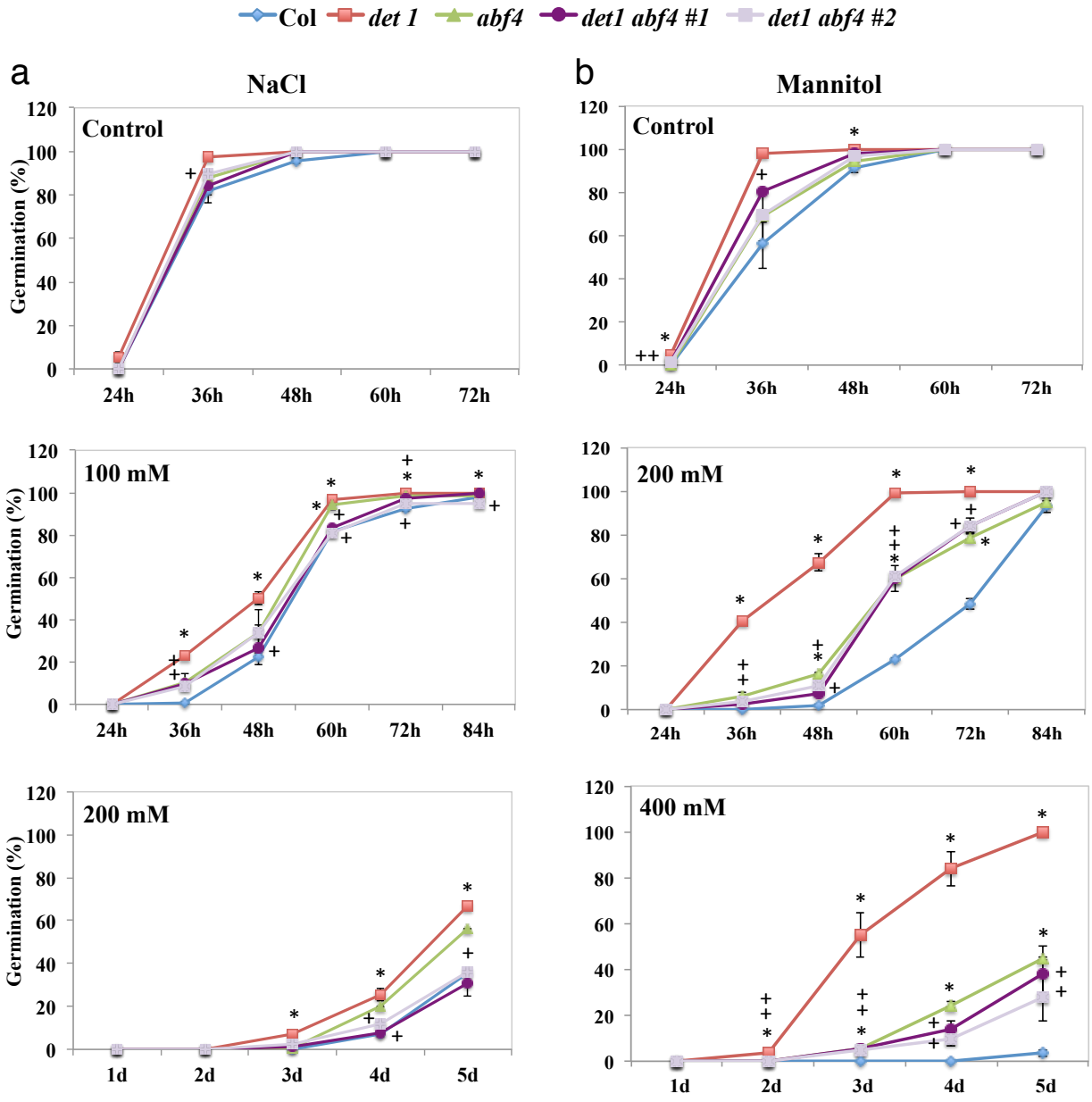

Figure 3.7 Germination in *det1 abf4* double mutants.

Germination (%) on (a) 0, 100mM, 200mM NaCl (b) 0, 200mM, 400mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

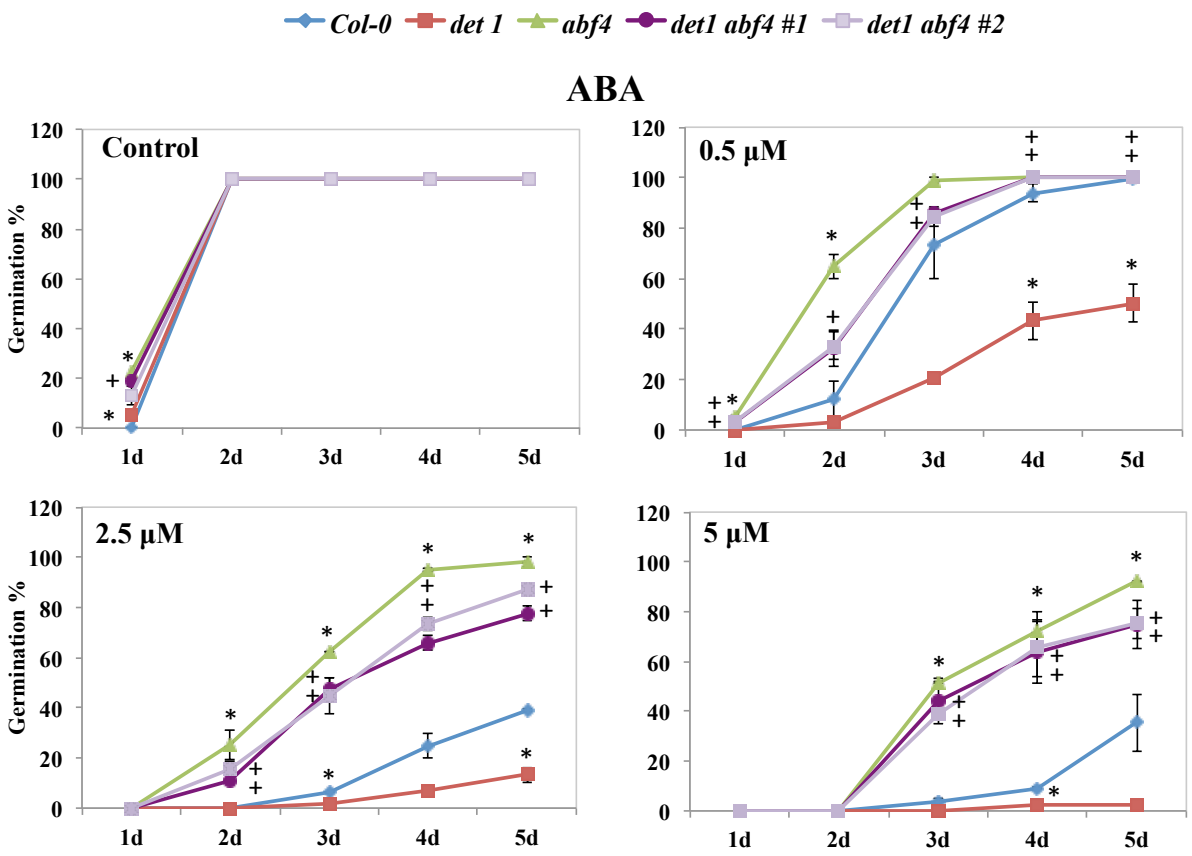

Figure 3.8 Germination in *det1 abf4* double mutants on ABA media.

Germination (%) on media supplemented with 0, 0.5 µM, 2.5 µM or 5 µM ABA. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

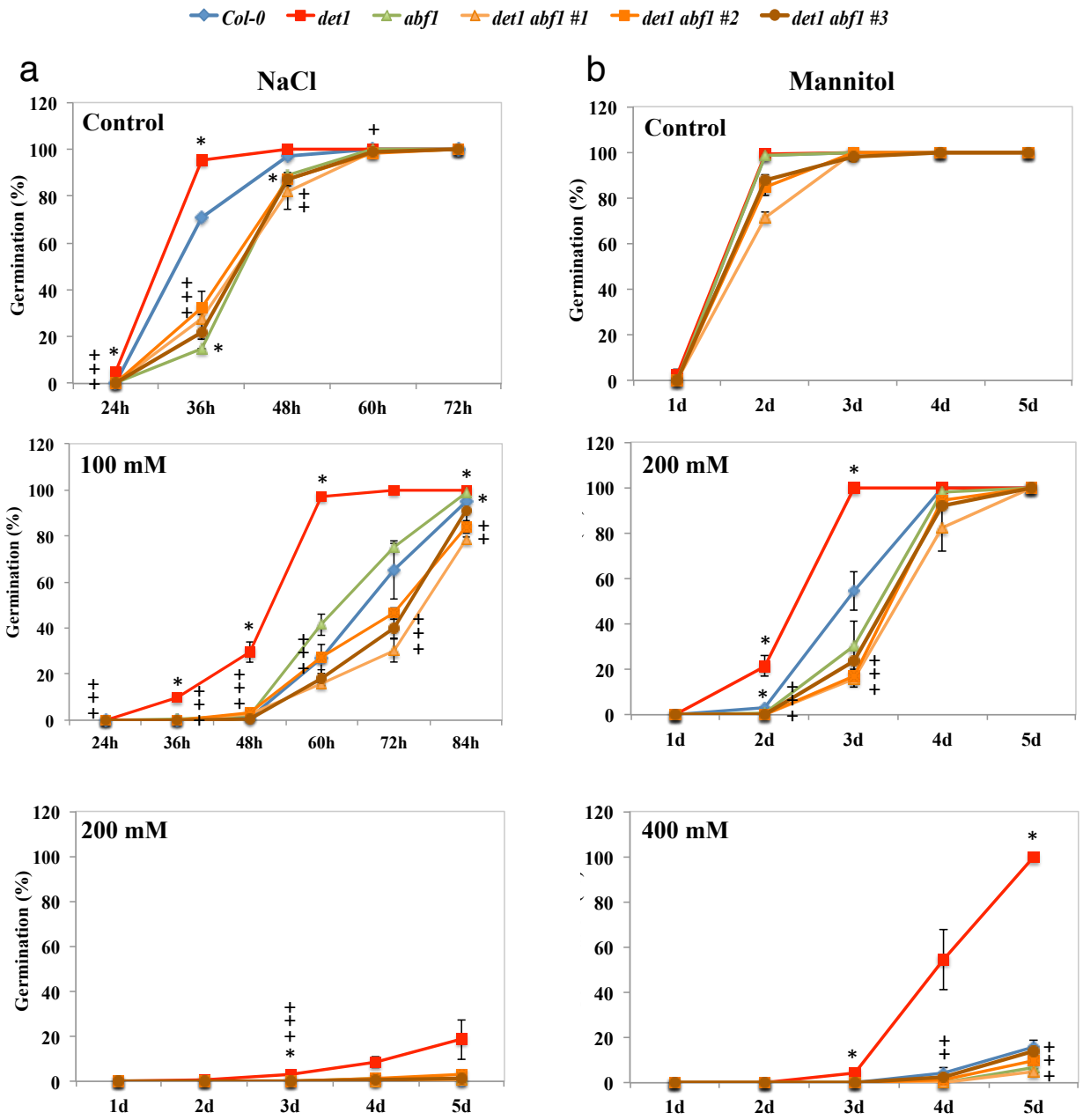

Figure 3.9 Germination in *det1 abf1* double mutants.

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

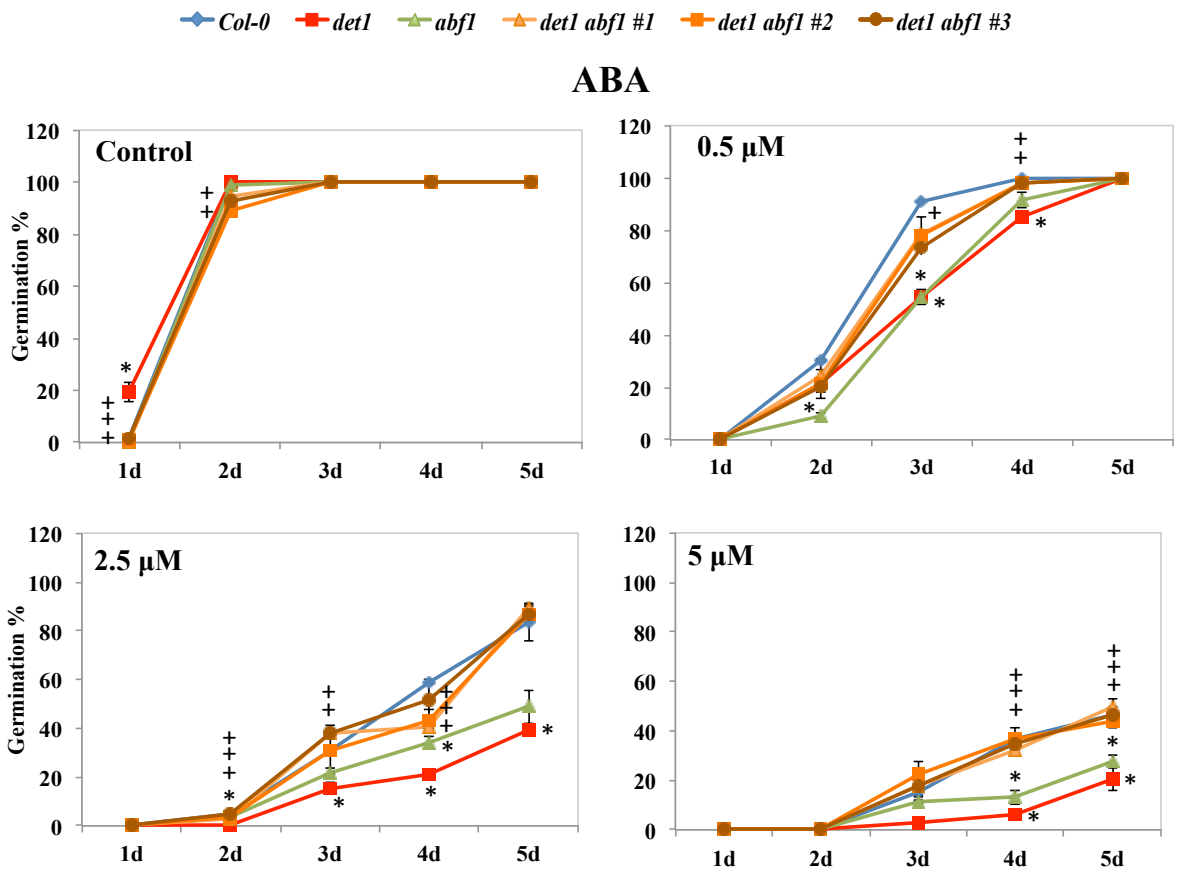

Figure 3.10 Germination in *det1 abf1* double mutants on ABA media.

Germination (%) on media supplemented with 0, 0.5 µM, 2.5 µM and 5µM ABA. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

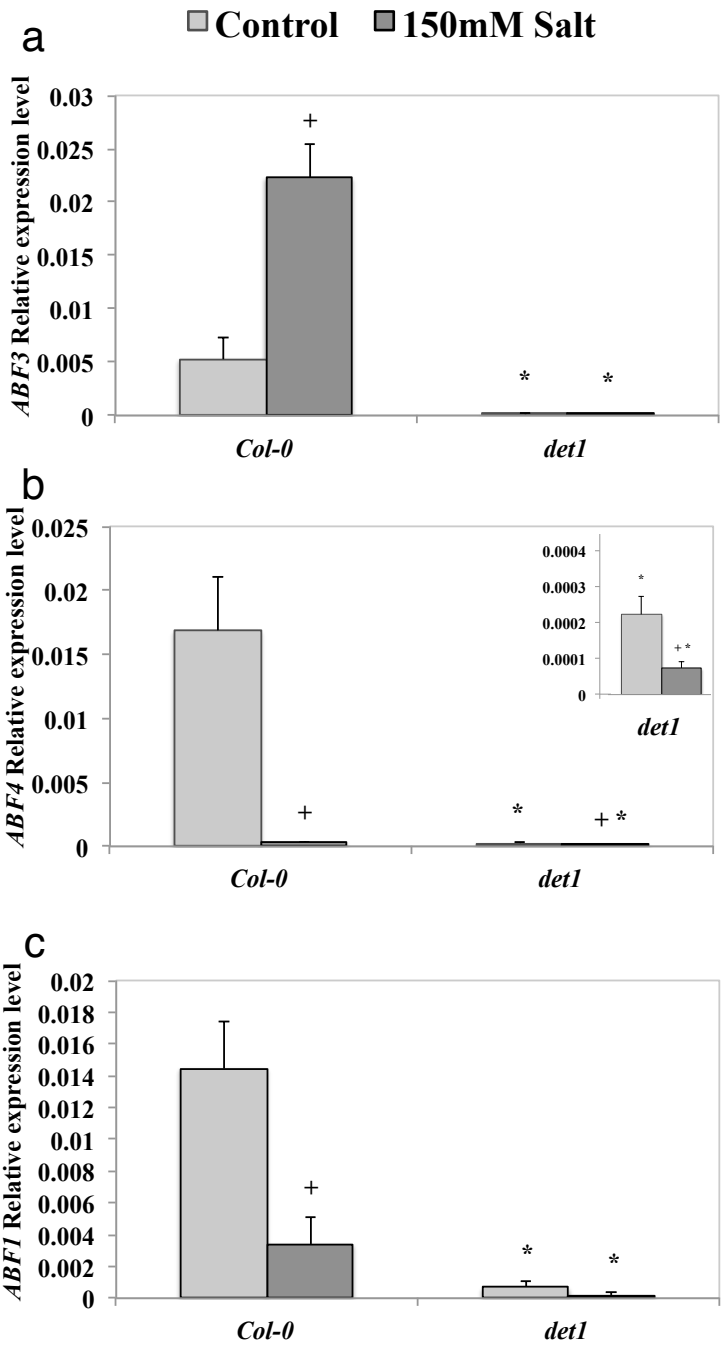

Figure 3.11 Effect of salt on *ABF* transcript levels in *det1* seeds

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 4^0C. Values are normalized relative to the reference gene *EF1α.* Error bars indicate SE of 6 technical replicates. *=P≤ 0.05 of *det1* vs Col-0 and + =P≤ 0.05 of + NaCl vs – NaCl

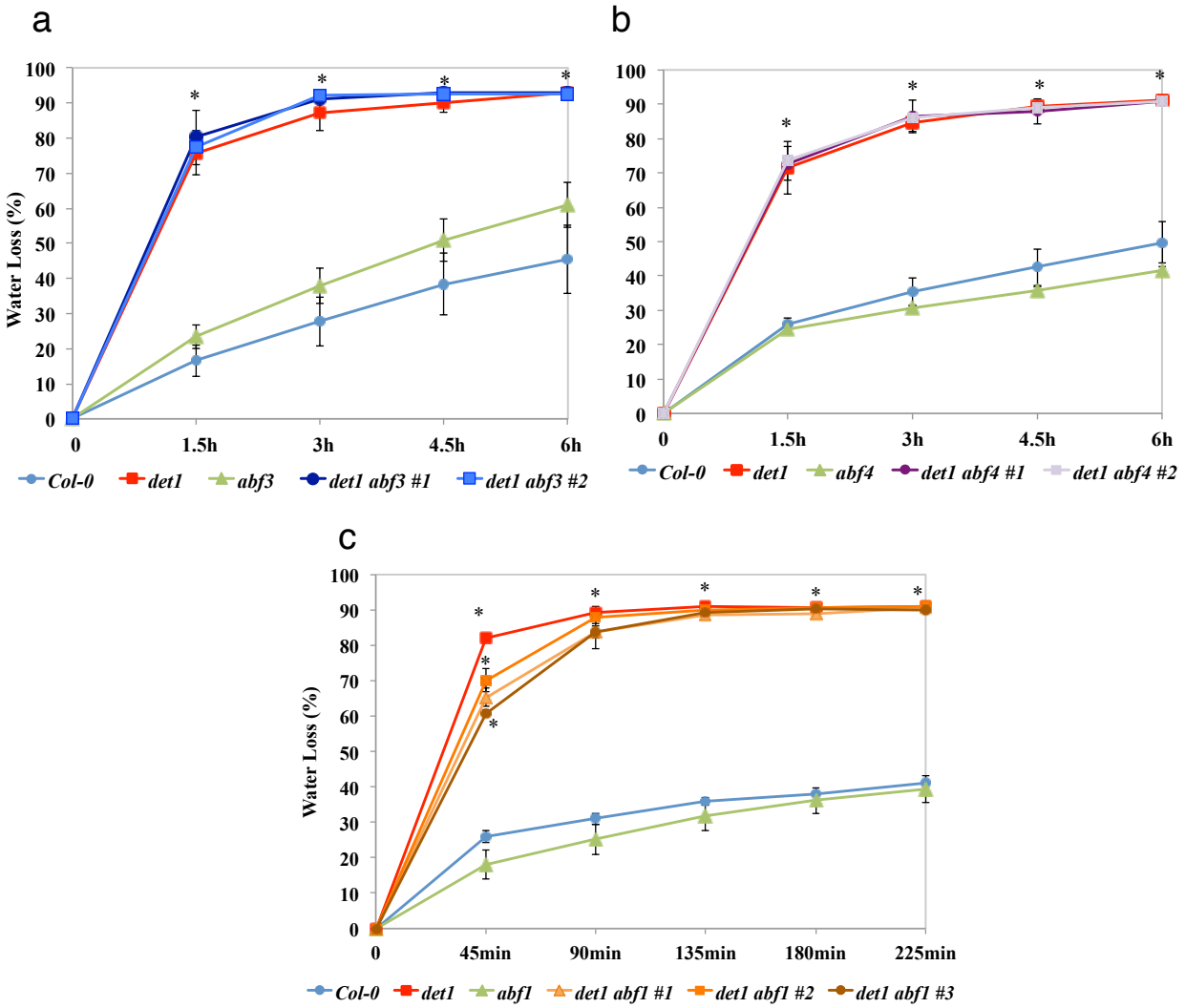

Figure 3.12 *det1* rapid water loss phenotype requires ABF1 but not ABF3 or ABF4

Water loss from detached leaves as a % loss of fresh weight. Values are means ± SE of 2 samples of 3-6 leaves, *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

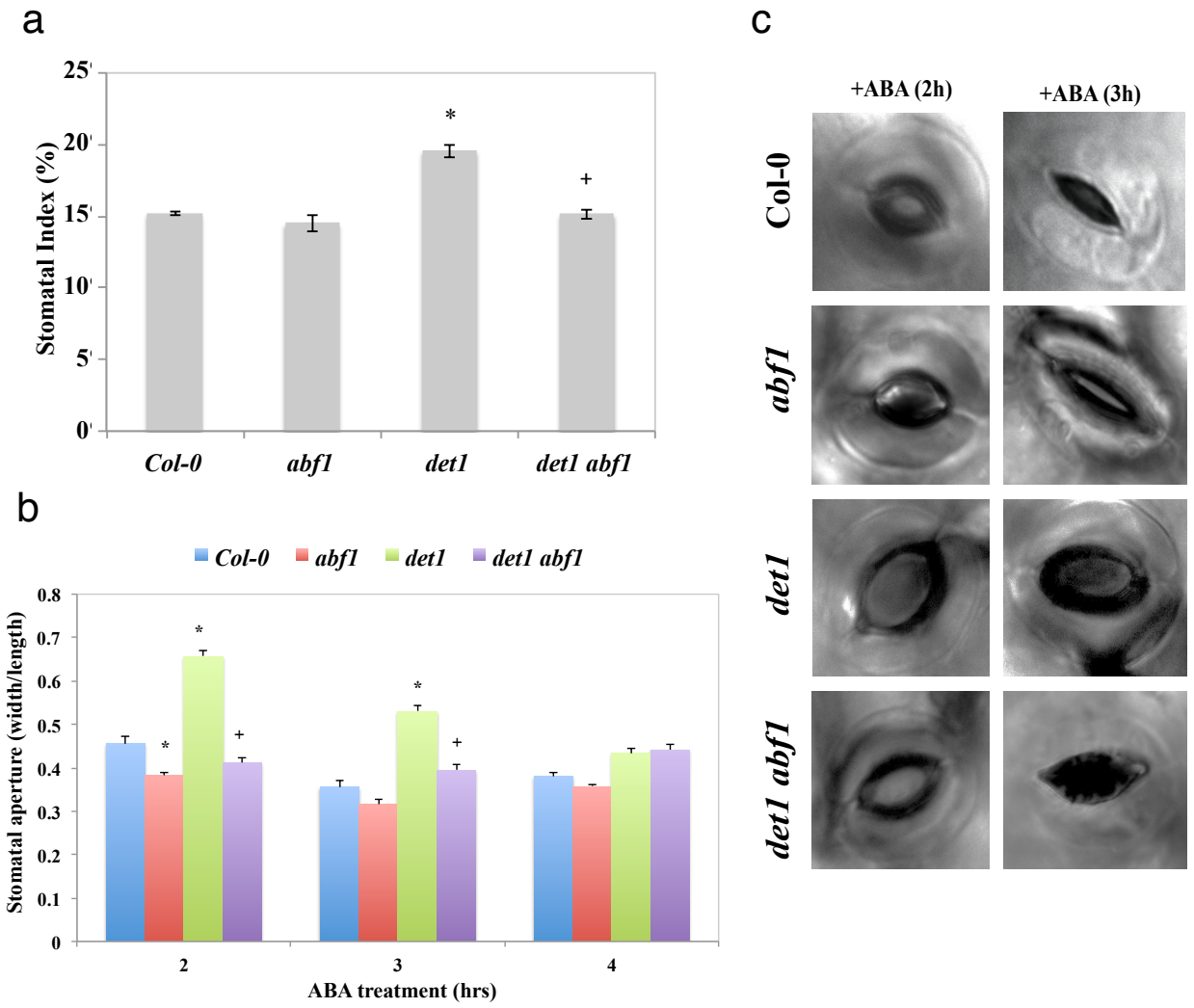

Figure 3.13 *det1* stomatal phenotypes are suppressed by *abf1*

(a) Stomatal Index (number of stomata/(number of stomata + number of pavement cells) X 100%) as mean ± SE of 5 areas of 3 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 hours (d) Stomatal pore phenotypes. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

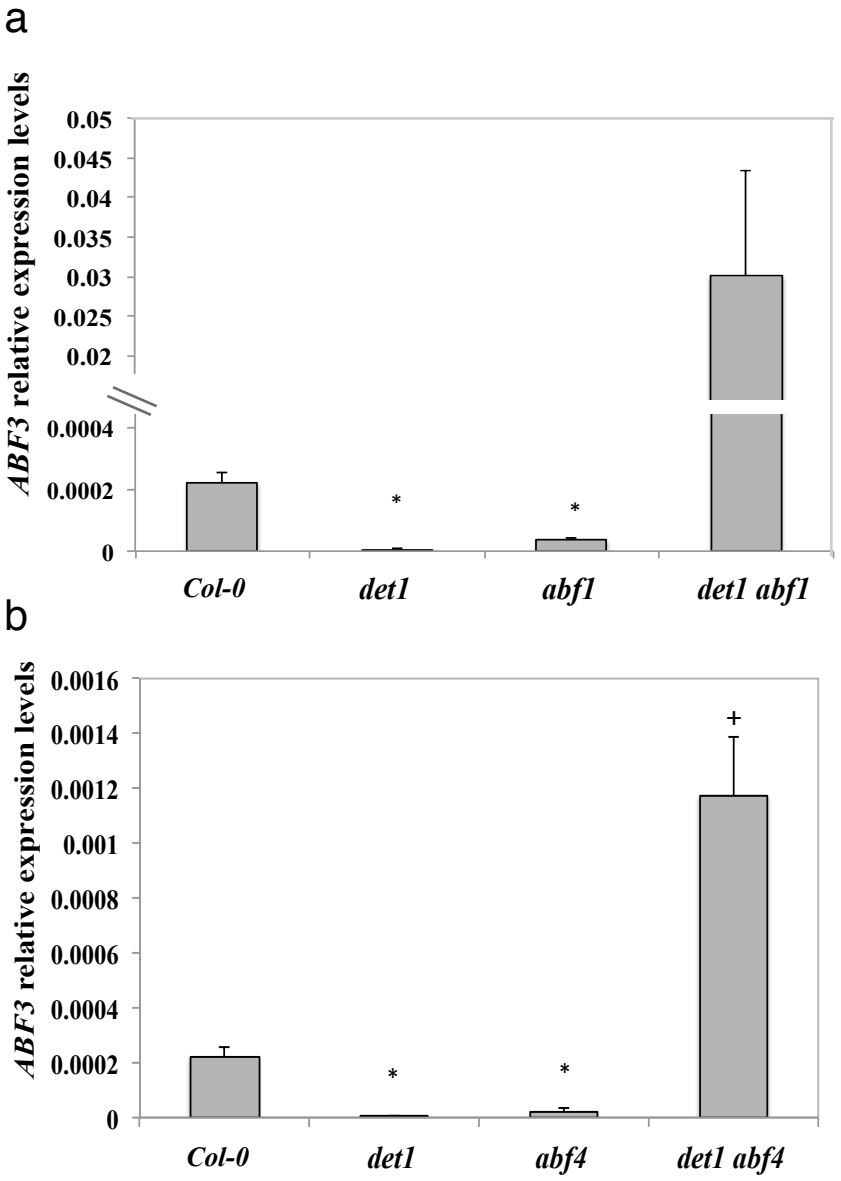

Figure 3.14 *ABF3* transcript levels are upregulated in salt treated *det1 abf1* and *det1 abf4* seeds

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 4^0C. Values are normalized relative to the reference gene *EF1α*. Error bars indicate SE of 3 technical replicates. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

3.5 Supplementary data

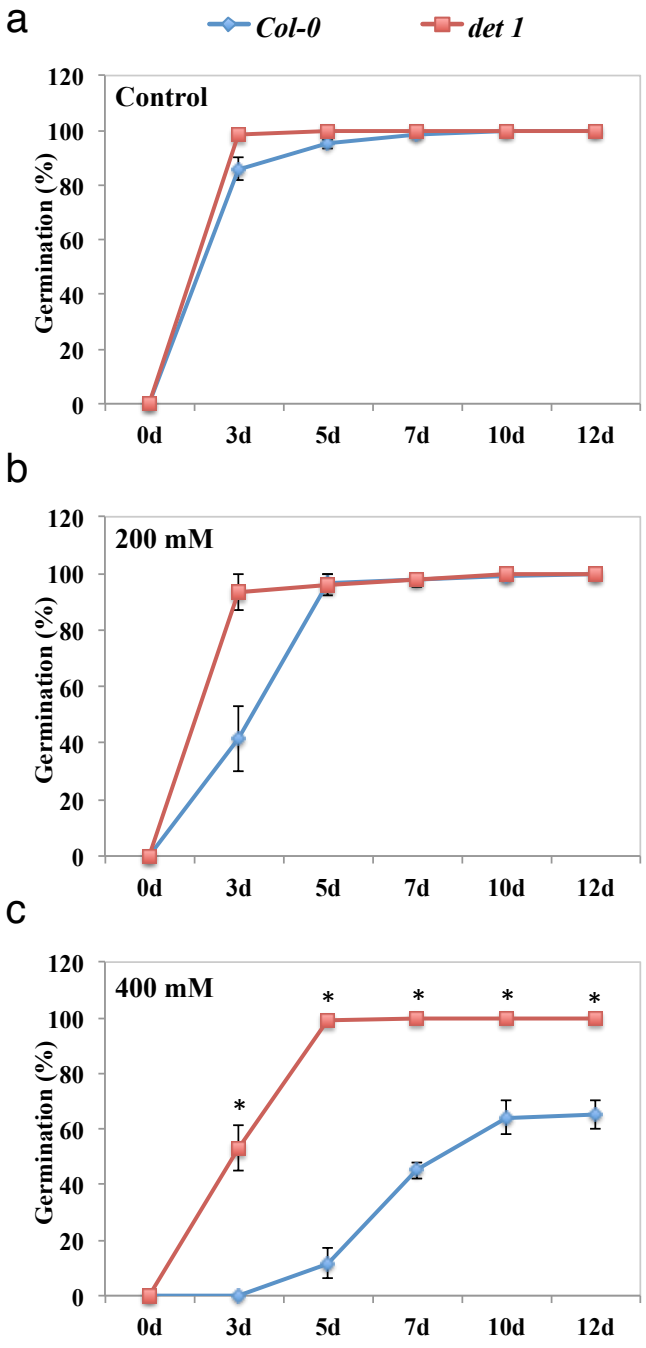

Figure S3.1 *det1* mutants exhibit sorbitol resistant germination

Germination of *det1* mutants on (a) control (b) 200 mM and (c) 400 mM sorbitol. Values are means ± SE of 2 replicates of 50-100 seeds, *=P< 0.05 of *det1* vs wild type.

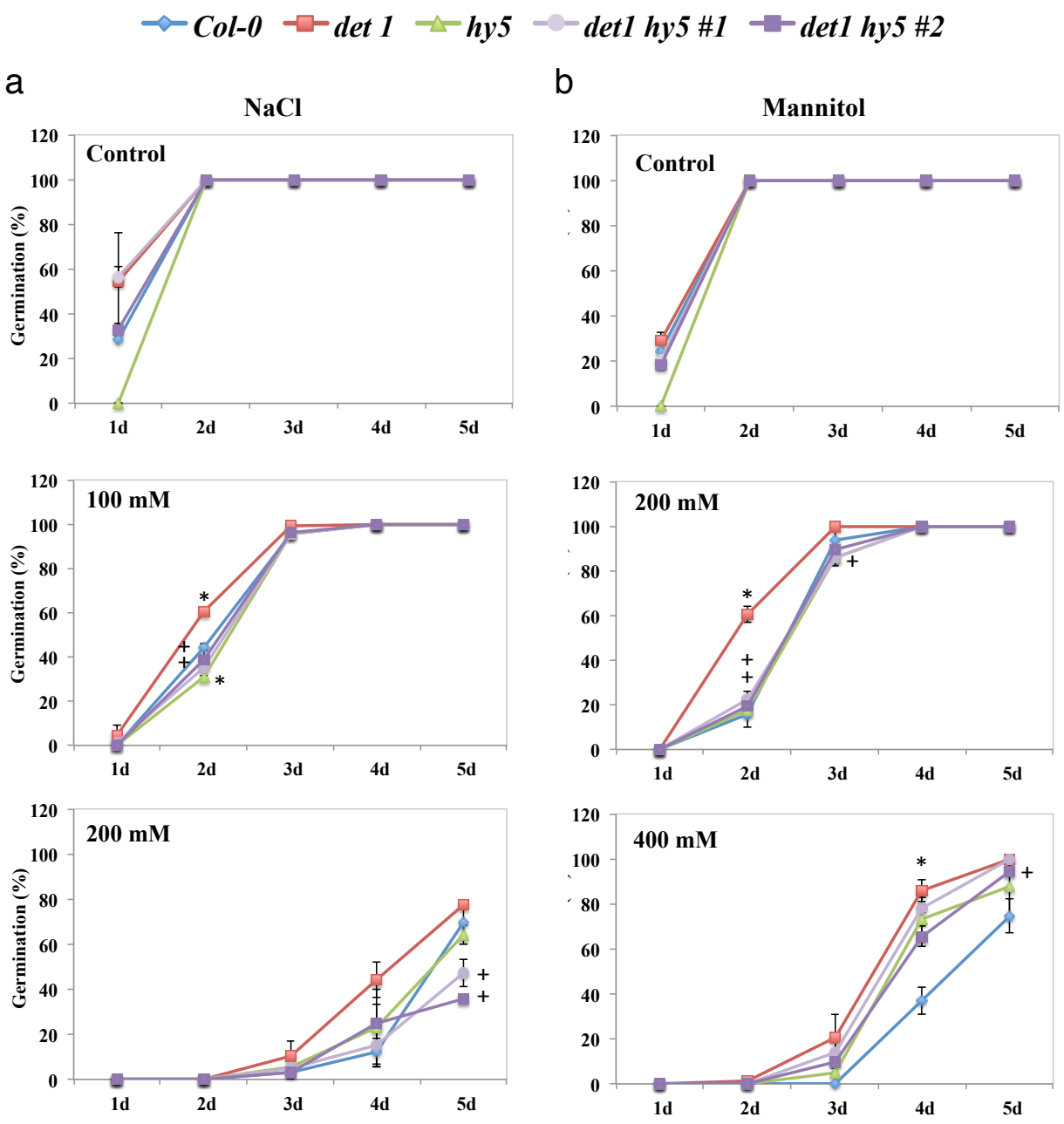

Figure S3.2 Germination in *det1 hy5* double mutants

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

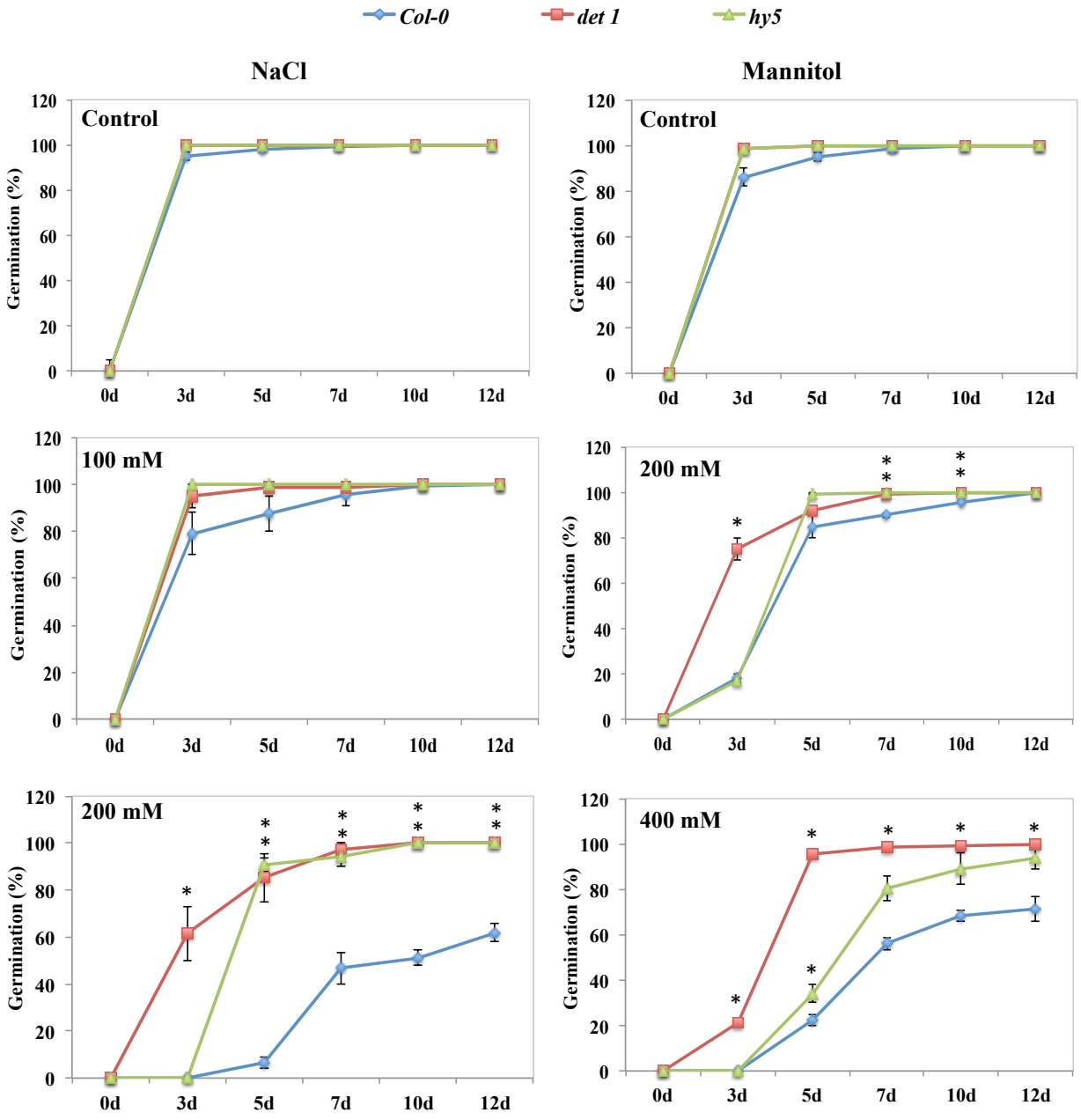

Figure S3.3 Germination in *hy5* single mutants on 0.6% sucrose supplemented media

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype.

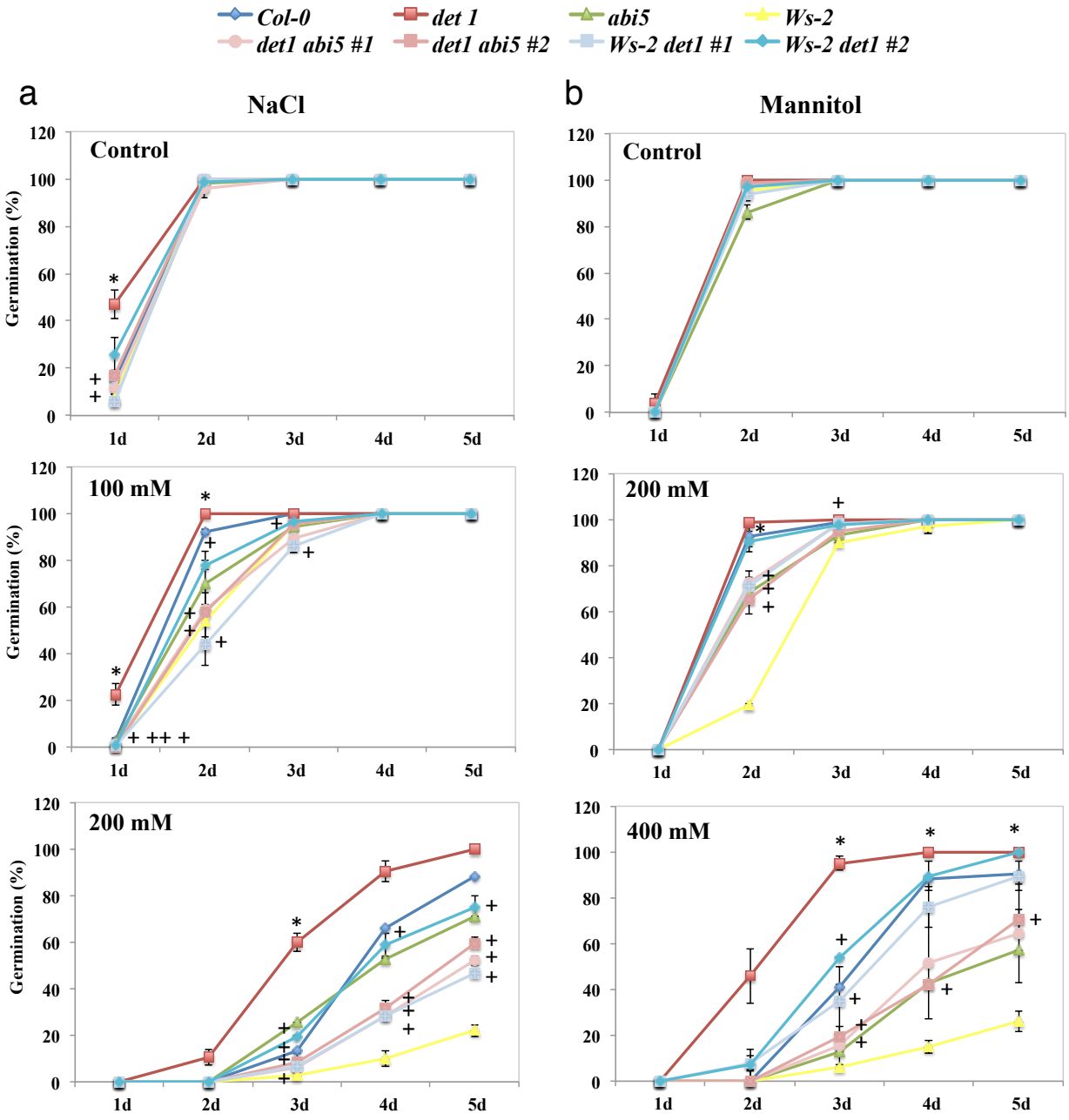

Figure S3.4 Germination in *det1 abi5* double mutants

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

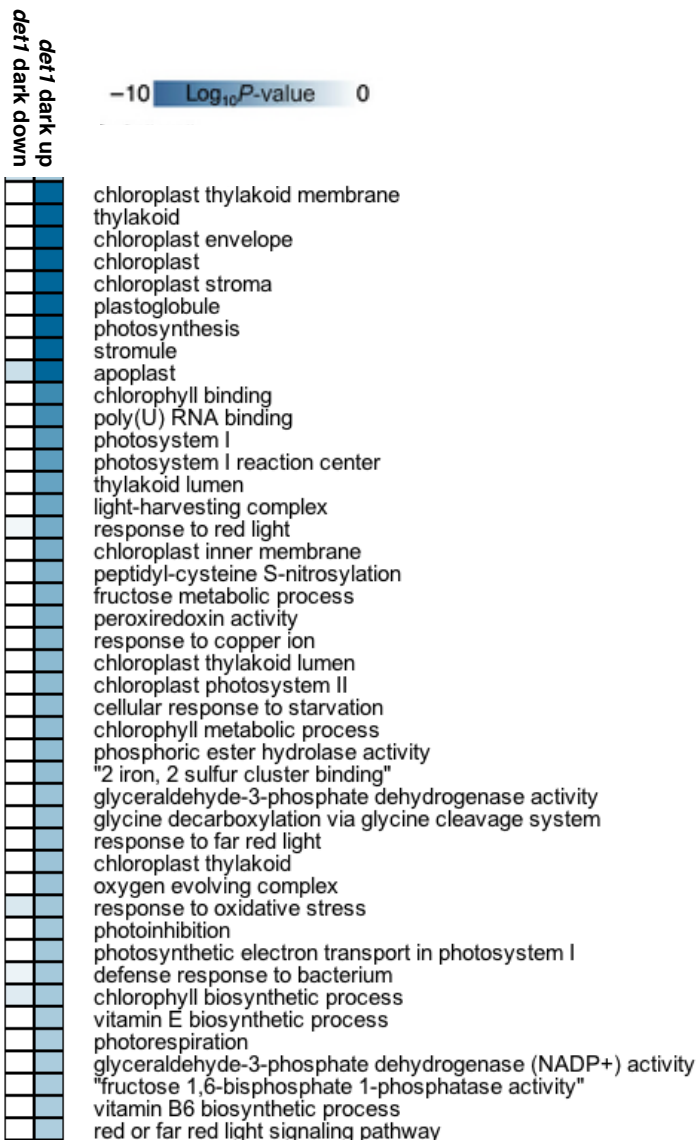

Figure S3.5 Heat map of enriched GO terms in microarray data of *det1* dark grown seedlings showing upregulated genes

Heat map of enriched GO terms (visualized in Multiple Expression viewer) upregulated in *det1* dark grown seedlings in publicly available microarray data. Left column indicates downregulated GO terms and right column represents upregulated GO terms in dark grown det1 seedlings. 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.

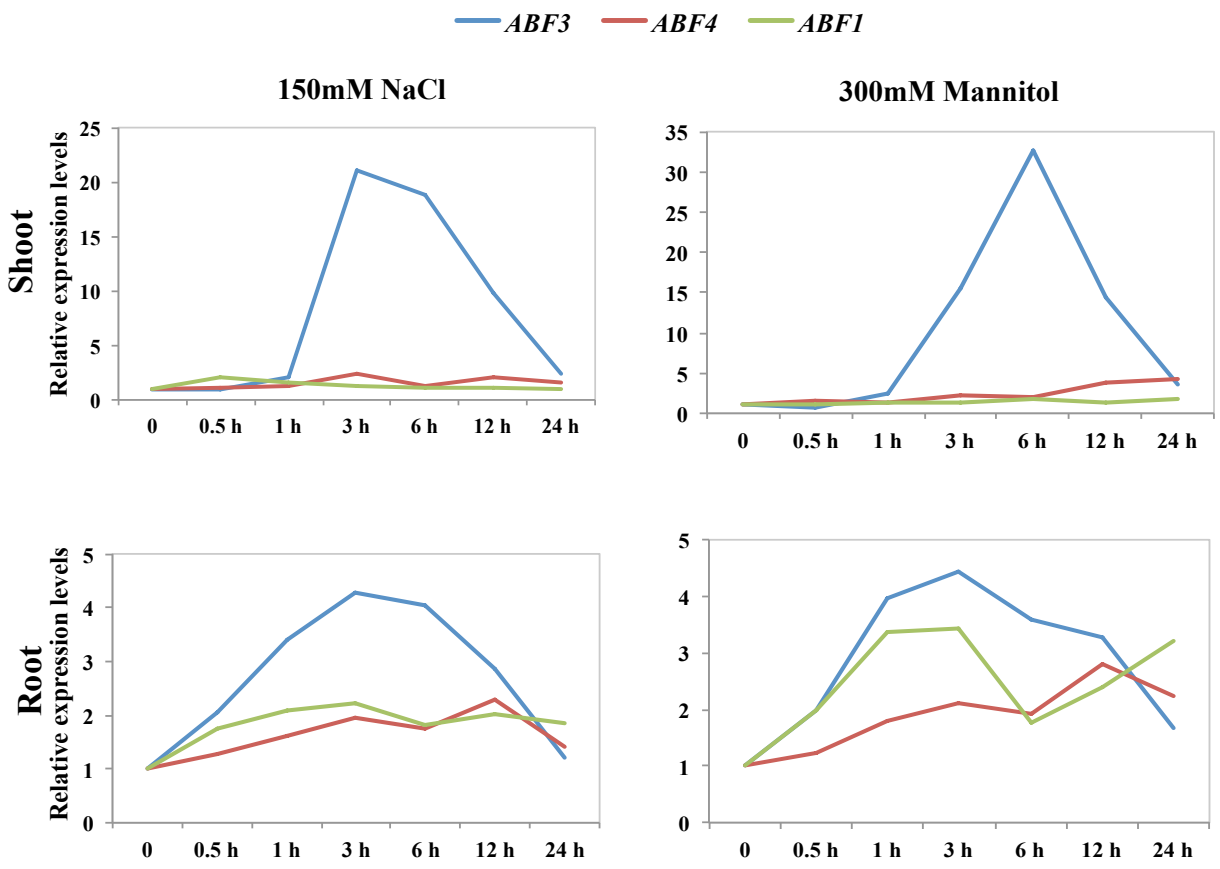

Figure S3.6 Relative expression levels of *ABF* genes under salt and osmotic stress

Relative expression levels of *ABF* genes in aerial parts under (a) salt and (b) osmotic stress and in roots under (c) salt and (d) osmotic stress (Kilian et al 2007) accessed via AtGenExpress. *ABF1, ABF3,* and *ABF4* are upregulated by salt and osmotic stress to varying degrees.

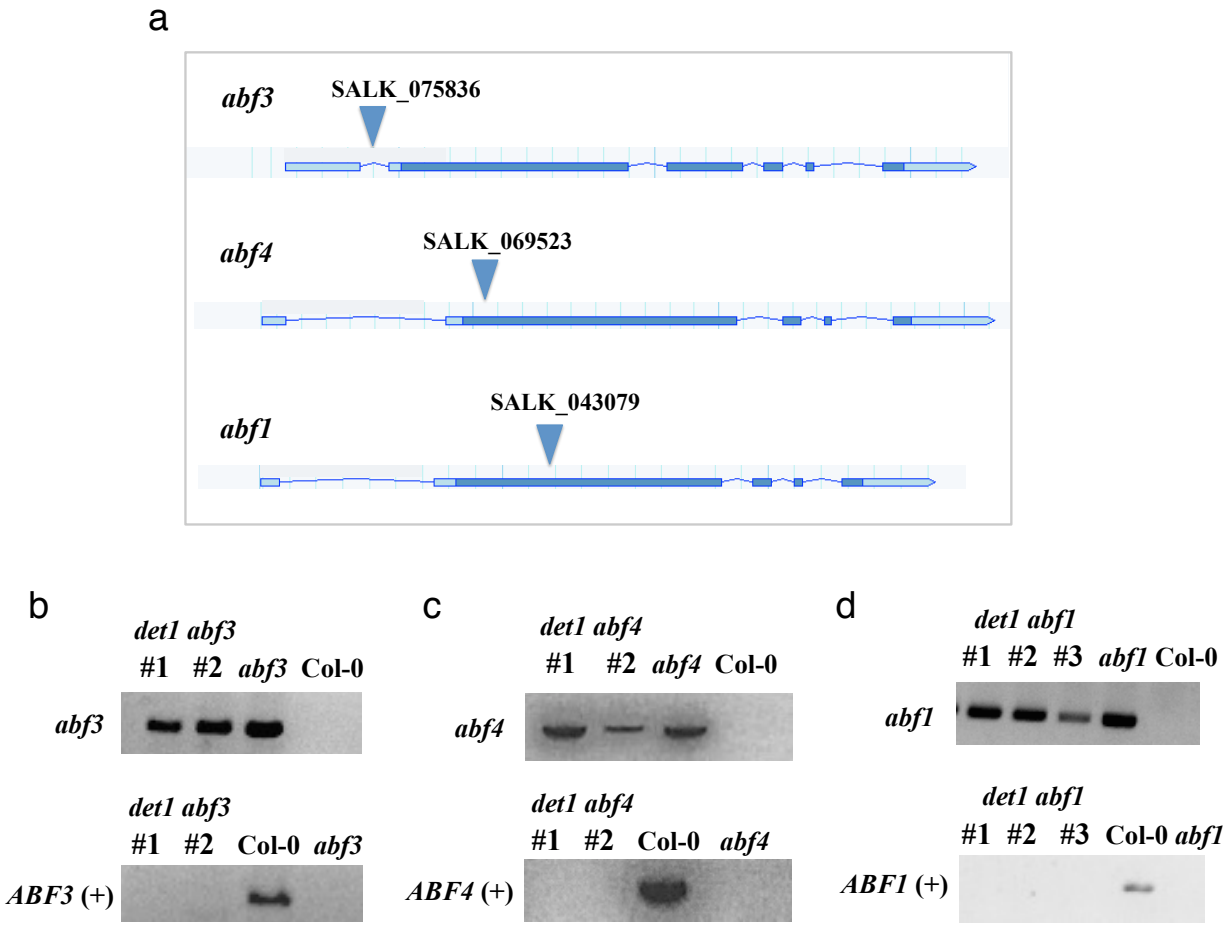

Figure S3.7 (a) T-DNA alleles of Arabidopsis mutants in *ABF3* (At4g34000), *ABF4* (At3g19290), and *ABF1* (At1g49720) used for the double mutant analysis. PCR genotyping of double mutant lines generated for this study (b) *det1 abf3* (c) *det1 abf4* and (c) *det1 abf1*.

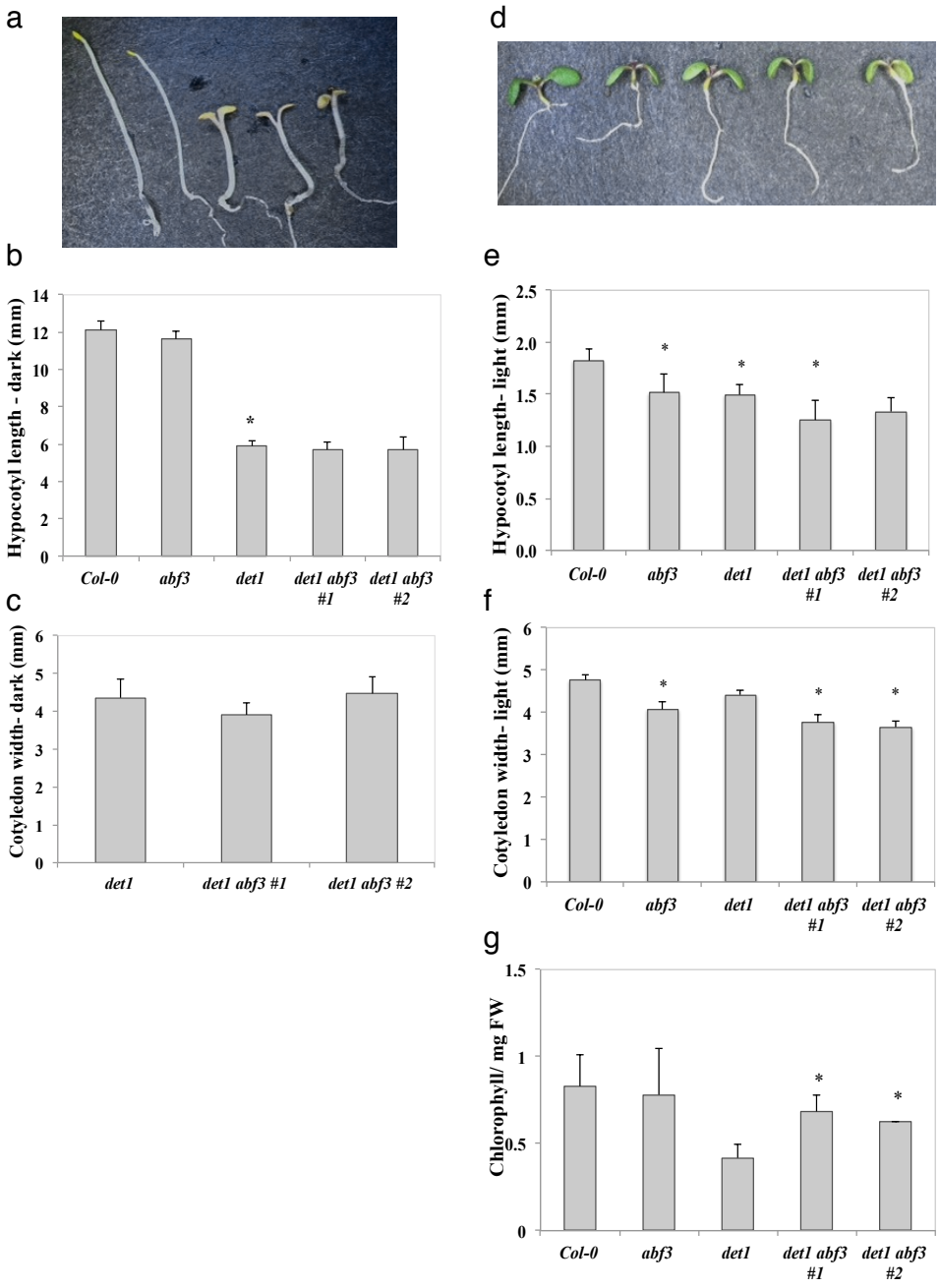

Figure S3.8 Seedling phenotypes of dark and light grown *det1 abf3* seedlings.

(a) Dark grown seedlings, from left: Col-0, *abf3*, *det1*, *det1 abf3 #1* and *det1 abf3 #2* (b) Hypocotyl length (n=10) and (c) cotyledon width (n=10) of dark grown seedlings. (d) Light grown seedlings, from left: Col-0, *abf3*, *det1*, *det1 abf3 #1* and *det1 abf3 #2*. (e) Hypocotyl length (n=10), (f) cotyledon width (n=10), and (g) chlorophyll content (n = 2) of light grown seedlings. Error bars indicate 95% CI, * = $P \leq 0.05$ of single mutants relative to Col-0 or of double mutant relative to *det1*.

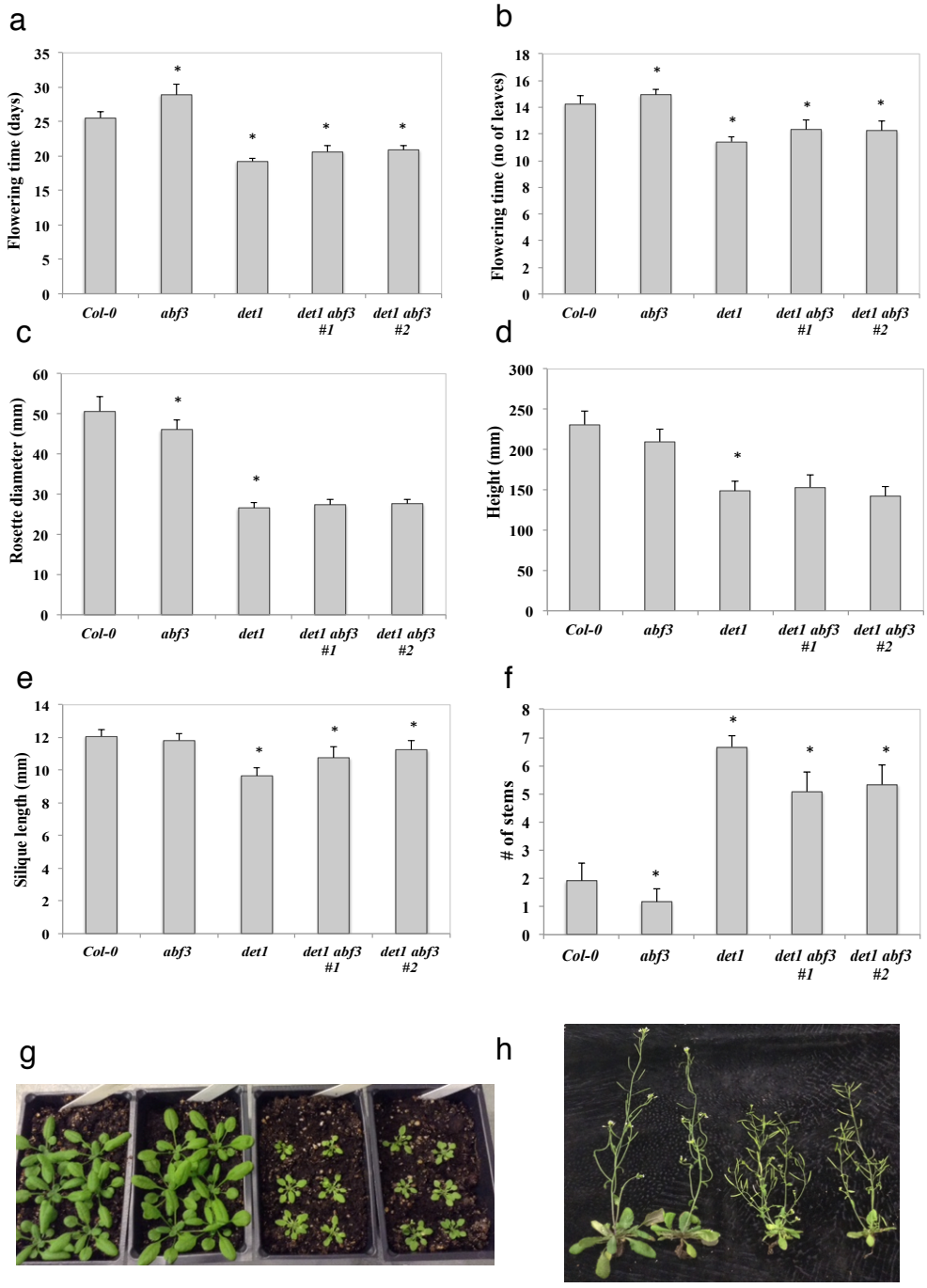

Figure S3.9 Adult phenotypes of *det1 abf3* double mutants.

(a) Flowering time (in days) (b) flowering time (in # of leaves) (c) rosette diameter (d) height (e) sillique length (f) Number of stems (n=12). Adult plants (g) showing rosette diameter at 4 weeks and (h) showing height and apical dominance at 6 weeks. From left: Col-0, *abf3*, *det1*, *det1 abf3*. Error bars indicate 95% CI. * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

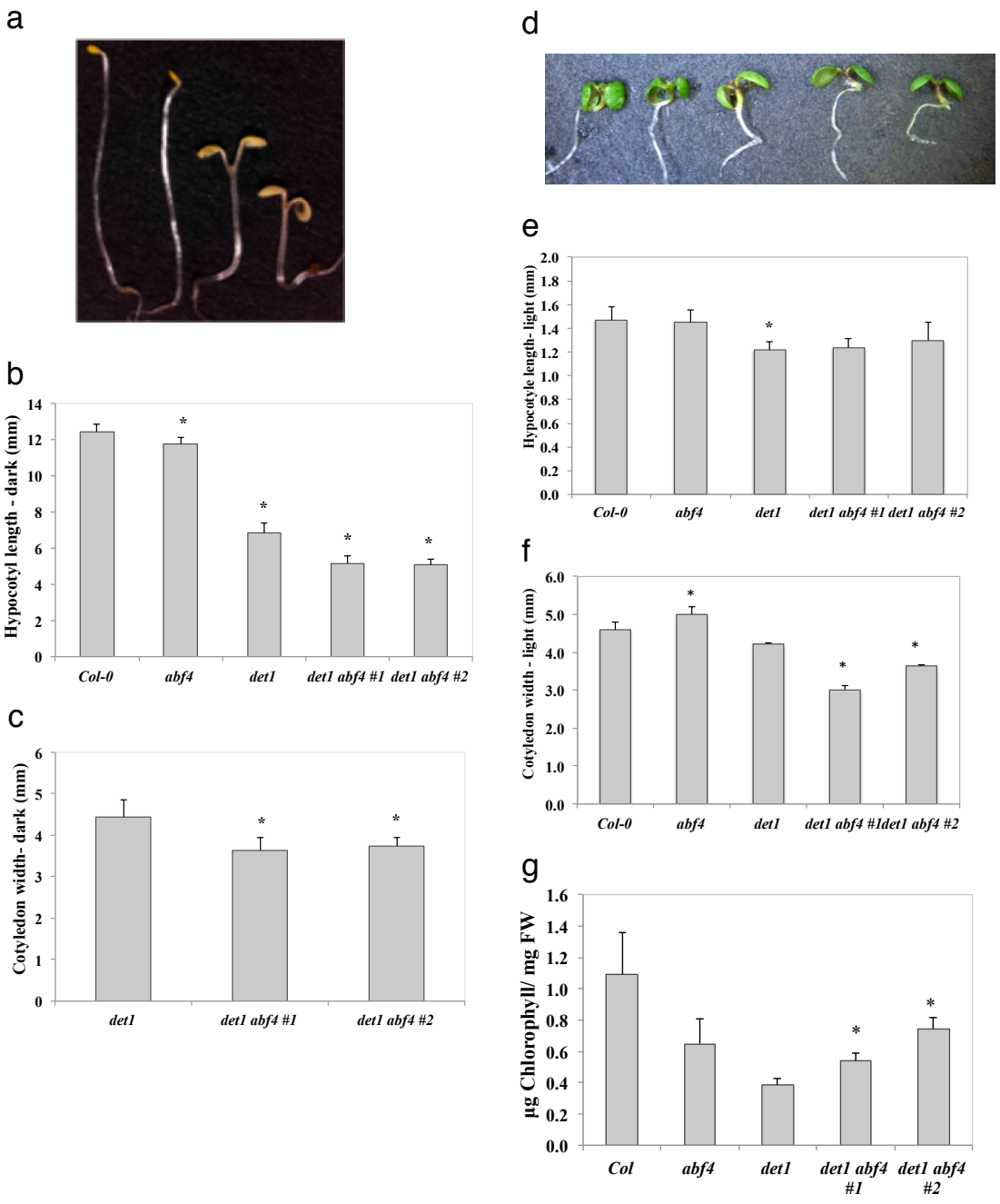

Figure S3.10 Seedling phenotypes of *det1 abf4* dark and light grown seedlings.

(a) Dark grown seedlings, from left: Col-0, *abf4*, *det1*, *det1 abf4 #1*. (b) Hypocotyl length (n=10) and (c) cotyledon width (n=10) of dark grown seedlings. (d) Light grown seedlings, from left: Col-0, *abf4*, *det1*, *det1 abf4 #1* and *det1 abf4 #2*. (e) Hypocotyl length (n=10) (f) cotyledon width (n=10) and (g) chlorophyll content (n=2) of light grown seedlings. Error bars indicate 95% CI, * = P ≤ 0.05 of single mutants relative to Col-0 or of double mutant relative to *det1*.

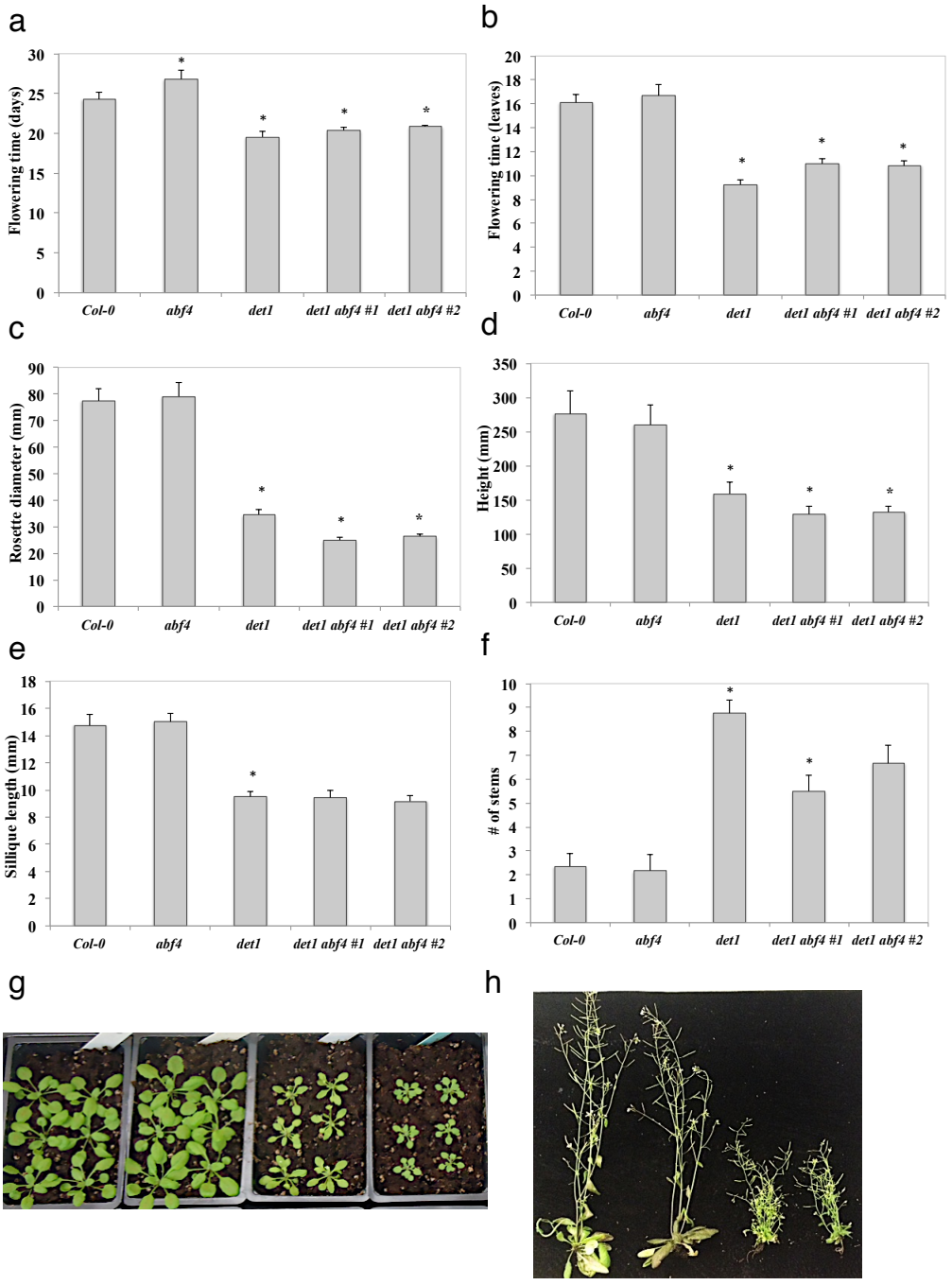

Figure S3.11 Adult plant phenotypes of *det1 abf4*

(a) Flowering time (in days) (b) flowering time (in # of leaves) (c) rosette diameter (d) height (e) sillique length (f) Number of stems (n=12) Adult plants (g) showing rosette diameter at 4 weeks and (h) showing height and apical dominance at 6 weeks from left: Col-0, *abf4, det1, det1 abf4*. Error bars indicate 95% CI. * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

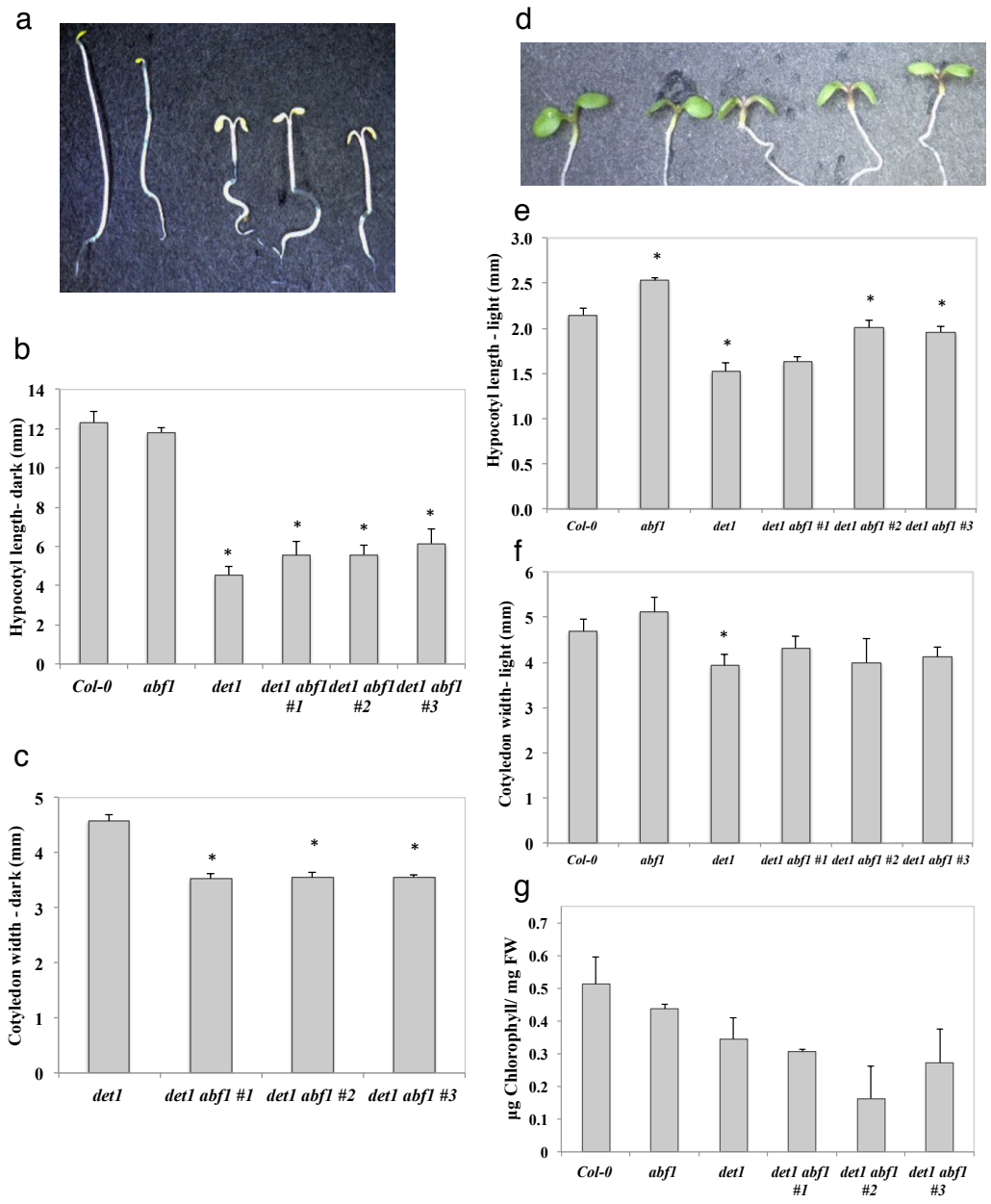

Figure S3.12 Seedling phenotypes of *det1 abf1* dark and light grown seedlings.

(a) Dark grown seedlings, from left: Col-0, *abf1*, *det1*, *det1 abf1 #1* and *det1 abf1* #2 (b) hypocotyl length (n=10) and (c) cotyledon width (n=10) of dark grown seedlings. (d) Light grown seedlings, from left: Col-0, *abf1*, *det1*, *det1 abf1 #1* and *det1 abf1* #2 (e) hypocotyl length (n=10) (f) cotyledon width (n=10) and (g) chlorophyll content (n=2) of light grown seedlings. Error bars indicate 95% CI, * = P ≤ 0.05 of single mutants relative to Col-0 or of double mutant relative to *det1*.

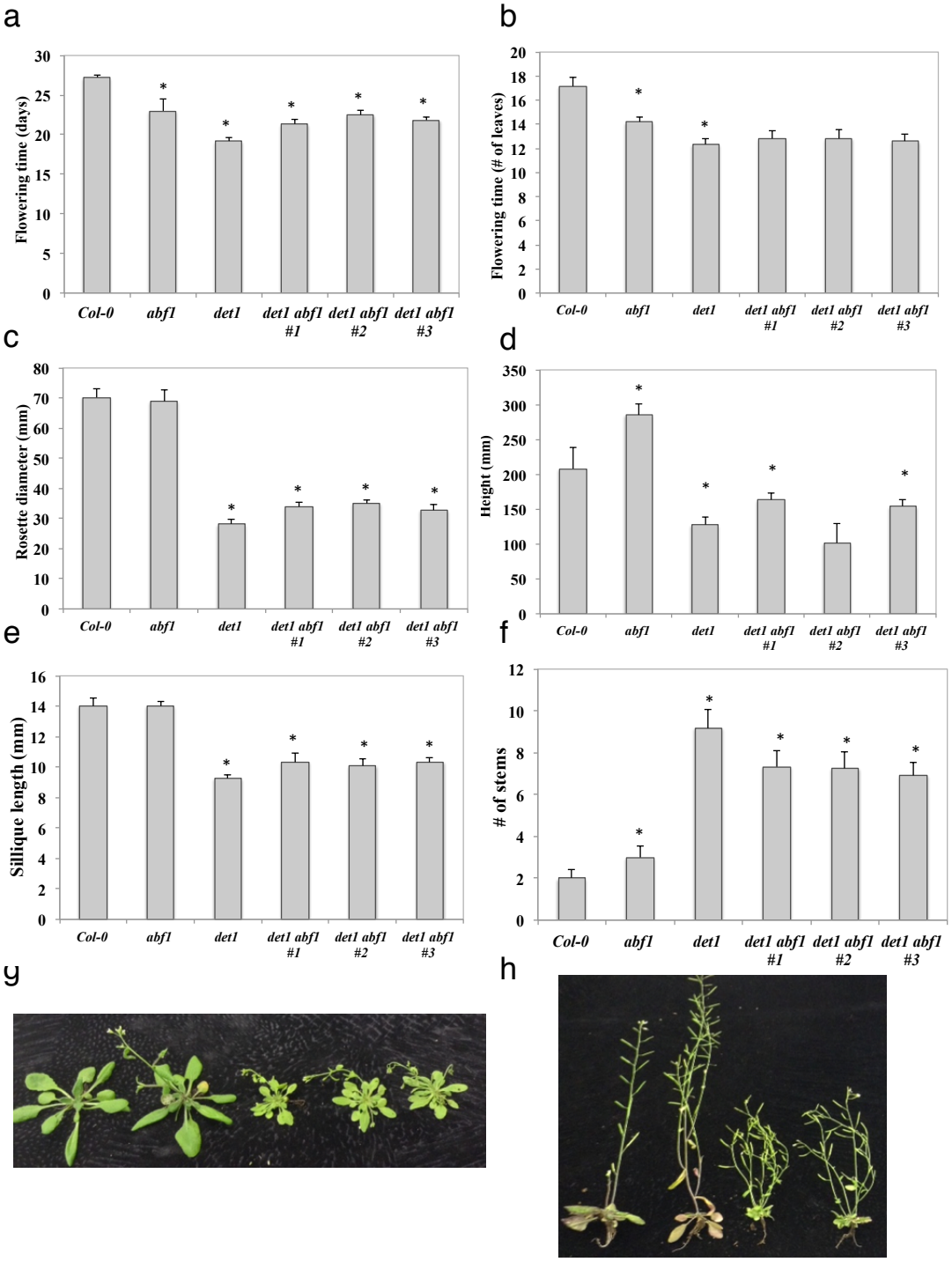

Figure S3.13 Adult plant phenotypes of *det1 abf1*

(a) Flowering time (in days). (b) Flowering time (in # of leaves) (c) Rosette diameter (d) height (e) sillique length (f) Number of stems (n=12) Phenotypes of adult plants (g) showing rosette diameter at 4 weeks and (h) showing height and apical dominance at 6 weeks from left Col-0, *abf1, det1, det1 abf1*. Error bars indicate 95% CI. * indicates $P \leq 0.05$ of single mutants relative to Col-0 and double mutants relative to *det1*.

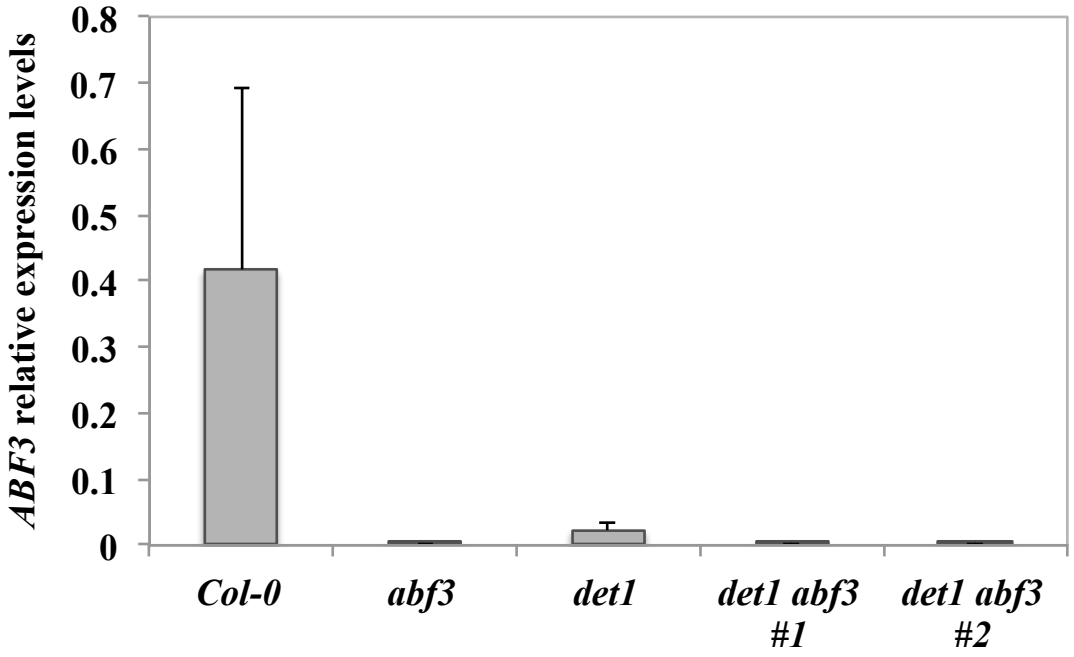

Figure S3.14 *ABF3* transcript levels in *det1abf3* dark grown seedlings

Real-time PCR analysis of *ABF3* mRNA levels in *det1 abf3* dark grown seedlings. Values are normalized relative to the reference gene *EF1α*. Error bars indicate SE of 3 technical replicates.

Chapter 4. Arabidopsis DDB1-CUL4 E3 ligase complexes in *det1* salt/osmotic stress resistant germination

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4. Arabidopsis DDB1-CUL4 E3 ligase complexes in *det1* salt/osmotic stress resistant germination

4.1 Abstract

A key regulatory mechanism in plant growth, development and stress signalling utilizes E3 ubiquitin ligases, which target a variety of substrates for degradation. DE-ETIOLATED 1 (DET1) forms a complex with DDB1 (DAMAGED DNA BINDING protein 1) and CUL4 (CULLIN 4), and negatively regulates light signalling. Another DDB1-CUL4 complex containing DWA1 and DWA2 (DWD hypersensitive to ABA 1 and 2) has been shown to negatively regulate abscisic acid (ABA) signalling. Since distinct DDB1-CUL4 complexes have been shown to influence each other, we analyzed genetic interactions between *DET1* and components of DDB1-CUL4 complexes during seed germination under salt and osmotic stress conditions. *det1* germination was resistant to salt and osmotic stress and *dwa1* and *dwa2* enhanced this phenotype. In contrast, *ddb1a* partially suppressed the *det1* germination phenotype on both salt and mannitol, while *ddb1b* had no effect. Mutations in DDB2, a DDB1-CUL4 complex component involved in DNA repair, also partially suppressed the *det1* germination phenotype while mutants in COP1, another light signalling component, completely suppressed the *det1* resistant germination phenotypes. Taken together these data suggest that components of E3 ubiquitin ligase complexes have variable but significant effects on *det1* salt/osmotic stress responses.

4.2 Introduction

Eukaryotes use ubiquitination as a means of regulating protein function. Ubiquitination is a process by which the 76 amino acid conserved protein Ubiquitin (Ub) is covalently attached to a target protein. Monoubiquitination regulates protein trafficking or activity while polyubiquitination targets proteins for degradation via the 26S proteasome. Three major enzymes are involved in this process, namely Ub activating enzymes (E1), Ub conjugating enzymes (E2), and Ub ligases (E3). Ub E3 ligases play important roles in ubiquitination by providing substrate specificity. E3 ligases transfer Ub from the E2 to the target protein and position it properly for Ub conjugation (Sadowski et al 2012). E3 Ub ligase complexes have diverse roles in plants and animals including regulation of growth and development and response to abiotic and biotic stress. There are approximately 1400 E3 ligases in *Arabidopsis thaliana* (Lee and Kim 2011, Stone 2014). During plant stress response, a large number of E3 ligases are implicated in the response to the stress hormone abscisic acid (ABA), regulating processes from biosynthesis to signalling. Thus E3 ligases play a critical role in ABA responses in plants (Kelley and Estelle 2012, Stone 2014).

Many E3 ligases employ one of the four CULLINs (CULs) as the scaffolding protein. CUL4 based E3 ligase complexes bind to a large number of substrates via the substrate adapter DAMAGED DNA BINDING protein 1 (DDB1), which interacts with a variety of substrate receptors (Biedermann and Hellmann 2011). Arabidopsis has two homologues of DDB1, DDB1A and DDB1B (Schroeder et al 2002). The substrate receptors in turn interact with specific substrates to be targeted for degradation (Figure 4.1). These substrate receptors are referred to as DDB1 BINDING WD40 (DWD) or DDB1-CUL4 ASSOCIATED FACTOR

(DCAF) factors (Biedermann and Hellmann 2011). Arabidopsis has 85 DWD proteins with the conserved 16 amino acid DWD motif (Lee et al 2008).

DE-ETIOLATED 1 (DET1), a central repressor of photomorphogenesis (light growth), interacts with DDB1, CUL4 and CONSTITUTIVE PHOTOMORPHOGENIC 10 (COP10) to form the CUL4-CDD complex (Figure 4.1a) (Yanagawa et al 2004, Schroeder et al 2002, Bernhardt et al 2006, Chen et al 2006). CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), another central repressor of light signalling, also acts as an E3 Ub ligase. COP1 targets photomorphogenesis promoting transcription factors like LONG HYPOCOTYL 5 (HY5) for degradation (Osterlund et al 2000, Huang et al 2014). COP1 interacts with SUPPRESSOR OF PHYTOCHROME A 1-4 (SPA1-4) and forms tetrameric COP1-SPA complexes that exhibit E3 ligase activity (Figure 4.1a) (Zhu et al 2008). COP1-SPA complexes act as E3 ligases alone in some instances and as part of CUL4-DDB1 E3 ligase complexes in others (Chen et al 2010). COP1-SPA and CDD are distinct CUL4 complexes that do not interact directly with each other (Chen et al 2010), however DET1 is required for COP1 nuclear localization (von Arnim et al 1997) and HY5 degradation, but the basis of this requirement is not known (Osterlund et al 2000, Huang et al 2014).

DAMAGED DNA BINDING protein 2 (DDB2) also interacts with CUL4-DDB1. The primary function of this E3 ligase complex is facilitating UV damaged DNA repair (Figure 4.1b) (Molinier et al 2008, Ganpudi and Schroeder 2011). In an example of CUL4-DDB1 complexes interacting with each other, examination of the effect of *det1* on CUL4-DDB1/2 complexes showed that DET1 is required for DDB2 degradation (Castells et al 2011). In addition, while the Arabidopsis *ddb2* single mutant has no significant developmental phenotypes, *ddb2* modifies *det1* phenotypes (Al Khateeb and Schroeder 2007).

During ABA signalling, another DDB1-CUL4 complex containing DWA1 and DWA2 (DWD hypersensitive to ABA 1 and 2) has been shown to regulate ABA response (Figure 4.1c). DWA1 and 2 interact with each other as well as directly interact with the ABA response promoting transcription factor ABSCISIC ACID INSENSITIVE 5 (ABI5), targeting it for degradation (Lee et al 2010). Thus DWA1/2 negatively regulate ABA signalling. We have previously shown that *DET1* interacts genetically with *DWA1* during plant development. *dwa1* affects *det1* seedling growth, flowering time and fertility phenotypes. In contrast, *dwa2* exhibited no consistent effects on *det1* growth phenotypes. However both *dwa1* and *dwa2* partially suppressed *det1* ABA sensitive germination (Fernando and Schroeder 2015). Therefore, these components of distinct DDB1-CUL4 E3 ligase complexes appear to interact with each other directly or indirectly during light signalling as well as in stress signalling.

Other DDB1-CUL4 ligase complexes have also been implicated in ABA signalling. DWA3 also forms DDB1-CUL4 complexes and is a negative regulator of ABA signalling, but its target is unknown. Although DWA3 accumulates ABI5, no interaction was found between DWA3 and ABI5. Therefore it was suggested that DWA3 suppresses a negative regulator of DWA1 and 2 (Lee et al 2011). ABA HYPERSENSITIVE DCAF 1 (ABD1) also interacts with CUL4-DDB1 and forms another E3 ligase complex that targets ABI5 for degradation (Figure 4.1d) (Seo et al 2014). DET1-DDB1-ASSOCIATED 1 (DDA1) interacts with the CDD complex to target the ABA receptor PYRABACTIN RESISTANCE LIKE 8 (PYL8) for degradation (Figure 4.1e) (Irigoyen et al 2014). Thus DDB1-CUL4 complexes are involved in many aspects of ABA signalling.

det1 exhibits ABA sensitive germination and *dwa1, dwa2, ddb1a, ddb1b,* and *ddb2* have been shown to either partially or completely suppress this phenotype, while *cop1* enhances it

(Fernando and Schroeder 2015). In this study we showed that, in contrast, *det1* mutants exhibit resistance to salt and osmotic stress induced inhibition of seed germination and examined the role of E3 ligase components in this phenotype.

4.3 Materials and methods

4.3.1 Plant materials

All *Arabidopsis thaliana* mutants in this study are in the Columbia-0 ecotype. *det1-1*, *cop1-4, ddb1a, ddb1b, ddb2* and their respective *det1* double mutants are as previously characterized (Chory et al 1989, Schroeder et al 2002, Al Khateeb and Schroeder 2007, Ganpudi and Schroeder 2013, Ly et al 2015). *dwa1* and *dwa2* and their double mutants in the *det1* background (*det1 dwa1* and *det1 dwa2*) used in this study are as described in Lee et al (2010) and Fernando and Schroeder (2015).

4.3.2 Seed germination assays

Sterilized Arabidopsis seeds from the above genotypes were sown on Linsmaier and Skoog (LS) media (Caisson), with 0.86% Phytoblend (Caisson) and 0% sucrose, supplemented with 100 or 200 mM NaCl (Fisher Scientific) or 200 or 400 mM Mannitol (Fisher Scientific). Plates were stratified at 4^0C for 2 days then transferred to 20^0C and long day conditions (16 h light/ 8 h dark) supplied by fluorescent bulbs (100 μM photons m^{-2} s^{-1}). Seed germination was scored every 24 hours as percentage of seeds with radical emergence for up to 5 days (Bolle 2009). Germination assays for *cop1 det1* and *ddb1a det1* were done by visibly identifying double mutants in a population of segregating *cop1 det1*/+ and *ddb1a det1*/+ heterozygotes, because the doubles are lethal and infertile, respectively (Ang et al 1994, Schroeder et al 2002, Ly et al

2015). Specifically, plates were scanned and germination date of every seed monitored using a colour coding system. Upon cotyledon emergence, distinctive purple cotyledons were used to identify the double mutants.

4.3.3 Statistical analysis

Each experiment included 2 replicates and every experiment was repeated at least 3 times. The results of a single representative experiment are presented here. Results are means ± SE compared using two-tailed student's t-test. $P \leq 0.05$ was considered statistically significant.

4.4 Results

While *det1* mutants have been shown to exhibit ABA sensitive germination (Fernando and Schroeder 2015, Irigoyen et al 2014), they were in fact resistant to salt and osmotic stress induced inhibition of germination (Chapter 3). We have previously examined the role of *DWA1, DWA2, DDB1A, DDB1B, COP1* and *DDB2* in *det1* ABA sensitive germination. Here we examined the role of these genes in *det1* salt/osmotic stress resistant germination by generating double mutants and assessing germination on salt and mannitol containing media.

4.4.1 *det1 dwa1* and *det1 dwa2* salt/mannitol germination responses

Since we observed partial rescue of the *det1* ABA sensitive germination phenotype in *det1 dwa1* and *det1 dwa2* double mutants (Fernando and Schroeder 2015), and plant responses to salt and mannitol often utilize ABA signalling, we examined the effect of *dwa1* and *dwa2* on *det1* salt/mannitol resistant germination. The germination of the *dwa1* single mutant was slightly delayed on salt and mannitol containing media, as expected for the loss of function of a negative

regulator of ABA signalling (Figure 4.2) (Lee et al 2010). On salt and 400 mM mannitol, *dwa1* did not exhibit any consistent significant effect on *det1* germination. On 200 mM mannitol, however, germination was enhanced in the *det1 dwa1* double mutants, implying a possible role for *DWA1* in *det1* osmotic stress resistant germination. The *dwa2* single mutant exhibited wildtype germination on both types of stress media (Figure 4.3). *dwa2* enhanced the *det1* salt resistant germination phenotype on 100 mM salt, but no consistent significant effects were observed on mannitol or 200 mM salt. Thus while DWA1 is not required for *det1* salt resistant germination but may be involved in osmotic stress response, DWA2 is involved in the *det1* salt, but not osmotic, germination phenotype.

4.4.2 *det1 ddb1a* and *det1 ddb1b* salt/mannitol germination responses

Since DET1 interacts physically and genetically with both DDB1A and DDB1B (Schroeder et al 2002, Chen et al 2006, Ganpudi and Schroeder 2013), and *ddb1a* completely suppresses and *ddb1b* partially suppresses the *det1* ABA sensitive germination phenotype (Fernando and Schroeder 2015), we examined the role of *DDB1A* and *DDB1B* in *det1* salt/osmotic resistant germination. Germination in the *ddb1a* single mutant was hypersensitive to both salt and mannitol (Figure 4.4), consistent with previously reported *ddb1a* germination phenotypes (Ganpudi and Schroeder 2013). The *det1 ddb1a* double mutant partially suppressed the *det1* salt resistant germination phenotype on 200 mM salt on day 3. On mannitol, complete rescue was observed at 200 mM and an intermediate phenotype at 400 mM mannitol. Therefore, DDB1A contributes to the *det1* salt and osmotic stress germination phenotypes. In contrast, the *ddb1b* single mutant did not show a phenotype on salt but was hypersensitive to mannitol, and

the *det1 ddb1b* double mutants did not differ significantly from *det1* (Figure 4.5). Thus *DDB1A* but not *DDB1B* contribute to *det1* stress resistant germination.

4.4.3 *det1 ddb2* salt/mannitol germination responses

Although they are components of distinct DDB1-CUL4 complexes, DET1 and DDB2 have been shown to interact genetically during both development and DNA repair (Al Khateeb and Schroeder 2007, Castells et al 2011). In addition, *ddb2* completely suppressed the *det1* ABA sensitive germination phenotype (Fernando and Schroeder 2015). Thus we examined the effect of *ddb2* on *det1* stress resistant germination. The *ddb2* single mutant was slightly sensitive to salt inhibition of seed germination at 100 mM but exhibited wildtype germination on mannitol (Figure 4.6). *ddb2* partially rescued both salt and osmotic stress resistant germination in *det1* mutants. Thus, as with the other phenotypes previously examined, *DDB2* contributes to *det1* stress resistant germination.

4.4.4 *det1 cop1* salt/mannitol germination phenotype

DET1 and COP1 are both negative regulators of photomorphogenesis. Developmentally, *det1* and *cop1* tend to enhance each other phenotypes, such as reduced hypocotyl length and increased anthocyanin content, and the *det1 cop1* double mutant is seedling lethal (Ang and Deng 1994). Therefore, *det1 cop1* germination was scored in a segregating population of *cop1 det1*/+. *cop1* enhanced *det1* ABA sensitive germination and the *cop1 det1* double mutant also exhibited reduced germination in control conditions (Fernando and Schroeder 2015). Here we found that *cop1* single mutants were hypersensitive to both salt and mannitol stress, exhibiting the opposite phenotype to *det1* (Figure 4.7). In control conditions, the germination of *cop1 det1* double mutants was delayed relative to both the *det1* and *cop1* single mutants, as previously

described (Fernando and Schroeder 2015). On salt containing media, *cop1* completely suppressed the *det1* resistant germination phenotype, indicating that *cop1* is epistatic to *det1*. On mannitol, *cop1* again completely suppressed the *det1* resistant phenotype. In fact, on 200 mM mannitol, germination in the double mutant was well below even that of the *cop1* single mutant. Thus COP1 is required for *det1* stress resistant germination.

4.5 Discussion

Components of CUL4-DDB1 complexes have previously been shown to genetically interact with *DET1* during Arabidopsis development and also play a role in *det1* ABA sensitive germination (Al Khateeb and Schroeder 2007, Irigoyen et al 2014, Fernando and Schroeder 2015). Here we investigated whether these components have a function in *det1* salt/osmotic stress resistant germination.

4.5.1 Role of *DWA1 and DWA2* in *det1* salt/mannitol resistant germination

dwa1 and *dwa2* have been shown to exhibit salt sensitive root growth and ABA sensitive germination and *dwa1 dwa2* double mutants exhibit enhanced phenotypes (Lee et al 2010). We observed that *dwa1* enhanced *det1* germination during osmotic stress, while *dwa2* enhanced *det1* germination under salt stress. DWA1 and DWA2 can interact with each other, suggesting they act as heterodimers, thus would be expected to have common loss of function phenotypes. However DWA1 and DWA2 can also interact with themselves, forming homodimers, and their loss of function phenotypes are additive, suggesting they also have independent functions (Lee et al 2010). Interestingly, eFP browser expression data indicates that in aerial tissues *DWA1* is

upregulated by osmotic stress, while *DWA2* is upregulated by salt, consistent with the phenotypes we observed (Figure 4.8) (Kilian et al 2007, Winter et al 2007)

Surprisingly the *det1 dwa1* and *dwa2* double mutants exhibited the opposite phenotype of what would be predicted based on the function of the CUL4-DDB1-DWA1/2 E3 ligase. In the presence of stress-induced ABA signalling, the absence of either DWA1 or DWA2 should increase ABI5 levels, repressing germination and thus rescuing the *det1* resistant germination phenotype, but we observed increased germination in the double mutants. Interestingly we observed the same trend on ABA, the *det1 dwa1/2* double mutants exhibited more germination than *det1*, in this case suppressing the *det1* ABA sensitive germination phenotype (Fernando and Schroeder 2015). Thus even though *det1* has opposite phenotypes on ABA and salt/mannitol, the effect of *dwa1/2* was the same, increased germination. These results suggest that in the *det1* background DWA1 and DWA2 act as positive, rather than negative, regulators of stress signalling. What might be the basis of this effect? Perhaps the downregulation of two CUL4-DDB1 complexes (DET1 and DWA1/DWA2) results in upregulation of other CUL4-DDB1 complexes that are negative regulators of ABA signalling, such as those containing DWA3, ABD1, or the recently described ALTERED SEED GERMINATION 2 (ASG2) (Lee et al 2011, Seo et al 2014, Dutilleul et al 2016). This would result in decreased ABA signalling and increased germination.

Alternatively, this effect may be due to transcriptional rather than post-transcriptional compensation. We have previously shown that *DWA1* mRNA levels are higher in *dwa2* mutants (Fernando and Schroeder 2015), thus perhaps *dwa1* mutants have increased levels of *DWA2*. Due to the redundant nature of their function perhaps in the absence of one DWA the other

compensates for it, resulting in reduced levels of ABI5 and enhanced germination. We have also previously observed variable levels of *DWA1* in the *det1 dwa2* double mutants, correlating with variable developmental phenotypes (Fernando and Schroeder 2015). This may also be the basis of the variable germination phenotypes observed here. Similarly, perhaps variable levels of *DWA2* in the *det1 dwa1* double mutants also result in the variation in germination phenotype observed.

4.5.2 Role of *DDB1A/B* in *det1* salt/mannitol resistant germination

ddb1a and *ddb1b* were previously shown to suppress both *det1* ABA sensitive germination and ABA resistant cotyledon emergence (Fernando and Schroeder 2015). Thus both DDB1A and DDB1B are required for these contrasting *det1* ABA phenotypes. Under salt and osmotic stress conditions, *ddb1a* partially suppressed the *det1* resistant germination phenotypes, while *ddb1b* did not. This could be due to the fact that *ddb1b* is a weaker allele than *ddb1a* (Bernhardt et al 2010, Ganpudi and Schroeder 2013). Nonetheless, we find that *DDB1A,* but not *DDB1B,* is required for the *det1* salt/osmotic resistant germination phenotypes. Although *ddb1a* and *ddb1b* generally suppress *det1* germination phenotypes, they enhance the majority of *det1* developmental phenotypes (Al Khateeb and Schroeder 2007, Schroeder et al 2002, Ganpudi and Schroeder 2013) indicating that DDB1A and DDB1B act antagonistically to DET1 during germination.

Given that there are at least five DDB1-CUL4 complexes that negatively regulate ABA signalling (DWA1/2, DWA3, ABD1, DDA1, ASG2), (Lee et al 2010, Lee et al 2011, Seo et al 2014, Irigoyen et al 2014, Dutilleul et al 2016) one would expect loss of DDB1A to result in increased ABA signalling and therefore less germination. This is in fact what we observed in salt

and mannitol conditions in both the *det1* and wildtype backgrounds. The *det1 ddb1a* salt and mannitol germination phenotypes could be interpreted as being additive, in that *det1* still results in increased germination even in the *ddb1a* background. This is in contrast to the *det1* ABA sensitive germination phenotype, which is completely suppressed by *ddb1a* (Fernando and Schroeder 2015). Thus a fraction of the *det1* stress resistant germination phenotype is DDB1A independent. In contrast, *ddb1b* mannitol sensitive germination is completely suppressed by *det1*, thus *ddb1b* mannitol sensitive germination is DET1 dependent.

4.5.3 Role of *DDB2* in *det1* salt/mannitol resistant germination

Germination in the *ddb2* single mutant was delayed on salt containing media but not on mannitol or ABA containing media (Fernando and Schroeder 2015). Interestingly, *ddb2* suppressed *det1* germination phenotypes on all the different stress media, completely suppressing *det1* ABA sensitive germination and partially suppressing *det1* salt and mannitol resistant germination. This suggests that DDB2 is required for *det1* germination phenotypes under stress conditions. *ddb2* suppresses not only *det1* germination phenotypes but also a number of other *det1* phenotypes, including chlorophyll content, anthocyanin content, and adult phenotypes (Al Khateeb and Schroeder 2007), indicating that DDB2 and DET1 act antagonistically. Although DDB2's primary role is in UV damaged DNA repair, it also seems to be involved in numerous other growth, developmental and stress phenotypes in the *det1* background. The effect of *ddb2* on *det1* phenotypes does not seem to be a general effect of lack of one CUL4-DDB1 complex on another, since *dwa1* and *dwa2* do not exhibit the degree of suppression of *det1* phenotypes that *ddb2* does (Al Khateeb and Schroeder 2007, Fernando and Schroeder 2015). Castells et al (2011) suggest that DET1 is required for DDB2 degradation. Perhaps in *det1* mutants there are

developmental consequences of this excess DDB2, which are rescued by the *ddb2* mutant. The basis of this interaction requires further investigation.

4.5.4 Role of *COP1* in *det1* salt/osmotic stress phenotype

COP1 is required for the *det1* early germination phenotype in control conditions as well as in salt and osmotic stress conditions. On ABA, both *cop1* and *det1* exhibited ABA sensitive germination, and germination was further impaired in the double mutant, suggesting that DET1 and COP1 act in the same pathway in response to ABA (Fernando and Schroeder 2015). In contrast, *cop1* and *det1* showed opposite phenotypes during salt/osmotic stress, where *det1* showed resistant germination while *cop1* was sensitive. These results indicate that COP1 and DET1 act antagonistically during seed germination in salt/osmotic stress conditions. The *det1 cop1* double mutant exhibited less germination than either single mutant on ABA and mannitol (Figure 4.7 and (Fernando and Schroeder 2015). This result suggests although *cop1* and *det1* exhibit opposite phenotypes during germination under salt/osmotic stress, *det1* still requires COP1 to exhibit the resistant germination phenotype. In addition, both COP1 and DET1 are required to execute wild type germination in control conditions.

Recently Yu et al (2016b) showed that COP1 negatively regulates salt inhibition of seed germination by post-translational regulation of HY5. They showed that salt inhibits COP1 nuclear localization in both light and dark and thus stabilizes HY5 accumulation in the nucleus. HY5 had previously been shown to be a positive regulator of *ABI5* transcription and therefore a negative regulator of germination (Chen and Xiong 2008). Salt inhibition of germination in *cop1* mutants was also shown to be via HY5 and ABI5 (Yu et al 2016b). Previous studies indicate that both *COP1* and *DET1* are involved in negative regulation of HY5 levels in the cell (Osterlund et

al 2000). We have previously shown that *det1* ABA sensitive germination requires HY5 and ABI5 (Fernando and Schroeder 2015). However other studies in our lab indicate that *det1* salt resistant germination requires HY5 but not ABI5 (Chapter 3). How *cop1* and *det1,* both acting via HY5, result in different germination phenotypes during salt stress is currently unclear.

In conclusion, *DET1* has genetic interactions with components of distinct E3 ligase complexes during salt/osmotic stress inhibition of seed germination. The interaction of *DET1* with *DWA1/2* showed the opposite effect of that expected, in that *dwa1/2* enhanced the *det1* salt/mannitol resistant germination phenotype. *ddb1a* partially suppressed the *det1* resistant germination phenotype, while *ddb1b* did not show a significant effect on this response. The *ddb2* single mutant was sensitive to salt, and *ddb2* partially suppressed *det1* resistant germination on both salt and mannitol. *cop1* and *det1* exhibit opposite phenotypes on stress media, but the absence of both COP1 and DET1 results in minimal germination, suggesting that *COP1* and *DET1* play important roles in stress signaling during seed germination. This study provides additional evidence of interactions between components of DDB1-CUL4 E3 ligase complexes (Al Khateeb and Schroeder 2007, Castells et al 2011, Fernando and Schroeder 2015).

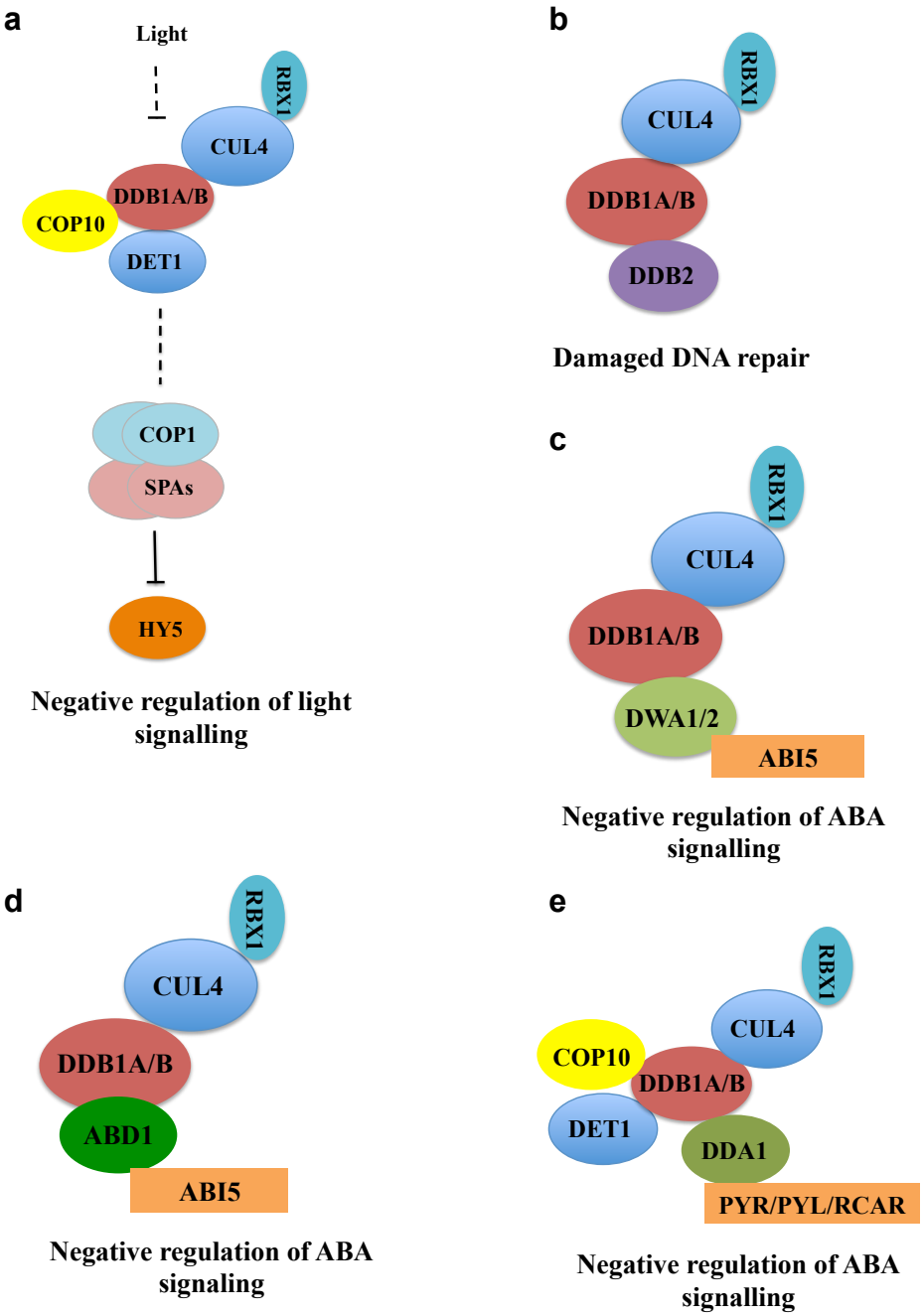

Figure 4.1 Selected CUL4 based E3 ligase complexes, their substrates and their regulatory roles

(a) CUL4-DDB1-CDD complex (b) CUL4-DDB1-DDB2 complex (c) CUL4-DDB1-DWA1/2 complex (d) CUL4-DDB1-ABD1 complex and (e) CUL4-CDD-DDA1 complex. Continuous lines indicate direct interactions, discontinuous lines indicate indirect interactions and T bars show negative regulation.

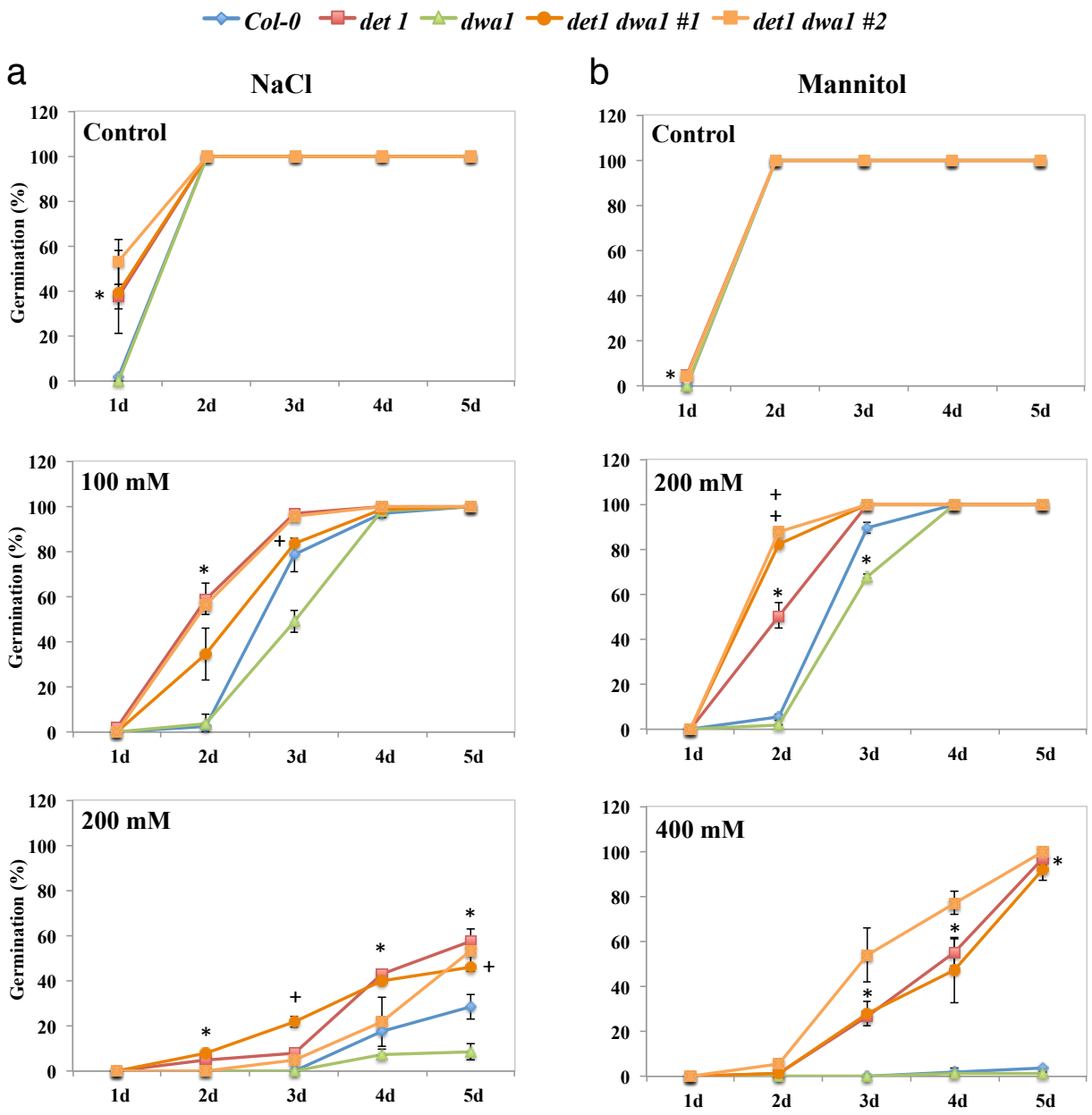

Figure 4.2 Germination in *det1 dwa1* double mutants.

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

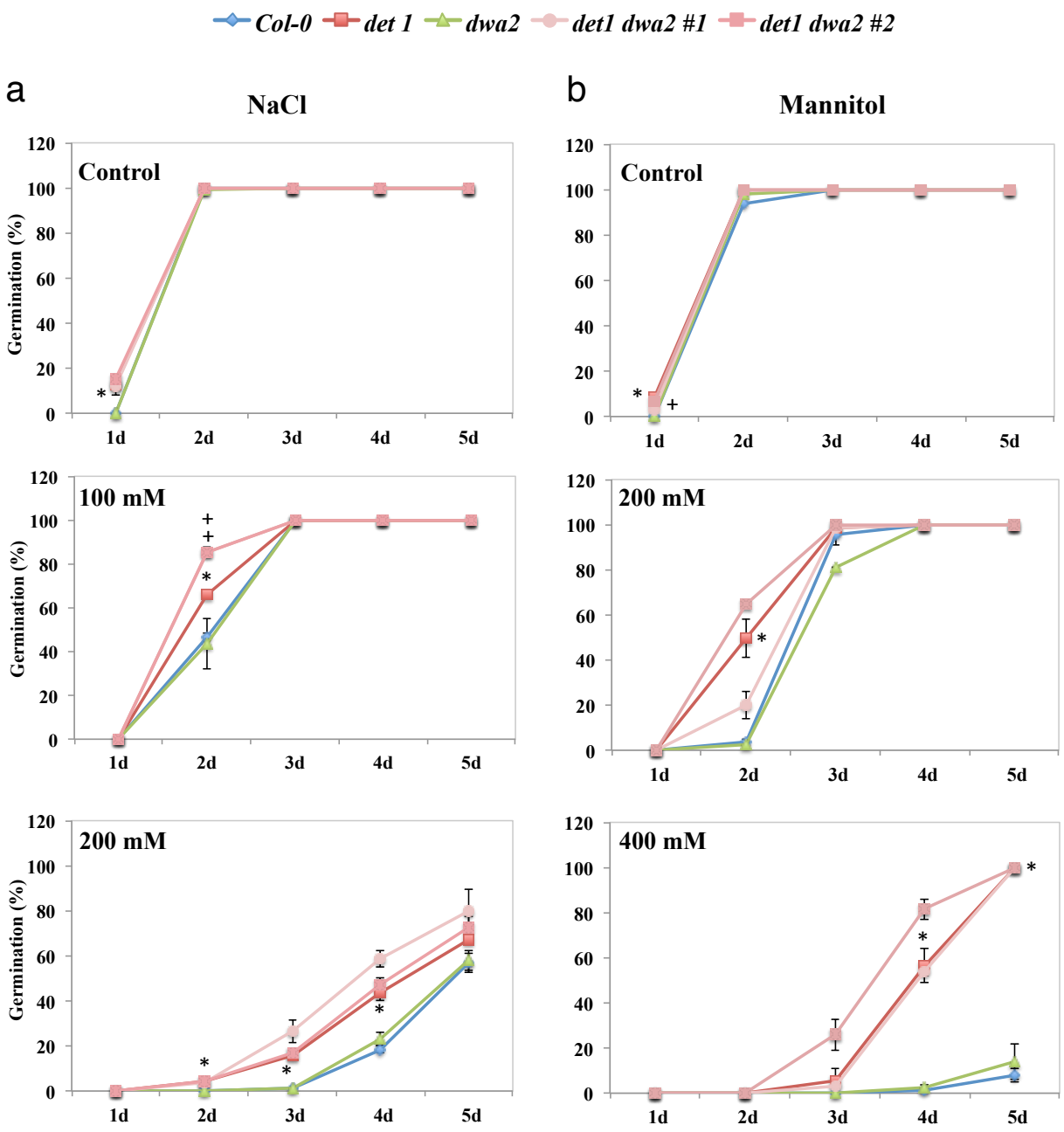

Figure 4.3 Germination in *det1 dwa2* double mutants

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

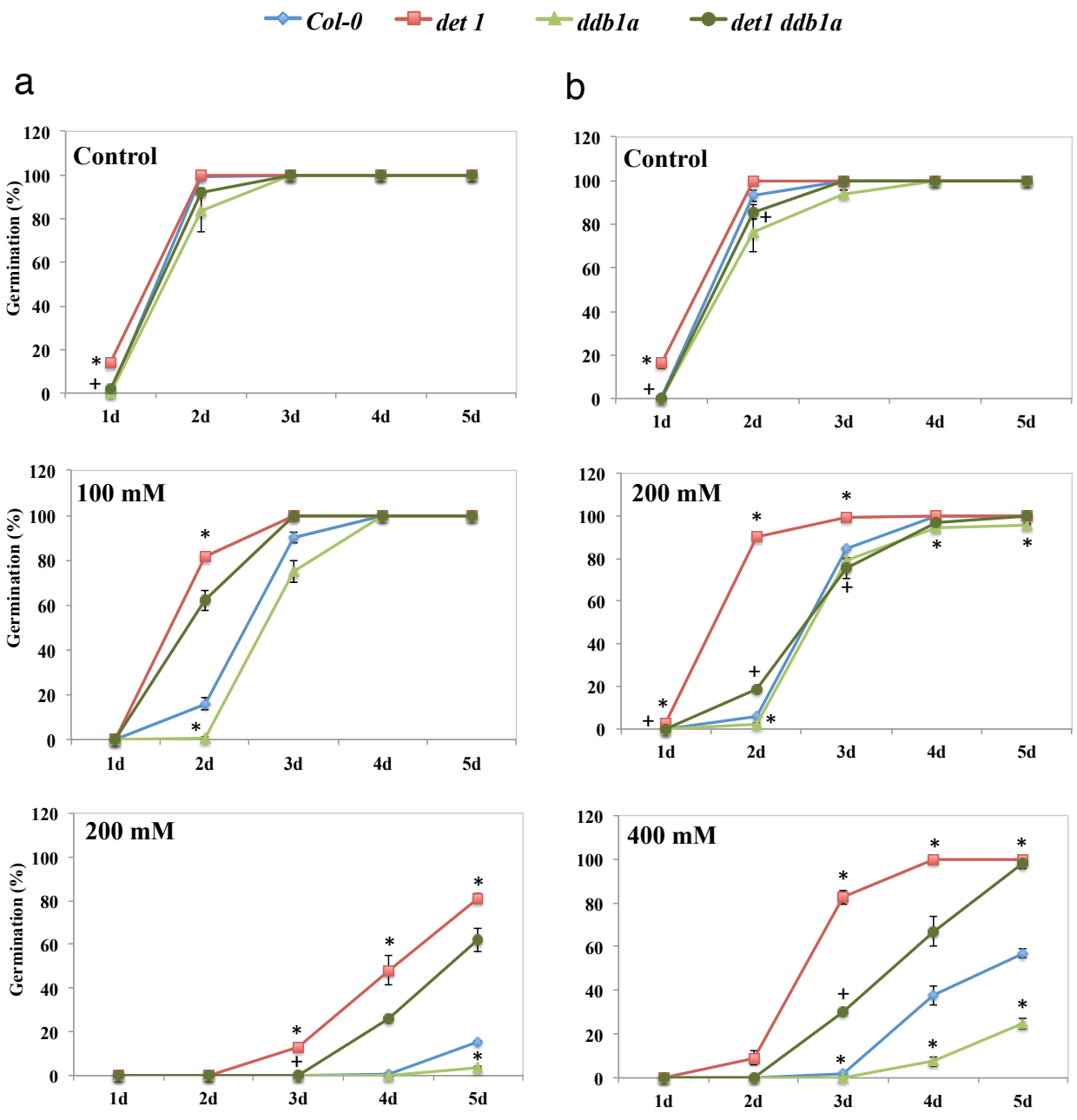

Figure 4.4 Germination in *det1 ddb1a* double mutants

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

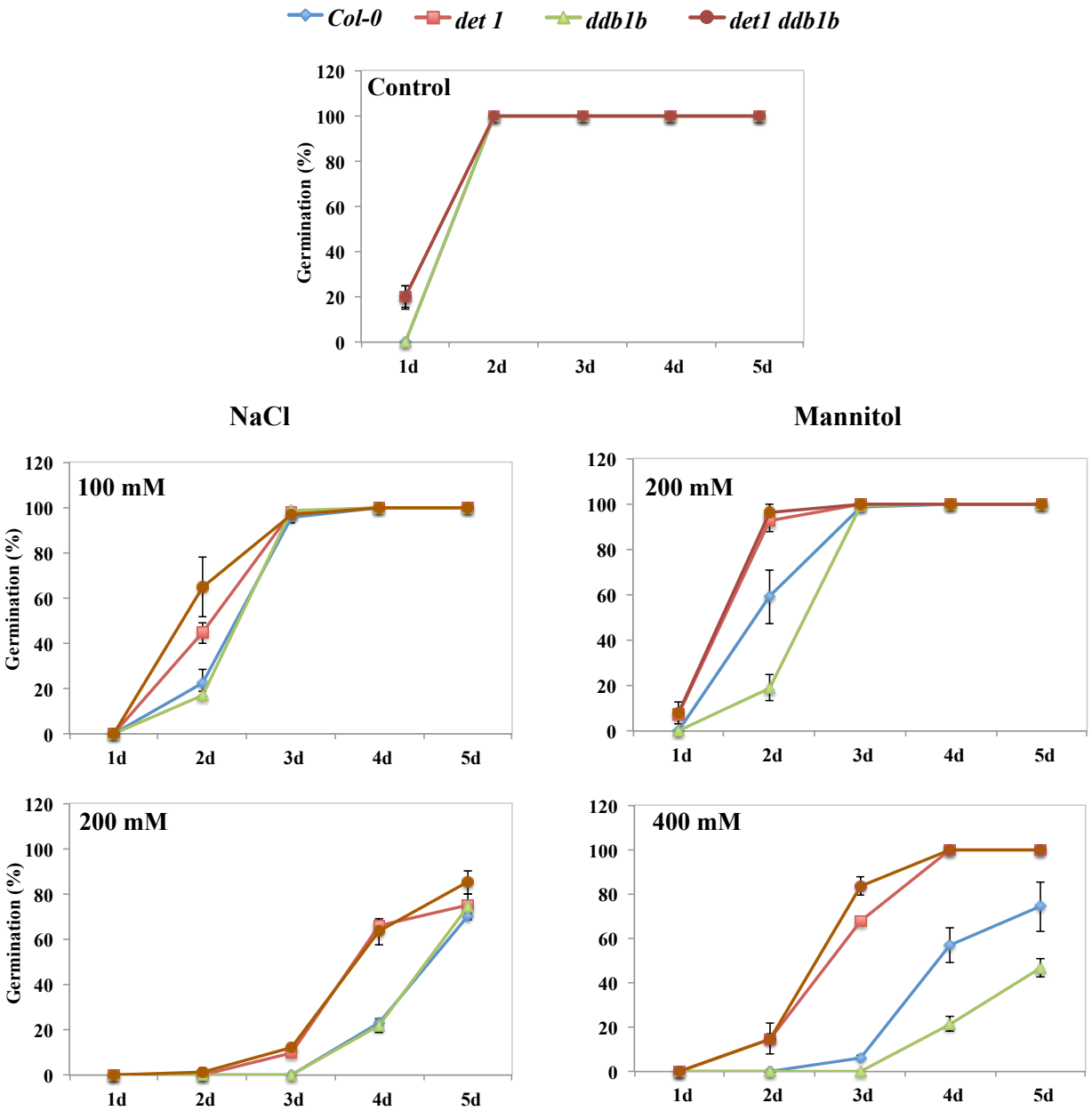

Figure 4.5 Germination in *det1 ddb1b* double mutants

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

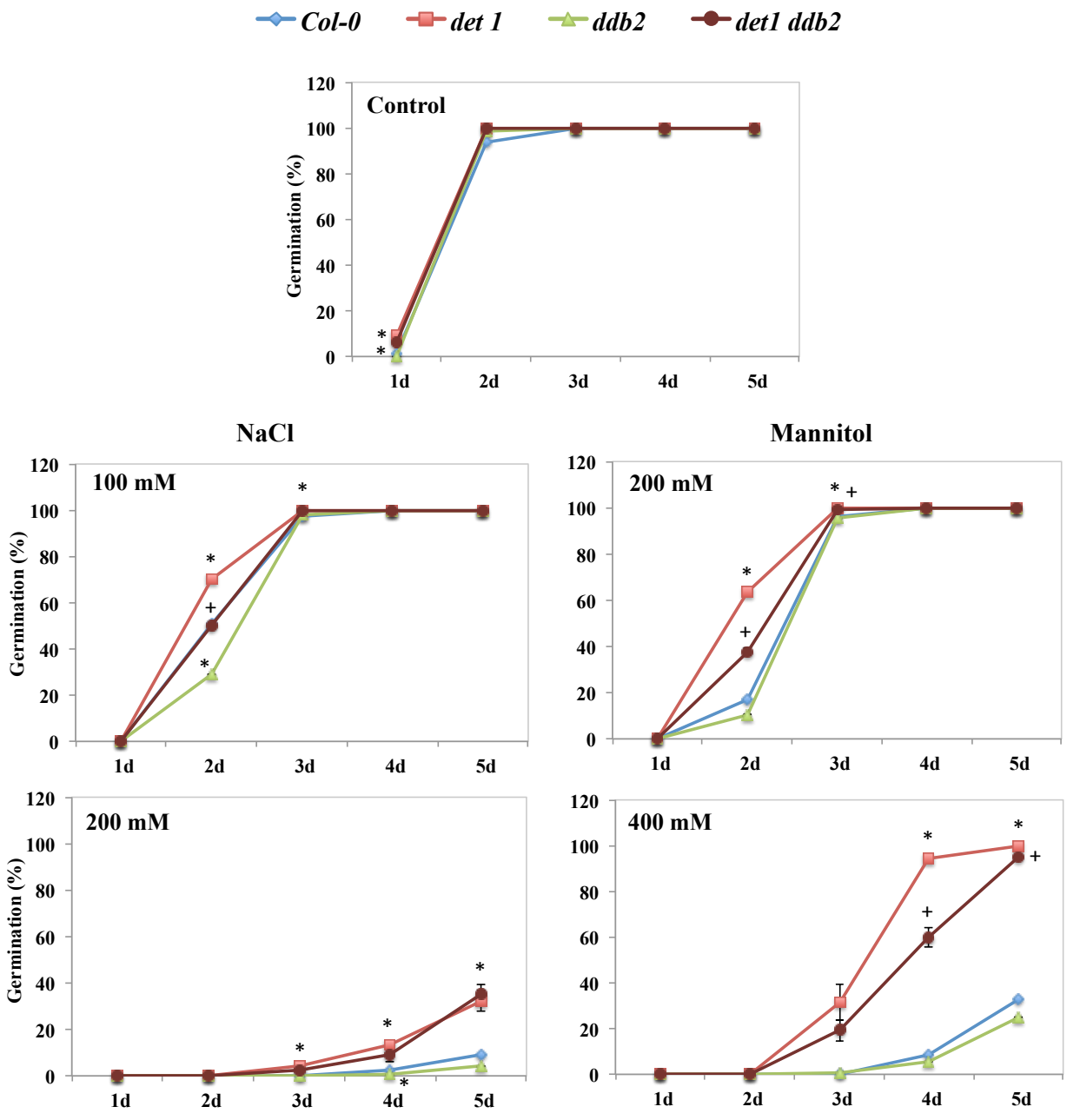

Figure 4.6 Germination in *det1 ddb2* double mutants

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

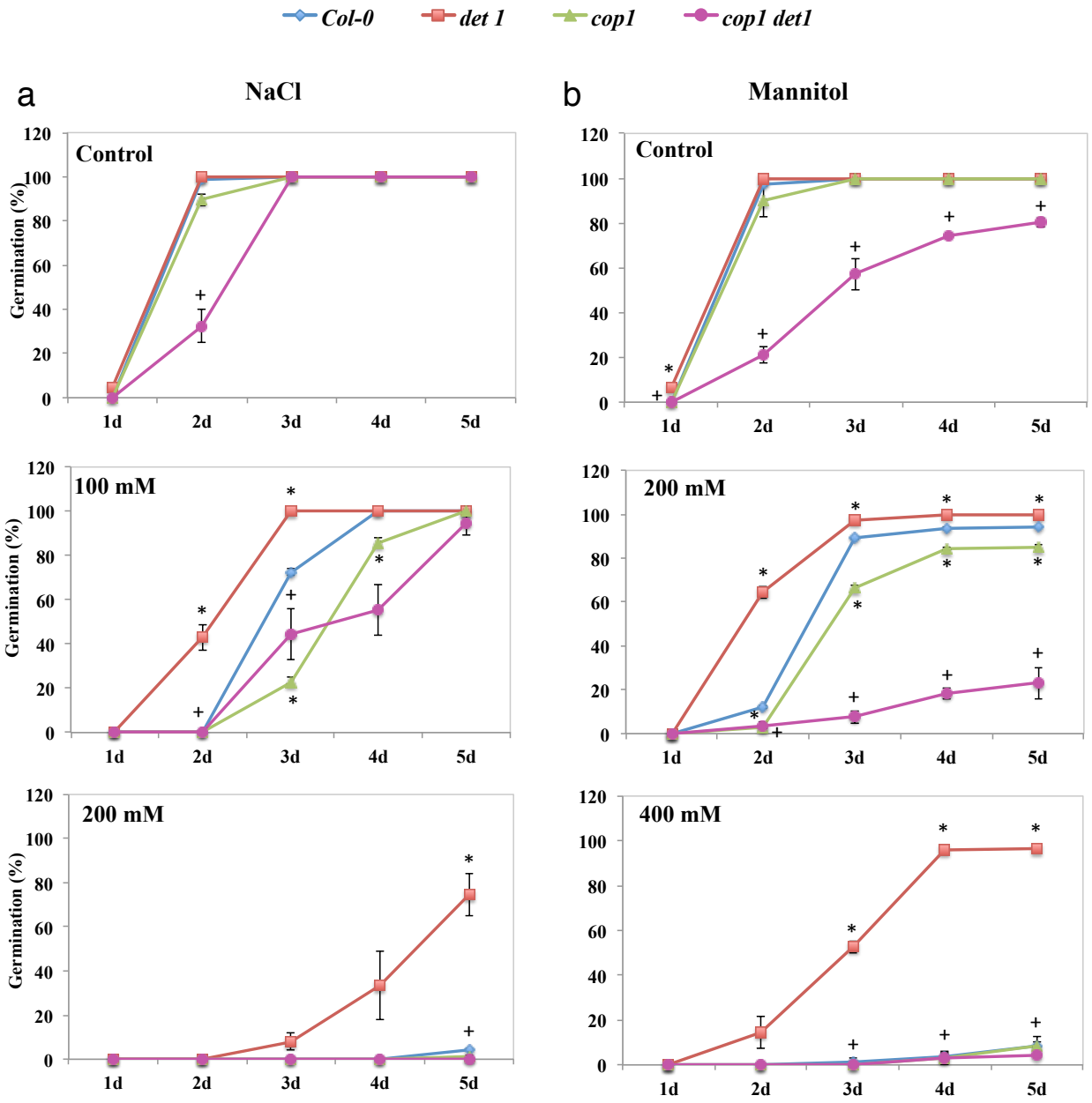

Figure 4.7 Germination in *det1 cop1-4* double mutants

Germination (%) on (a) 0, 100 mM, 200 mM NaCl (b) 0, 200 mM, 400 mM Mannitol. Values are means ± SE of 2 replicates of 50-100 seeds. *=P ≤ 0.05 of single mutants vs wildtype, + =P ≤ 0.05 of doubles vs *det1*.

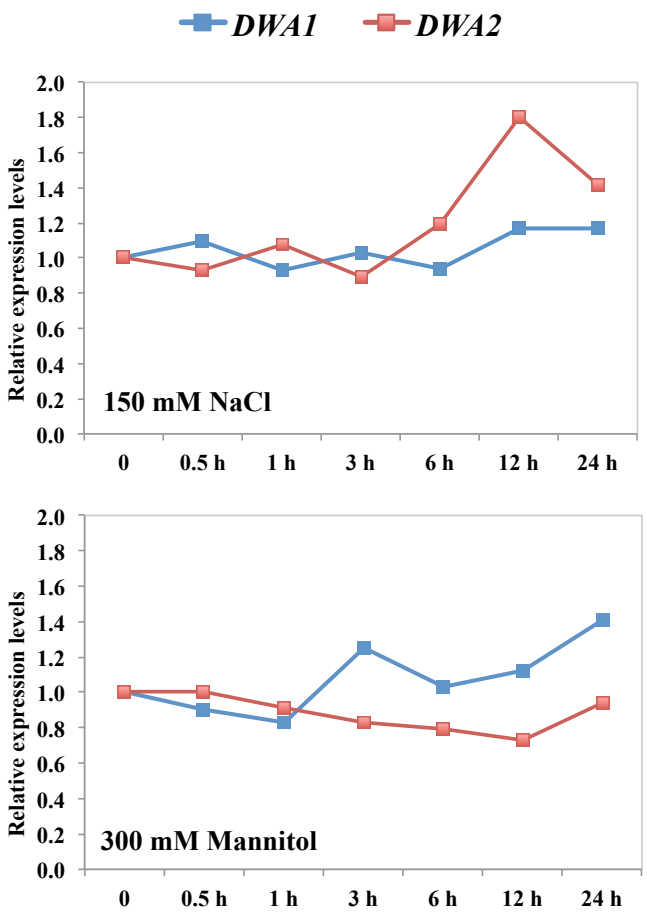

Figure 4.8 Relative expression levels of *DWA1* and *DWA2* under salt and osmotic stress

Relative expression levels of *DWA1* and *DWA2* in shoots of 18 day old plants following treatment with (a) 150 mM NaCl and (b) 300 MM Mannitol accessed via the Arabidopsis eFP browser (Kilian et al 2007, Winter et al 2007).

Chapter 5. Summary and Conclusions

5. Summary and Conclusions

A number of environmental factors affect plant growth and development, of which light could be argued to be one of the most important. In order to adapt to environmental conditions, plants have adopted a variety of developmental strategies. If the external environment is not favourable, seed germination is arrested as well growth is slowed down at the adult stage. These changes in response to environmental stresses occur as a result of genetic, biochemical and physiological interactions within the plant system. Endogenous hormonal cues shape these mechanisms. Thus plants complete their life cycles only if they are able to successfully adapt to environmental stresses by manipulating their developmental mechanisms accordingly. Seed germination is severely affected by abiotic stresses such as salt and osmotic stress, which subsequently influence plant yield (Finch-Savage and Leubner-Metzger 2006). Therefore, understanding the genetic basis of seed germination and abiotic stress tolerance is of significant importance in improving plant performance.

This study mainly focused on the effect of light and the hormone abscisic acid (ABA) on seed germination by investigating genetic interactions between the light and ABA signalling pathways. In addition, we examined ABA mediated salt and osmotic stress responses in mutants of our genes of interest. Our primary gene of interest, *DE-EIOLATED 1* (*DET1*), is a negative regulator of light signalling. There is a lot of crosstalk between light and hormone signalling. We investigated whether *DET1* has any role in ABA signalling and therefore, light and ABA signal integration in seed germination.

This study had 3 main objectives, the first of which was to describe the stress responses of *det1* mutants and to investigate the genetic interactions between *DET1* and intermediate genes in ABA signalling (*HY5*, *ABI5*, *DWA1*, *DWA2*) in terms of seed germination, seedling and adult

growth. Then we examined the genetic interactions between *DET1* and the *ABF* genes in order to elucidate genes involved in salt and osmotic stress responses. Finally, we studied the effect of components of CUL4 E3 ligase complexes on *det1* stress germination phenotypes. An overall summary of all the assessed phenotypes is presented in Table 5.1 and Table 5.2.

5.1 Double mutant analysis reveals genetic interactions between *DET1* and ABA signalling genes during Arabidopsis development

DET1 and intermediate genes in the ABA signalling pathway show a range of genetic interactions. In dark grown seedlings, *abf4, dwa2, ddb1a, ddb1b, ddb2,* and *cop1* all enhance the *det1* short hypocotyl phenotype. *abf4* and *cop1* also exhibit short hypocotyls in the wildtype background, so this may be an additive effect. *hy5* and *abf1* suppress the *det1* dark hypocotyl phenotype. For dark cotyledon width, *hy5, abf1, abf4, dwa1, ddb1a, ddb2, cop1*, and the Ws-2 background all suppress the *det1* cotyledon width phenotype, that is, make the cotyledons smaller. In light grown seedlings, *abf3, ddb1a,* and *cop1* enhance both the *det1* reduced hypocotyl length and cotyledon width phenotypes, potentially additively in the case of *abf3* and *cop1*, while *abf4* enhances cotyledon width only. *hy5, abf1, dwa1,* and *dwa2* suppress the *det1* light hypocotyl length phenotype, *hy5* and *abf1* apparently additively. *cop1* enhances the *det1* decreased chlorophyll phenotype, while *hy5, abf3, abf4, ddb1b,* and *ddb2* suppress it.

In adults, the *det1* early flowering time (days) phenotype is suppressed by all backgrounds examined. In the case of *hy5, abf3, abf4,* and *dwa2* this appears to be an additive effect. The *det1* early flowering time (leaves) phenotype was enhanced by *dwa1* and *ddb1a,* but suppressed by everything else. The *det1* decreased rosette diameter and height phenotypes showed similar trends where, *abi5, abf4*, *ddb1a,* and *ddb1b* enhance while *hy5, abf1,* and *ddb2*

suppress. The *det1* small silique length phenotype is enhanced by *abi5*, *ddb1a,* and *ddb1b* and suppressed by *hy5*, *abf3*, *abf1*, *dwa1, dwa2,* and *ddb2*. In the case of *ddb1a, hy5, dwa1,* and *dwa2* these are additive effects. Finally the *det1* reduced apical dominance phenotype is enhanced by the Ws-2 background and suppressed by *hy5, abf3, abf4,* and*. abf1*.

In examining general trends in this data, several things become evident. Firstly, both *hy5* and *abf1* suppress nearly all the *det1* growth and developmental phenotypes, as well as germination phenotypes, examined. HY5 is known to act downstream of DET1, and DET1 is a negative regulator of HY5 (Osterlund et al 2000, Nixdorf and Hoecker 2010). 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 regulates *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. We don't however see increased *ABF1* levels in *det1* seeds, suggesting other mechanisms are acting at this stage.

The mutant that exhibits the next highest levels of *det1* suppression is surprisingly *ddb2*. *ddb2* suppresses the majority of *det1* phenotypes, only enhancing dark hypocotyl length, indicating that DDB2 and DET1 act antagonistically. Al Khateeb and Schroeder (2007) generated triple mutants to see whether the *ddb2* effects on *det1* are dependent on *DDB1A* or not. We could do a similar analysis to see whether *ddb2 det1* germination phenotypes are dependent on *DDB1A*. Nonetheless the effect of *ddb2* on *det1* phenotypes does not seem to be a general effect of lack of one CUL4 DDB1 complex on another, since *dwa1* and *dwa2* do not exhibit the degree of *det1* suppression that *ddb2* does. Castells et al (2011) suggest that DET1 is required for

DDB2 degradation. Perhaps in *det1* mutants there are developmental consequences of this excess DDB2, which are rescued by the *ddb2* mutant.

On the other end of the spectrum, *cop1, ddb1a*, and *ddb1b* enhance the majority of *det1* developmental phenotypes. This is consistent with their known roles of working with DET1 to regulate photomorphogenesis (Huang et al 2014). However, these three mutants in general suppress, rather than enhance, *det1* germination phenotypes, suggesting that COP1, DDB1A and DDB1B are acting antagonistically to DET1 during germination. Surprisingly, *abi5* exhibits a pattern similar to *cop1, ddb1a*, and *ddb1b,* enhancing *det1* growth phenotypes but suppressing germination phenotypes. Lack of DDB1 would be expected to result in increased ABI5 levels, due to reduction of several complexes that negatively regulate ABI5. However, the majority of the effects observed in *det1 abi5* are also detected in the *det1* Ws-2 lines, suggesting that a modifier of *det1* segregating in the Ws-2 background may be the source of these effects.

abf3 and *abf4* exhibit similar effects on *det1* developmental phenotypes, consistent with the similar patterns of *ABF3* and *ABF4* expression in *det1* mutants in light and dark grown seedling (Figure 3.4a,b). In contrast, *abf3* and *abf4* have opposite effects on *det1* germination phenotypes, with *abf3* either enhancing or having no effect on *det1* phenotypes and *abf4* suppressing them. These contrasting effects in seeds are consistent with the effect of salt on gene expression in seeds (Figure 3.14), where *ABF3* is induced but *ABF4* is repressed by salt in wildtype seeds.

5.2 *det1* shows different germination phenotypes under different conditions

This study shows that *det1* mutants show a variety of germination phenotypes in different conditions. In control conditions, *det1* germinates earlier than the wildtype. Similarly, Ang and Deng (1994) showed that *cop1-6* mutants germinate early in R/FR and FR/R light conditions. However, we did not see a clear early germination phenotype in *cop1-4* mutants (Figure 2.9a and 4.7). Previous studies have shown that at least 50% of *det1* seeds germinate even in the dark (Shinomura et al 1994). This indicates that DET1 plays an important role in inhibition of seed germination in the dark and exposure to light suppresses DET1 and initiate seed germination (Shi et al 2015). On the other hand, germination in *det1* mutants is sensitive to ABA and *cop1* mutants are also sensitive to ABA. Thus both *COP1* and *DET1* act in the same pathway with regards to germination on ABA consistent with literature that COP1 And DET1 act in the same pathway in light regulated seed germination responses (Ang and Deng 1994). Moreover, both *cop1* and *det1* exhibit ABA resistant cotyledon emergence suggesting photomorphogenic mutants exhibit similar phenotypes on ABA. Although *det1* mutants are sensitive to ABA they show resistant germination to salt and osmotic stress. However, *cop1* is sensitive to salt/osmotic stress, showing an opposite phenotype to *det1,* suggesting that they act in different pathways in salt/osmotic stress inhibition of seed germination. Therefore, *det1* mutants show complex germination phenotypes in different conditions and the genes required for these responses is quite distinct from each other as discussed below.

5.3 Regulation of *det1* precocious germination in control conditions

Double mutants of *hy5*, *cop1*, *abf1* and *abf4* suppress the *det1* early germination phenotype in control conditions, indicating the requirement of these genes for this phenotype (Table 5.1, 5.2). On the other hand, *ddb1b* and *abf3* enhance this phenotype.

Since HY5 levels are negatively regulated by *DET1*, the *det1* mutant has excess HY5, thereby we would expect *det1* to have more ABI5 and less ABF3. However, our molecular analysis indicates that germination inhibiting *ABI5* levels are low in *det1* seeds (Figure 2.10a), consistent with the *det1* early germination phenotype. In addition, double mutant analysis indicates that the *det1* early germination on control media does not require ABI5. Perhaps there is little difference in *ABI5* levels between *det1* and *det1 abi5* since *ABI5* levels are already low in *det1*. In contrast, if ABF3 was involved in germination under control conditions, we would predict to see enhanced germination in *det1 abf3,* which is what we observe.

A functional relationship between the PIFs and COP1-SPA was identified recently. PIFs enhance COP1-SPA interaction and the ubiquitination activity of COP1, thus mediating degradation of HY5 (Dong et al 2015). In addition, DET1 directly interacts with PIFs, promoting their protein stability (Dong et al 2014). Collectively these findings reveal that DET1, COP1 and PIF interactions negatively regulate HY5 levels in the cell.

The *det1* early germination on control media may also be via PIF1/PIL5. *ABI5* is also a direct target of PIF1/PIL5 (Oh et al 2009). DET1 stabilizes PIF1/PIL5 protein levels in the dark, upregulating ABI5, repressing GA signalling, and inhibiting germination. Light destabilizes PIF1, promoting germination (Oh et al 2009). Perhaps in *det1* seeds reduced levels of PIF1 result in a decreased *ABI5* levels, resulting in increased germination (Figure 5.1). Thus DET1 is a

positive regulator of PIF1 and a negative regulator of HY5, which are both positive regulators of *ABI5*. In control conditions, PIF1 regulation of *ABI5* appears to be the dominant effect.

5.4 *det1* germination on ABA and salt/osmotic stress follows two different pathways

We observed that *det1* mutants exhibit opposite germination phenotypes on ABA and salt/osmotic stress. We propose that the basis of these two different *det1* phenotypes is HY5-

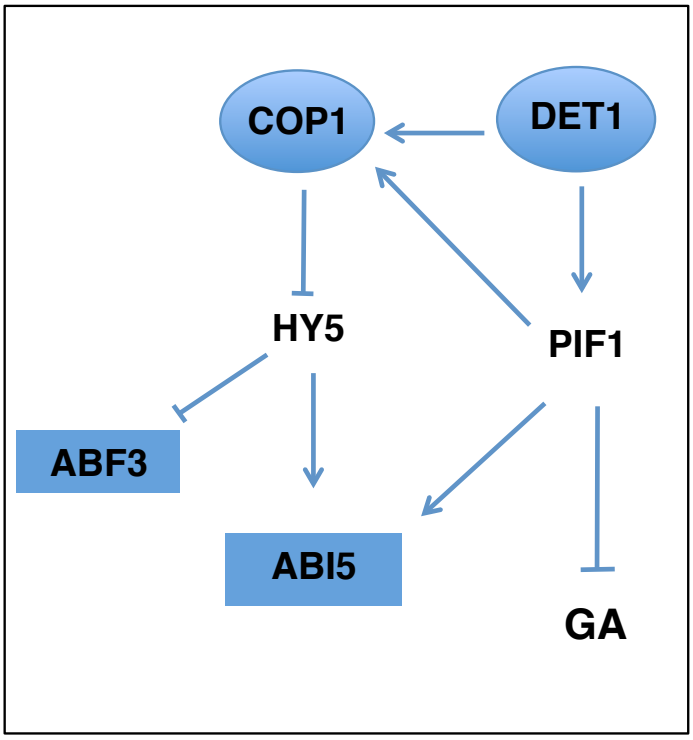

Figure 5.1 Model showing the role of DET1 in germination phenotypes

Interaction of DET1 with light and ABA signalling genes during germination. Arrows indicate positive interactions, whereas T-bars indicate negative effects. DET1 indirectly negatively regulates HY5 levels in the cell. On ABA, HY5 upregulates *ABI5* transcription. *det1* mutants have excess HY5, and thereby ABI5, resulting in ABA sensitive germination. In contrast, under salt/osmotic stress, HY5 downregulates *ABF3* transcription, thus *det1* mutants show salt/osmotic stress resistant germination. In addition, DET1, as a component of the CUL4-CDD complex, prevents PIF degradation, stabilizing PIF1 in the dark. PIF1 regulation of *ABI5* appears to

mediate germination in control conditions. Thus in *det1* mutants, reduced levels of PIF1 and thereby ABI5 results in early seed germination.

mediated upregulation of *ABI5* during ABA inhibition of germination (Chapter 2), while downregulation of *ABF3* results in resistance to salt/osmotic stress (Chapter 3). Thus, the effect of ABA on *det1* germination is via HY5-ABI5 regulation, whereas salt/osmotic stress effect is via HY5-ABF3 regulation (Figure 5.1).

Plants have ABA dependent and ABA independent mechanisms for salt and drought tolerance. Although salt/osmotic stress signalling in *det1* mutants during seed germination follows a different pathway than ABA signalling, it is still ABA dependent as it is via *ABF* genes. Both pathways involve light signalling component HY5 as the convergence point and DET1's role in repression of HY5 is probably the basis of *det1* stress germination phenotypes. However if HY5 was the only factor regulating *det1* phenotypes, we would expect to see similar phenotypes in *det1* and *cop1* mutants, since they both negatively regulate HY5. While this is true on ABA, on salt/osmotic stress they exhibit opposite phenotypes (Figure 4.7). A recent study on the role of COP1 during salt inhibition of germination showed that COP1 regulates seed germination via HY5 and ABI5 during salt stress (Yu et al 2016b), similar to what we observe with regard to DET1 during ABA treatment. How COP1 and DET1, both acting via HY5, result in different patterns of gene expression and different phenotypes during salt stress in currently unclear.

5.5 Overview of role of DET1 in seed germination

Recently LONG HYPOCOTYL IN FAR RED1 (HFR1) and PHYTOCHROME INTERACTING FACTOR 1 (PIF1) were identified as the main positive and negative regulators

of light induced seed germination respectively. DET1 enhances HFR1 degradation while it represses PIF1 degradation, thus DET1 is a key regulator of seed germination in control conditions (Shi et al 2015). Our work shows DET1 also has a role in seed germination under stress conditions. On ABA, DET1, acting as a repressor of HY5 and thereby germination inhibiting ABI5, regulates ABA inhibition of seed germination (Chapter 2). In addition, DET1 suppresses seed germination in salt/osmotic stress conditions by regulating ABF3 levels via HY5 (Chapter 3). These findings further strengthen the idea that DET1 is an important regulator of seed germination not only under normal conditions but also in stress conditions.

5.6 Future research

Much research is being carried out to elucidate the mechanisms behind light and hormone signal integration. The main goal of this study was to investigate whether the light signalling component DET1 has a role in ABA signalling and thereby integration of light and ABA signalling pathways. In this study we have shown the role of Arabidopsis *DET1* in seed germination under different conditions as well as the interactions between DET1 and ABA signalling genes during development. However, there are several experiments that can be done in order to validate and further examine the models we propose for the basis of *det1* phenotypes.

- Although we predicted enhanced germination in *det1 abf4* and *det1 abf1* double mutants relative to *det1* single mutants on salt and mannitol, we observed a suppressed phenotype instead (Figure 3.7, 3.9). Perhaps these differences are due to increased levels of *ABF3* in the *abf4 det1* and *abf1 det1* double mutants. Therefore, qRT-PCR analysis will be performed in order to test this prediction.

- The *ABF* genes have been reported to have redundant functions, particularly at the vegetative stage. Thus, generating an *abf1 abf3 abf4* triple null mutant in the *det1* background would enable us to distinguish the effect of *ABF* genes on *det1* salt/osmotic stress phenotypes, developmental phenotypes and water loss. In addition it will be interesting to investigate the effect of overexpression of *ABF3* in *det1*, to see whether *det1* resistant germination is rescued under salt/osmotic stress conditions.
- Next Generation Sequencing techniques could be used with the double mutant lines established in this study to uncover additional regulatory networks in ABA and other signaling cascades.
- We have shown that *DWA1* and *DWA2* do not have any significant role in *det1* salt/ osmotic stress germination but show partial rescue of the *det1* ABA germination phenotype. Both *det1 dwa1* and *det1 dwa2* phenotypes are different than what we predicted and therefore, it is not clear how *DWA1* and *DWA2* contribute to *det1* stress germination phenotypes. Although not homologous, *DWA1* and *DWA2* genes show redundant function (Lee et al 2010). Also our molecular analysis showed that *DWA1* mRNA levels were upregulated in *det1 dwa2* double mutants and maybe one *DWA* compensates in the absence of the other. Therefore it may be beneficial to construct *dwa1 dwa2* double mutants in the *det1* background (*det1 dwa1 dwa2* triple mutants) and assess their germination phenotypes under ABA and salt/mannitol conditions, and perform developmental analysis and water loss assays to find out whether DWA1 and DWA2 have any role in the above phenotypes.
- Immunoblot analysis with anti-ABI5 and anti-ABF antibodies to allow us to detect ABI5 and ABF protein levels in the *dwa1 dwa2 det1* triple mutant will enable us to understand the function of DWA1/2 at the post translation level in the absence of DET1. This will also show

whether ABF proteins are additional targets of DWA1/DWA2 substrate receptors in the CUL4-DDB1 E3 ligase complex.

- It is also interesting to elucidate the interaction between PIF and DET1 in germination under stress conditions. We could use *det1 pif1* double mutants and PIF1 overexpression in *det1* lines to look at the effects of PIF1 on *det1* germination using similar germination experiments. Moreover we can extend this analysis and use Western blots to detect PIF1 and ABI5 protein levels in ABA and salt/osmotic stress induced *det1* seeds to see whether PIF1 and ABI5 levels are different in *det1* mutants. We can also detect the *PIF1* and *ABI5* mRNA levels by quantitative Real time PCR to see whether there is any difference at the transcriptome level. These findings will further uncover the role of PIF1 in regulating GA and ABA hormone levels in stress conditions.
- *ABI5* exhibits genetic interactions with *DET1*, however analysis of these effects is complicated by modifiers of *det1* phenotypes in the Ws-2 background. Therefore in future experiments it is important to include a *abi5* mutant in the Col-0 ecotype in our analysis.

The outcome of this research provides a comprehensive assessment of the role of DET1 in seed germination under stress conditions as well as insight into the role of DET1 in light and ABA signal integration. Understanding the role of DET1 in genetic models such as Arabidopsis is an important start so that the *DET1* orthologues in tomato and other systems can be studied in future with the intention of improving plant performance in stress conditions. Recently it was found that the rice orthologue of *DET1* (*OsDET1*) is very important for normal growth and development. In addition they found OsDET1 is involved in mediating ABA signaling in rice

(Zang et al 2016). Overall the results of this study provide further evidence for the important regulatory role of DET1 in plants in terms of stress signaling via ABA.

Table 5.1 Summary of phenotypic effects of transcription factors on *det1*. 0: no significant effect, - : significant decrease, +: significant increase in parameter relative to appropriate control (Col-0 for single mutants and *det1* for double mutants). , ND: Not determined. In double mutants one symbol represents each line assessed. In salt and mannitol assays " / " indicates phenotype on different concentrations.

	det1	*hy5*	*det1 hy5*	*abi5*	*det1 abi5*	*det1 Ws-2*	*abf3*	*det1 abf3*	*abf4*	*det1 abf4*	*abf1*	*det1 abf1*
7 day dark												
Hypocotyl length	-	0	+ +	0	0/0	0/0	0	0/0	-	- -	0	+ + +
cotyledon width	+		- -		- 0	- 0		0/0		- -		- - -
Anthocyanin content	+	0	0 0	ND	ND	ND	0	0/0	0	0 0	0	0 0 0
7 day light												
Hypocotyl length	-	+	+ +	ND	ND	ND	-	- 0	0	0 0	+	0 + +
cotyledon width	-	0	0 0	ND	ND	ND	-	- -	+	- -	0	0 0 0
chlorophyll content	-	0	+ 0	ND	ND	ND	0	+ +	0	+ +	0	0 0 0
Anthocyanin content	+	0	0 0	ND	ND	ND	0	- 0	0	0 0	0	0
Adults												
Flowering time (days)	-	+	+ +	0	+ +	+ +	+	+ +	+	+ +	-	+ + +
Flowering time (leaves)	-	+	+ +	0	0 0	+ 0	+	+ +	0	+ +	-	0 0 0
Rosette diameter	-	0	+ +	0	- 0	+ -	-	0 0	+	- -	0	+ + +
Height	-	0	+ +	0	- 0	+ 0	0	0 0	0	- -	+	+ 0 +
Silique length	-	+	+ +	0	- 0	- -	0	+ +	0	0 0	0	+ + +
Number of stems	+	0	- -	0	+ +	0 +	-	- -	0	- -	+	- - -
Germination												
control	+	-	-	0	0	-	0	0 +	0	0 -	-	-
NaCl (100/200 mM)	+	0	-/-	+	0/0	-/-	+	- 0/+ -	+	--/--	0/0	---/---
Mannitol (200/400 mM)	+	+	-/0	+	0/-	-/-	+	- +/0+	+	--/--	0/0	---/---
ABA	-	+	+ +	+	+ +	0 0	0	0 0	+	+ +	-	+ + +
ABA cotyledon emerg	+	0	+ +	+	0 0	- -	ND	ND	ND	ND	ND	ND
Water loss	+	0	0 0	0	0 0	0 0	0	0 0	0	0 0	0	+

Enhancement of mutant phenotype
Suppression of mutant phenotype

Table 5.2 Summary of phenotypic effects of E3 ligase components on *det1*. 0: no significant effect, - : significant decrease, +: significant increase in parameter relative to appropriate control (Col-0 for single mutants and *det1* for double mutants). , ND: Not determined. NA: Not applicable/adult lethal. In double mutants one symbol represents each line assessed. In salt and mannitol assays " / " indicates phenotype on different concentrations.

	det1	*dwa1*	*det1 dwa1*	*dwa2*	*det1 dwa2*	*ddb1a*	*det1 ddb1a*	*ddb1b*	*det1 ddb1b*	*ddb2*	*det1 ddb2*	*cop1*	*det1 cop1*
7 day dark													
Hypocotyl length	-	-	0 0	0	- 0	0	-	0	-	0	-	-	-
cotyledon width	+		- -		0 0		-		0			+	-
Anthocyanin content	+	0	0 0	+	0 0	0	+	0	0	0	-	0	+
7 day light													
Hypocotyl length	-	0	+ +	0	+ +	0	-	0	0	0	0	-	-
cotyledon width	-	0	+ +	0	0 0	0	-	0	0			-	-
chlorophyll content	-	0	0 0	ND	ND	0	0	0	+	0	+	(-)	-
Anthocyanin content	+	0	0 0	+	0 0	0	+	0	+	0	0	0	+
Adults													
Flowering time (days)	-	-	+ +	+	0 0 +	0	+	0	+	0	+	0	NA
Flowering time (leaves	-	-	- -	+	0 0 0	0	-	0	+	0	+	0	NA
Rosette diameter	-	0	0 0	-	0 0 0	0	-	0	-	0	+	-	NA
Height	-	+	0 0	-	+ 0 -	0	-	0	-	0	0	-	NA
Silique length	-	+	+ +	+	+ + 0	-	-	0	-	0	+	-	NA
Number of stems	+	+	+ 0	0	+ + 0	0	0	0	0	0	0	0	NA
Germination													
control	+	-	0	0	0 +	0	0	0	+	-	0	0	-
NaCl (100/200 mM)	+	0	00/00	0	++/00	-	0/-	0	0/0	-	-/0	-	-/-
Mannitol (200/400 mM	+	-	++/00	0	00/00	-	-/-	-	0/0	0	-/-	-	-/-
ABA	-	0	+ +	0	+ +	0	+	0	+	0	+	-	-
ABA cotyledon emerg	+	0	00	0	0 -	0	-	0	-	ND	ND	+	-
Water loss	+	0	0 0	0	0 0	ND	ND	0	0 0	ND	ND	ND	NA

Enhancement of mutant phenotype
Suppression of mutant phenotype

Seedling and adult developmental data for *ddb1a, ddb1b, ddb2, cop1* and their double mutants are from Schroeder et al 2002; Al Khateeb and Schroeder 2007; Ganpudi and Schroeder 2013; Ly et al 2015.

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