The Role of Arabidopsis *Damaged DNA Binding Protein 1B* and genetic interactions with *DDB1A*, *DDB2*, *De-etiolated1 (DET1)* and *Constitutive Photomorphogenic1 (COP1)*

by

Ashwin L. Ganpudi

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Department of Biological Sciences

University of Manitoba

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Abstract

Damaged DNA Binding Protein 1 (DDB1) – CULLIN4 E3 ubiquitin ligase complexes have been implicated in a variety of biological processes in a range of organisms. Uniquely, *Arabidopsis thaliana* encodes two homologs of DDB1, DDB1A and DDB1B. In this study we utilize a viable partial loss of function allele of *DDB1B, ddb1b-2,* to examine genetic interactions with *DDB1A, DET1* and *COP1*. While the *ddb1b-2 ddb1a* double mutant is lethal, *ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+* heterozygotes do not exhibit any developmental phenotypes. These heterozygotes do however exhibit decreased UV tolerance. In addition, germination in *ddb1a* and *ddb1a ddb1b-2/+* was found to be sensitive to salt and mannitol, and both *DDB1* single mutants as well as the heterozygotes exhibited heat sensitivity. DE-ETIOLATED1 (DET1) and CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) are negative regulators of light development which interact with DDB1A. While *ddb1a* enhances *det1* phenotypes in both dark and light grown seedlings, *ddb1b-2* did not affect *det1* dark phenotypes but enhanced anthocyanin levels and suppressed the *det1* low chlorophyll phenotype in light grown seedlings. In adults, *ddb1a* suppresses *det1* early flowering and enhances the *det1* dwarf phenotype. A similar trend was observed in *ddb1b-2 det1* double mutants, although the effects were smaller in magnitude. In *cop1* mutants, *ddb1b-2* enhanced the *cop1-4* short hypocotyl phenotype in the dark, and enhanced anthocyanin levels and suppressed the short hypocotyl phenotype in *cop1-1* in the light, but had no effect in adults. Hence *DDB1B* and *DDB1A* vary in their importance to different complexes during development.

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I wish to thank members of the Schroeder lab both past and present, my committee members Dr. Sumner and Dr. Stasolla, my “Canadian” parents Snehil and Alok Dua who continue to host a bunch of us until today, as well as friends in both India and Canada and my extended family members.

I also wish to thank my mom for enormous emotional support throughout the course of my program, for some reason it made the 12,000 odd Km away from home seem closer.

List of Abbreviations

6-4PPs	(6-4) pyrimidinone dimers
95% C.I.	95% confidence interval
CDD	COP1-DET1-DDB1
COP	Constitutive Photomorphogenic
CPD	Cyclobutane pyrimidine dimers
CSA/B	Cockayne Syndrome A and B
CSN	COP9 Signalosome
CUL4	Cullin 4
DCAF	DDB1-CUL4 Associated Factor
DDB	Damaged DNA Binding Protein
DET	De-etiolated
DWD	DDB1-binding WD40 protein
GG-NER	Global Genomic – Nucleotide Excision Repair
HY5	LONG HYPOCOTYL 5 transcription factor
$J.m^{-2}$	Joule per square meter
LD/SD	Long Day/Short Day
PIF	Phytochrome Interacting Factor

RBX1 Ring Box 1

RT-PCR Reverse Transcriptase – Polymerase Chain Reaction

T-DNA Transfer DNA

TC-NER Transcription Coupled – Nucleotide Excision Repair

Ub Ubiquitin

UV Ultraviolet

XP Xeroderma Pigmentosum

Table of Contents

List of Figures

List of Tables

1. LITERATURE REVIEW

Plants, being sessile, must cope with a variety of environmental conditions. Protein degradation is central to regulation of plant environmental response. E3 ubiquitin ligases regulate the degradation of proteins specific to distinct responses. E3 ubiquitin ligases often include Cullin (CUL) proteins which interact with specific adaptor proteins to mediate the degradation activity. Of interest to us is the CUL4 based E3 ligase which employs Damaged DNA Binding protein 1 (DDB1) as its substrate adaptor. This CUL4-DDB1 system is capable of interacting with many DCAF proteins (DDB1-CUL4 Associated Factors). Examples of DDB1 interacting proteins of interest include UV damaged DNA repair proteins Damaged DNA Binding Protein 2 (DDB2) and Cockyne Syndrome A (CSA), repressors of photomorphogenesis Constitutive Photomorphogenic 1 (COP1) and De-etiolated 1 (DET1) and proteins involved in abiotic stress response and ABA signalling, DWA1, DWA2 and DWA3.

My work focuses on the genetic interactions between the two Arabidopsis *DDB1* homologs: *DDB1A* and *DDB1B*. We employ a reverse genetic approach to tease apart the redundant yet distinct roles played by the two genes in overall growth and developmental response. In addition, we characterize the genetic interactions of *DDB1B* with its interactors, *DDB2*, *DET1* and *COP1* (Fig 1.1). The following introductory sections provide basic background on the above mentioned players in light signalling and UV-damaged DNA repair in plants.

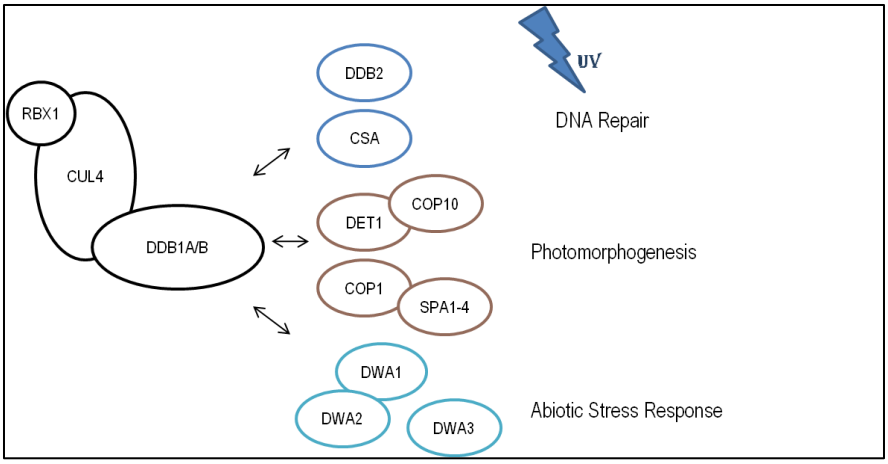

Fig 1.1: DDB1-CUL4 Associated Factor (DCAF) interactions

1.1 GENETICS OF LIGHT SIGNALLING

Plant growth is influenced by many environmental stimuli such as temperature, light, touch, water, wind and gravity. Of these, light in particular plays a crucial role. It serves as an activation signal for multiple transduction cascades, alters endogenous hormonal activities, and initiates tissue biogenesis and differentiation, thereby triggering variations in global gene expression (Chen et al. 2004).

Apart from serving as a primary substrate for photosynthesis (CO_2 fixation catalyzed by light photons), light is crucial for photoperiodism (biological clock), phototropism (directional growth), and photomorphogenesis (dark to light transitional development). The molecular bases of the above responses are distinct, yet have interlinked signalling pathways (Jiao et al. 2007).

Sunlight can be classified into three broad spectral domains: Ultra-Violet (<400 nm), Visible (400-700 nm) and Far-red (>700 nm). Plants are capable of interpreting and

transducing light signals from all three spectral domains through specialized proteins called photoreceptors. The three major classes of plant photoreceptors are: Red (R) / Far Red (FR) light (600-750 nm) absorbing phytochromes, blue / UV-A (320-500 nm) absorbing cryptochromes and phototropins (Chen et al. 2004) and the very recently characterized UV-B photoreceptors (Rizzini et al. 2011). Light mediated signal transduction initiates with light perception by these photoreceptors followed by signalling cascades resulting in altered expression of several thousand genes enabling downstream physiological response (Jiao et al. 2007).

1.1.1 Positive regulators in light signalling

1.1.1.1 Plant photoreceptors

1.1.1.1.1 Phytochromes

Arabidopsis has five phytochromes (PhyA - PhyE) which absorb red – far red light. In Arabidopsis, all five phytochromes are cytosol localized in the dark and nuclear localized under light conditions. PhyA, classified as a "Type-I" phytochrome, is predominantly expressed in etiolated seedlings and negatively regulated by light (its level drops almost a 100 fold). PhyB-E constitute the "Type II" class wherein PhyB is most predominantly expressed in de-etiolated seedlings and PhyC-E are less abundant (Franklin and Quail 2010). Phytochromes, soluble chromoproteins with linear tetrapyrrole chromophores, exist in two spectrally distinct forms: the unstable red light absorbing form (Pr) and the stable far-red light absorbing form (Pfr), except for PhyA,

where the Pfr form is degraded within minutes of light exposure, thus the Pr form is more active (Rubio and Deng 2005).

Red light activates phytochromes B-E by converting Pr to the Pfr form, while upon perceiving far red light, the active Pfr is interconverted to the inactive Pr form. Upon perceiving red light, the active Pfr forms are nuclear localized and regulate several downstream transcriptional networks (Fankhauser and Chen 2008). Phytochromes are homodimeric proteins, consisting of an N-terminal photosensory domain, which binds to a bilin chromophore to confer light absorption, and a C terminal domain, which is essential for dimerization, protein-protein interaction and nuclear localization (Nagatani 2010). Different phytochromes have distinct, redundant, antagonistic and synergistic roles. Phytochromes in general regulate many physiological and developmental processes in plants (Reviewed in Kami et al. 2010).

Nuclear localization of the Pfr form of PhyB appears as speckles co-localizing with a specific class of basic Helix Loop Helix (bHLH) transcription factors called Phytochrome Interacting Factors (PIFs) (Huq and Quail 2002). PIFs function as negative regulators of various photomorphogenic (light growth) characteristics such as hypocotyl growth inhibition, seed germination and chlorophyll accumulation (Quail 2000), as evident from the quadruple mutant which exhibits a photomorphogenic growth pattern in the dark. In the dark, PIFs are active and repress light responsive growth, but are degraded in the light. PIF turnover is catalysed by phosphorylation upon direct interactions with the photoactivated phytochromes. Nuclear localized PhyB appearing as speckles are proposed to be the sites of PIF degradation. However, other proteins involved in this process remain unclear (Fankhauser and Chen 2008). The recently

identified mutant *hemera*, which represents a new class of mutants, lacks this PhyB - PIF interaction, as well as PhyA, PIF1 and PIF3 degradation in light (Chen et al. 2010b).

1.1.1.1.2 UV/Blue light photoreceptors: cryptochromes, phototropins, and UV-B photoreceptors

Blue light activates specific signalling cascades downstream of the cryptochrome and phototropin blue light photoreceptors. Cryptochrome signalling is pivotal in seedling photomorphogenesis, activating several genetic and physiological responses. In addition, cryptochromes are essential for resetting the circadian rhythm and co-ordinate with phytochromes in several instances. Arabidopsis encodes two cryptochromes – Cry1 (dominant under high blue light fluence rate) and Cry2 (more dominant during low blue light fluence rates). Cryptochromes share close structural similarity to the DNA repair enzyme photolyase but lack repair activity. Cry2 is exclusively nuclear localised but Cry1 localization is light dependant (nuclear in dark but cytoplasmic in light). Cry1 and Cry2 interact specifically with PhyA and PhyB respectively, suggesting synergistic interactions in light mediated transcription. While *Cry1* is expressed constitutively, *Cry2* levels are downregulated by blue light (Lin 2000; Lin and Shalitin 2003). Cry1 plays a prevalent role in the de-etiolation response to high intensities of blue light, while Cry2 requires much lower intensities. Cryptochromes 1 and 2 possess two domains: an N terminal photolyase related domain (PHR) (responsible for binding to chromophores enabling light perception) and a C terminal DAS domain (transduces signals from PHR domain,

responsible for nuclear/cytosol trafficking and mediating protein-protein interactions) (Nagatani 2010).

Phototropins are photoreceptors mediating light dependent directional growth. Two genes have been identified in Arabidopsis – *Phot1* and *Phot2*. Apart from phototrophism, this specific set of photoreceptors are redundantly involved in regulating photosynthesis, chloroplast movement, stomatal opening, cotyledon/leaf expansion, electrophysiological responses (Ca^{2+} flux rates) and blue light induced transcriptional regulation. Both Phot1 and Phot2 are plasma membrane embedded. While Phot1 is more specific for low fluence rates of blue light, Phot2 requires higher fluence rates. The double mutant *phot1 phot2* is completely insensitive to both fluences. The phototropins are composed of two distinct domains: a C terminal Ser/Thr protein kinase domain and an N terminal domain containing two LOV (low oxygen voltage) subdomains. LOV non-covalently binding to Flavin Mononucleotide (FMN) permits light sensing (Demarsy and Fankhauser 2009). Interestingly, upon blue light perception, a portion of Phot1 detaches from the plasma membrane and streams into the cytosol (Sakamoto and Briggs 2002). Any such functional localization for Phot2 is yet to be determined.

Recently, Rizzini et al. (2011) characterized the UV-B photoreceptor system in plants. They provide strong evidence that UV specific protein UVR8, under UV-B irradiation, monomerizes and interacts with Constitutive Photomorphogenic 1 (COP1) to relay the UV-B specific downstreaming signalling.

1.1.1.2 HY5

Other key positive regulators of photomorphogenesis include HY5 (Hypocotyl elongated 5), a bZIP transcription factor which serves as a convergence point of multiple light and hormone signalling networks (Lau and Deng 2010). HY5 directly regulates the transcription of light responsive genes. HY5 levels are COP1 regulated and light dependent (Osterlund et al. 2000).

1.1.2 Negative regulators of photomorphogenesis

1.1.2.1 Photomorphogenesis and photomorphogenic mutants

During seed germination in the dark, seedlings grow heterotrophically in the soil following a skotomorphogenic (or etiolated) growth pattern (rapidly elongated hypocotyls and unexpanded cotyledons protected by an apical hook). In contrast, on perceiving light once above the soil surface, the seedling switches to the photomorphogenic (or de-etiolated) growth pattern (short hypocotyls and expanded open cotyledons with active chloroplasts participating in photosynthesis) (Fig. 1.1).

This transition from etiolation to de-etiolation is controlled by the *Constitutively Photomorphogenic / De-etiolated / Fusca (COP / DET / FUS)* genes. Loss of function recessive mutations in all *COP/DET/FUS* loci generate a phenotypically de-etiolated plant in the absence of light suggesting that these functional proteins repress photomorphogenic development in the dark and that light ceases this repressive function (Chory 1993). The mutants were initially generated from genetic screens that sought to

identify seedlings that display characteristics of light grown phenotypes in complete darkness (Chory et al. 1989; Deng et al. 1991). Hence COP/DET/FUS can be collectively referred to as negative regulators of photomorphogenesis and function downstream of the photoreceptor pathways. At least 11 genetic loci have been identified and mapped for the *COP/DET/FUS* genes. Biochemically, the COP/DET/FUS associate into three distinct protein complexes *in vivo* to repress photomorphogenesis: the COP1 complex; the CDD complex; and the COP9 Signalosome (CSN) complex (Table 1.2).

Dark Light

Fig. 1.2 Dark and light grown phenotype of 7 day old Arabidopsis seedlings.

Knockout or knockdown mutations in all these loci are recessive and result in seedlings with a photomorphogenic response in the absence of light, in addition to expressing a wide array of light regulated genes (Ma et al. 2003; Schroeder et al. 2002).

All three functional complexes have been identified as components of the *Arabidopsis* cullin based E3 Ubiquitin ligase machinery, catalyzing target protein turnover.

Gene locus name	**Other protein names**	**Protein M. Wt**	**Complex formation**
COP1		76 kDa	COP1 E3 ligase
DET1		62 kDa	CDD complex
COP10		20 kDa	CDD complex
COP11	CSN1	50 kDa	COP9 signalosome
FUS12	CSN2	51 kDa	COP9 signalosome
FUS11	CSN3	47 kDa	COP9 signalosome
COP8	CSN4	45 kDa	COP9 signalosome
AJH1, AJH2	CSN5	40 kDa	COP9 signalosome
CSN6a, CSN6b	CSN6	35 kDa	COP9 signalosome
FUS5	CSN7	25 kDa	COP9 signalosome
COP9	CSN8	22 kDa	COP9 signalosome

Table 1.1 Summary of the COP/DET/FUS proteins in Arabidopsis

1.1.2.1.1 Constitutive Photomorphogenic 1 (COP1)

COP1, a 76 kDa protein, consists of a N-terminal RING finger domain, a coiled coil domain and C terminal domain containing seven WD40 motifs (40 amino acid repeats initiated by glycine-histidine dipeptide and terminated by tryptophan-aspartate (WD) dipeptide which facilitates protein-protein interactions) (McNellis et al. 1994). The RING finger domain is similar to E3 ubiquitin ligases, the coiled coil domain is responsible for homo or hetero dimerization of COP1 and WD40 motifs mediate protein interactions (Torii et al. 1998). Within and adjacent to the coiled-coil domain is the nuclear localized signal (NLS). Under dark conditions, COP1 accumulates in the nucleus as a 700 kDa complex catalyzing the degradation of photomorphogenesis promoting transcription factors such as HY5, HYH, CIP7, and LAF1, but localizes to the cytoplasm within minutes of exposure to light. This light influenced localization is COP9 signalosome (CSN) and CDD complex dependent, suggesting synergistic interactions among the entire family of *COP/DET/FUS* genes in regulating photomorphogenesis (Holm et al. 2002; Osterland et al. 1999; von Arnim and Deng 1994). Recently functional COP1 was shown to interact with SPA1-4 (Supressor of PhyA). The quadruple mutant *spa1 spa2 spa3 spa4* exhibits a photomorphogenic response similar to the pleiotrophic *cop/det/fus* mutants (Chen et al. 2011). The human ortholog of Arabidopsis COP1 has been shown to be an important negative regulator of the human tumor suppressor P53 (Yi and Deng 2005) and in concert with DET1, is capable of degrading the proto-oncogene transcription factor c-JUN (Wertz et al. 2004).

1.1.2.1.2 COP9 Signalosome (CSN)

Six of the *COP/DET/FUS* genes encode subunits of the COP9 Signalosome (CSN). Two other redundant genes encode the remaining two subunits of the CSN complex (as described in Table 1.1). Structurally, the CSN complex exhibits high homology to the lid sub complex of the 19S regulatory subunit of the 26S proteosome, consistent with the CSN's role in protein degradation (Wei et al. 2008). COP1 nuclear accumulation in darkness is abolished in the *csn* mutants indicating its importance in COP1 regulation (Chamovitz et al. 1996). Furthermore, the CSN plays a prominent role in post-translational modification of CULLIN based E3 ligases through deconjugating/conjugating RUB (in plants) or NEDD8 (in mammals), a ubiquitin variant. Details on the functional role and requirements of the CSN in overall plant development are reviewed in Schwechheimer and Isono (2010).

1.1.2.1.3 COP10-DET1-DDB1 (CDD) complex

The *cop10* mutation results is a constitutively photomorphogenic phenotype in the dark with defects in COP1-mediated degradation of HY5 (Osterlund et al. 2000). Wildtype levels of COP10 protein requires a functional COP9 Signalosome. The amino acid sequence of COP10 exhibits high homology with E2 (Ubiquitin conjugating enzymes) such as UBC4/UBC5 from *S. cerevisiae* and UBC8/UBC9 from Arabidopsis (Suzuki et al. 2002). COP10, similar to DE-ETIOLATED 1 (DET1), is exclusively nuclear localized.

DET1 encodes a 62 kDa protein lacking any known DNA binding domain. DET1 is constitutively nuclear localized and capable of interacting with the non-acetylated core histone proteins (Benvenuto et al. 2002; Pepper et al. 1994). Recently Lau et al. (2011) have identified DET1 as a transcriptional repressor of circadian rhythms which interacts with the morning specific transcription factors LHY and CCA1 to repress TOC1 expression. Schroeder et al. (2002) discovered biochemical and genetic interactions of DET1 with DDB1A. Damaged DNA Binding Protein 1 (DDB1) consists of two homologs – DDB1A and DDB1B in Arabidopsis. Biochemical data confirm that COP10 exists as a complex with DET1-DDB1 *in vivo* designated the CDD complex (Yanagawa et al. 2004). The CDD complex is further capable of interacting with COP1 and the CSN signalosome. Recently, it has been shown that the COP1-SPA1-4 complex also interacts with DDB1 and subsequently with CUL4 to regulate photomorphogenesis (Chen et al. 2010a) (Fig. 1.3).

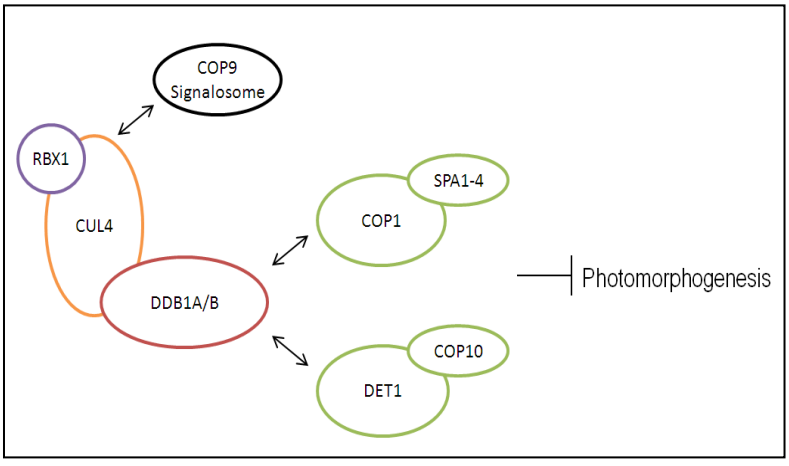

Fig. 1.3 Interactions of COP1-SPA, DET1-COP10, and CSN with the DDB1-CUL4 complex

1.1.3 Overview of light signalling cascades

Upon light absorption, the photoreceptors, phytochromes and cryptochromes, suppress two pathways: 1) the COP/DET/FUS mediated E3 ligase pathway (which catalyzes the turnover of positive regulators of photomorphogenesis) and 2) PIFs (transcriptional regulators which negatively regulate photomorphogenesis), thereby promoting a light responsive growth (Fig. 1.4).

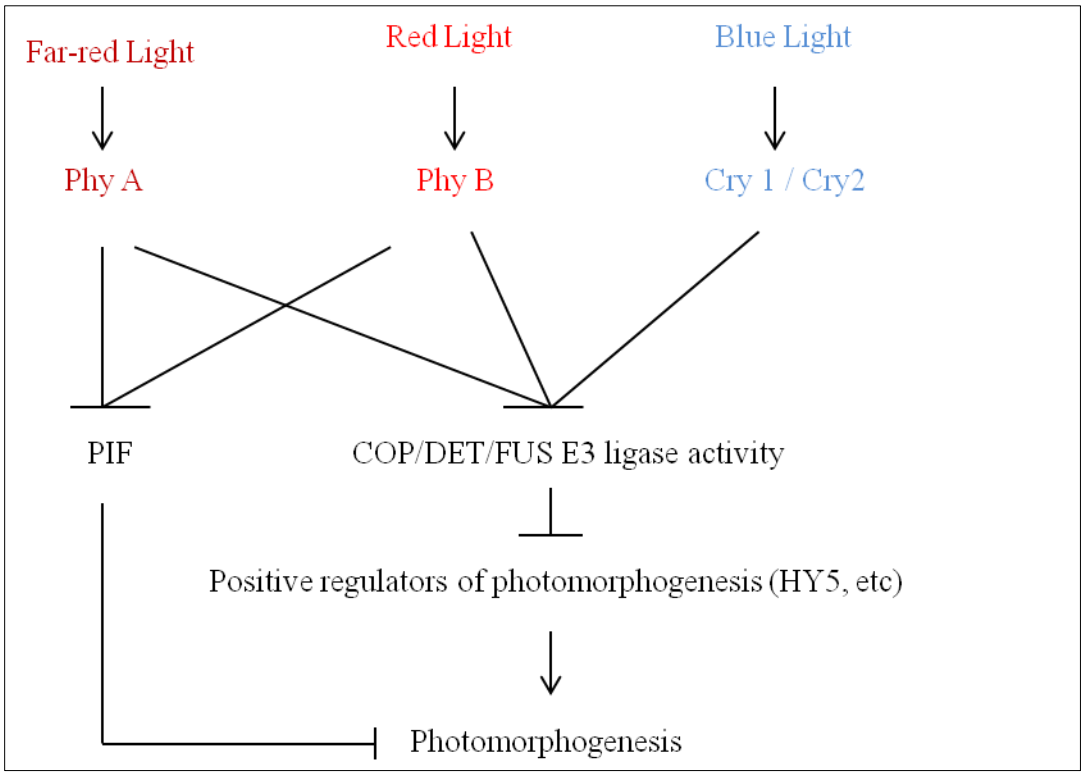

Fig. 1.4 Overview of the photomorphogenesis pathway (Modified from Lau and Deng 2010).

1.2 UV DAMAGED DNA REPAIR & TOLERANCE IN PLANTS

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1.2.1 Introduction

In both natural and agricultural conditions, plants are frequently subjected to unfavourable environments, resulting in varying degrees of stress. UV irradiation, drought, heat shock, chilling/freezing, salinity and oxygen deficiency are a few of the major abiotic factors restricting plant growth and development. An important consequence of stratospheric ozone depletion is increased transmission of solar Ultra Violet (UV) radiation through the earth's atmosphere. This increased incidence of UV irradiation causes detrimental effects to all life forms on earth.

1.2.2 UV irradiation

The spectrum of solar electromagnetic radiation striking the earth's atmosphere ranges from 100 nm to 1 mm. This includes the UV spectrum (100-400 nm), visible spectrum (380-780 nm) and infrared spectrum (700 nm-1 mm). The UV spectrum is further subdivided into three catogories: UV-C (100-280 nm), UV-B (280-315 nm), and UV-A (315-400 nm) (Ballaré 2003**)**. The shortest of these wavelengths, UV-C, is blocked completely by the ozone layer and atmospheric oxygen. In contrast, UV-A is weakly

absorbed and directly transmitted to the earth's surface. Wavelengths in the UV-B range are absorbed efficiently though not completely by ozone, as a very small percentage may pass through holes in the ozone. UV-C is extremely harmful, followed by UV-B, while UV-A has milder effects (Batschauer 1999).

1.2.3 Plants and UV radiation

Plants, due to their non-motile nature, generally have a higher rate of UV tolerance than animals. Plant secondary metabolites aid in defence against both abiotic and biotic stress factors. Plants are capable of reflecting or absorbing harmful UV rays via thick layers of waxy cutin or suberin on the cell walls and intracellular accumulation of chemical substances such as flavanols or phenolics. The biological effects of UV radiation on plants include altered growth responses, reproductive deformities, epigenetic variations, plant susceptibly to biotic factors, premature senescence, damage to the photosynthetic apparatus, and altered conformation of membrane structures. A wide array of genes were found to be induced upon prolonged exposure to low doses of UV-B in the model plant *Arabidopsis thaliana* (Frohnmeyer and Staiger 2003; Mackerness 2000; Ries et al. 2000). Upregulated transcripts include: antioxidant/free radical scavenging enzymes, proteins involved in: DNA repair, translation, E3 ligase system, cell cycle, signal transduction and secondary metabolites, as well as several other genes with unknown function (Brosché et al. 2002; Jansen et al. 2008). UV-B also results in numerous changes in plant morphology. This signalling cascade is well reviewed

elsewhere (Jenkins 2009). Here we focus on plant responses to UV-induced DNA damage.

1.2.4 UV induced DNA damage

UV-C/B radiation is directly absorbed by DNA, inducing lesions which inhibit vital cellular functions such as transcription and DNA replication. UV-A is comparatively less efficient in lesion induction but triggers the production of reactive oxygen species (ROS) (Kunz et al. 2006). The primary UV induced DNA lesions include cyclobutane pyrimidine dimers (adjacent pyrimidines covalently linked between C-5 and C-6 carbon atoms) and secondary lesions 6-4 pyrimidine-pyrimidone photoproducts (6-4 PP) (covalent linkage between the C-4 position of a pyrimidine to the C-6 position of an adjacent pyrimidine) (Fig. 1.5). In order to respond to this damage, plants employ specific mechanisms (Britt 1999). In light conditions, photoreactivation catalyses dimer monomerizations while during dark conditions, Nucleotide Excision Repair (NER) excises these helix-distorting lesions. Finally, residual lesions are bypassed via translesion synthesis (TLS).

1.2.5 Photoreactivation

Photoreactivation is a blue light dependant DNA repair mechanism catalysed by the photolyase (E.C 4.1.99.3) class of enzymes. Pyrimidine dimers are split by the action of two photoactive damage specific DNA repair enzymes – CPD photolyase and 6-4 PP

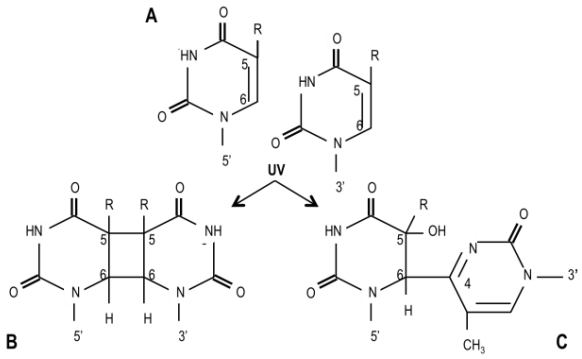

Fig. 1.5 UV induced pyrimidine dimers. A) Normal adjacent pyrimidine residues. B) UV-induced Cyclobutane Pyrimidine Dimer (CPD) and C) 6-4 Pyrimidine- Pyrimidone photoproduct (6-4 PP).

photolyase. Both classes of photolyase require two co-factors, one being the two electron reduced form of Flavin Adenine Dinucleotide (FAD) and the second chromophore, a blue light harvesting photoantenna, being either 5,10- methenyltretrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deazariboflavin (8-HDF). FAD is an essential co-factor for regulating DNA binding and repair. In contrast, the second chromophore has a higher extinction co-efficient and an absorption maximum at longer wavelengths hence regulates the rate of repair depending on the external light intensity. MTHF or 8-HDF absorbs the photoreactivating blue light photons and transfers this excitation energy to the reduced FAD. The $FADH^{-}$ in turn transfers electrons to the lesions, catalyzing the cleavage of the cyclobutane rings and dimer monomerization (Deisenhofer 2000; Sancar 2003, 2008). Multiple sequence alignment reveals that conserved homology between prokaryotic and eukaryotic CPD photolyases is limited to the C-terminal FAD binding site. It has been suggested that a common ancestor gave rise to both type I and type II photolyases but

diverged at an early evolutionary stage (Yasui et al. 1994). CPD photolyases have been classified into Class I (microbial) and Class II (higher eukaryotes excluding placental mammals) groups, respectively. The 6–4 photolyases from Drosophila and Arabidopsis have strong sequence similarity to class I CPD photolyases (Nakajima et al. 1998; Todo et al. 1996). Similarly cryptochromes, the plant blue light photoreceptors, are 30% similar to the class I microbial photolyases, but demonstrate no photolyase activity (Ahmad and Cashmore 1993). Thus, microbial Class I CPD photolyases, eukaryotic 6–4 photolyases, and blue light photoreceptors constitute the class I photolyase/photoreceptor family.

Genes encoding CPD photolyases and 6-4 PP photolyases have been identified and characterized in a range of prokaryotic and eukaryotic systems (Sancar 2003). In plants genes encoding CPD photolyases have been identified in *Arabidopsis thaliana (*Ahmed et al. 1997*)*, cucumber (Takahashi et al. 2002), rice (Hirouchi et al. 2003), spinach (Yoshihara et al. 2005), and soybean (Yamamoto et al. 2008). Genes encoding 6-4 PP photolyases have been identified in Arabidopsis and rice (Chen et al. 1994; Singh et al. 2010). In Arabidopsis, the highest levels of both photolyases are associated with floral tissues, which may presumably serve to minimize lesions in germline cells. While expression of the CPD photolyase is light/UV-B regulated, 6-4 PP photolyase is constitutively expressed (Takahashi et al. 2002; Waterworth et al. 2002). The Arabidopsis CPD photolyase gene (*AtPHR1*) encodes a class II CPD photolyase. An Arabidopsis mutant (*uvr2*) lacking this gene is hypersensitive to UV. AtPHR1 is efficient in CPD photoreactivation but deficient in 6-4 photoproduct repair (Ahmed et al. 1997). *AtPHR1* is upregulated several fold in a UV insensitive mutant of Arabidopsis (*uvi1*) irrespective of light conditions, conferring constitutive protection (Tanaka et al. 2002).

Overexpression of CPD photolyase in Arabidopsis and rice results in enhanced CPD removal (Hidema et al. 2007; Kaiser et al. 2009; Ueda et al. 2005). Genetic complementation of photolyase deficient *E.coli* strains with soybean, rice, spinach and Arabidopsis CPD photolyase genes restored photoreactivation activity (Yamamoto et al. 2007, 2008; Yoshihara et al. 2005). CPD photolyase activity in Arabidopsis (Pang and Hays 1991; Waterworth et al. 2002), soybean (Yamamoto et al. 2008) and rice (Hidema et al. 2000) has been reported to be temperature sensitive. Rice CPD photolyase, encoded by a single copy gene in the nuclear genome, translocates to chloroplasts, mitochondria and nuclei to repair UV-induced CPDs in all three genomes (Takahashi et al. 2011), a phenomenon not observed in spinach chloroplasts (Hada et al. 2000) or young Arabidopsis seedlings (Chen et al. 1996). However, upon exposure to photoreactivating blue light, Arabidopsis seedlings did exhibit efficient repair of CPDs in the extracellular organelles (Draper and Hays 2000). The Arabidopsis 6-4 PP photolyase, encoded by the *UVR3* gene, encodes a 62 kDa protein with 45% identity to Drosophila 6-4 PP photolyase and 17% identity to the Class II CPD photolyases. AtUVR3 is proficient in 6-4 photoproduct removal but deficient in CPD repair. Both *uvr2* and *uvr3* are nonsense mutations, and the double mutants are extremely sensitive to UV relative to the single mutants (Nakajima et al. 1998). Photolyases appear to be the sole repair mechanism active in non-proliferating plant tissues (Kimura et al. 2004). Hence, photolyases play an important role in plant repair of UV damaged DNA.

1.2.6 Nucleotide excision repair

Nucleotide excision repair (NER) is a light independent repair process involving a series of reactions: initial damaged DNA recognition, DNA unwinding, dual incision bracketing the lesion, repair synthesis and final ligation to seal the repaired site. NER initiates with specific sub-pathways for transcriptionally active (Transcription Coupled Repair (TC-NER)) or silent (Global Genomic Repair (GG-NER)) DNA. TC-NER and GG-NER exhibit different damage recognition strategies followed by a common repair pathway (Gillet and Scharer 2006) (Fig. 1.6). Defects in human NER genes result in the photosensitive syndromes Xeroderma pigmentosum (XP) or Cockayne syndrome (CS). Eight genetic complementation groups for XP have been identified (XPA-G, V) as well as two for CS (CSA and CSB). While the XP-V mutation uniquely results in defects in translesion synthesis, XP -A, -B, -D, -F, and -G mutation results in both TC-NER and GG-NER defects, while XP –C and -E mutation results in GG-NER defects only. CSA and CSB mutation results exclusively in TC-NER defects (Hoeijmakers 2001; Svejstrup 2002). Bioinformatic analysis of the plant NER protein machinery suggests the molecular mechanisms are largely but not entirely conserved with that of the extensively studied yeast *S. cerevisiae* and mammalian cells (Kimura and Sakaguchi 2006; Kunz et al. 2005, 2006). NER in plants has been studied primarily in rice and Arabidopsis (Singh et al. 2010). Many Arabidopsis NER related genes were initially isolated by analysis of UV hypersensitive (*uvh*) and UV repair defective (*uvr*) mutants which were subsequently mapped to homologues of the human XP genes (Table 1.2).

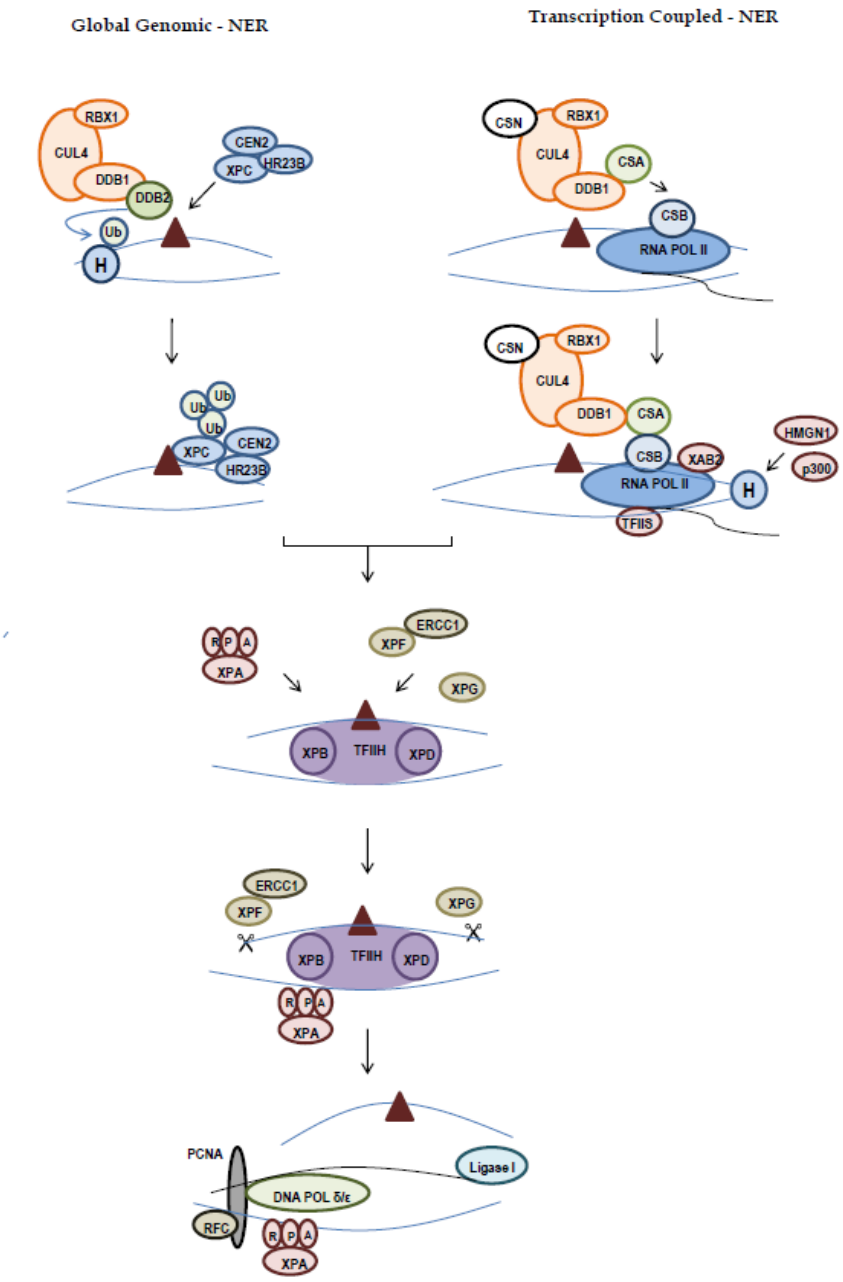

Fig. 1.6 Overview of mammalian nucleotide excision repair. In GG-NER, DDB2-CUL4 mediated histone (H) and XPC ubiquitination facilitates lesion binding. In TC-NER, stalled RNA POL II recruits CSB and the CSA-CUL4-CSN complex, followed by recruitment of other TCR specific factors. In both cases, NER core players follow suit: XPB and XPD helicases of the TFIIH complex, XPF-ERCC1 and XPG endonucleases, and the ssDNA binding XPA-RPA complex. The fragment encompassing the lesion is excised, followed by repair synthesis and ligation. Repair synthesis requires DNA POL δ/ε in concert with accessory proteins PCNA, RFC and RPA. See text for details.

Human	Yeast	Function	ATG no.	Arabidopsis
Photoreactivation:				
ND	ND	6-4 PP Photolyase	At3g15620	*UVR3*
ND	*PHR1*	Class II CPD Photolyase	At1g12370	*PHR1/UVR2*
Nucleotide Excision Repair:				
DDB1	ND	Substrate adaptor for CUL4. Interacts with DCAF proteins	At4g05420 At4g21100	*AtDDB1a* *AtDDB1b*
DDB2/XPE	ND	Damaged DNA binding (DCAF)	At5g58760	*AtDDB2*
CUL4	*CUL4*	Scaffolding subunit of E3 Ub ligase	At5g46210	*AtCUL4*
XPC	*RAD4*	GG-NER damage recognition	At5g16630	*AtXPC*
HR23B	*RAD23*	Binds to XPC	At1g79650 At1g16190 At3g02540 At5g38470	*AtRAD23A* *AtRAD23B* *AtRAD23C* *AtRAD23D*
CEN2	*CEN2*	Stabilizes XPC-HR23B complex	At3g50360	*AtCEN2*
XPB	*RAD25*	Subunit of TFIIH. 3′->5′ helicase	At5g41370 At5g41360	*AtXPB1* *AtXPB2*
XPD	*RAD3*	Subunit of TFIIH. 5′->3′ helicase	At1g03190	*AtXPD/UVH6*
GTF2H1 *GTF2H2* *GTF2H3* *GTF2H4* *GTF2H5*	*TFB1* *SSL1* *TFB4* *TFB2* *TFB5*	Core TFIIH subunits	At1g55750 At3g61420 At1g05055 At1g18340 At4g17020 At1g12400 At1g62886	*AtTFB1-1* *AtTFB1-2* *Atp44* *AtTFB4* *AtTFB2* *AtTFB5-1* *AtTFB5-2*
XPA	*RAD14*	ssDNA binding	ND	ND
XPG	*RAD2*	3′ endonuclease	At3g28030	*AtXPG/UVH3*
ERCC1	*RAD10*	5′ endonuclease with XPF	At3g05210	*AtERCC1/UVR7*
XPF	*RAD1*	5′ endonuclease with ERCC1	At5g41150	*AtXPF/UVH1*
PCNA	*PCNA*	RFC dependant sliding clamp	At1g07370 At2g29570	*AtPCNA1* *AtPCNA2*
RFC1 *RFC2* *RFC3* *RFC4* *RFC5*	*RFC1* *RFC2* *RFC3* *RFC4* *RFC5*	DNA-dependent ATPase required for DNA replication and repair	At5g22010 At1g21690 At1g77470 At1g63160 At5g27740	*AtRFC1* *AtRFC2* *AtRFC3* *AtRFC4* *AtRFC5*
RPA70 *RPA32* *RPA14*	*RFA1* *RFA2* *RFA3*	ssDNA binding protein required for architectural role in dual lesion incision and repair synthesis	At2g06510 At4g19130 At5g08020 At5g45400 At5g61000 At2g24490 At3g02920 At3g52630 At4g18590	*AtRPA70A* *AtRPA70B* *AtRPA70C* *AtRPA70D* *AtRPA70E* *AtRPA32A* *AtRPA32B* *AtRPA14A* *AtRPA14B*
CSA	*RAD28*	TC-NER specific DCAF protein	At1g27840 At1g19750	*AtCSA1* *AtCSA2*
CSB	*RAD26*	SWI/SNF2 like ATPase	At2g18760	*AtCSB*
XAB2	*SYF1*	Complex stabilization	At5g28740	*AtXAB2*
TFIIS	*TFIIS*	TCR elongation factor	At2g38560	*AtTFIIS*
HMGN1	ND	Nucleosome binding	ND	ND

Table 1.2 Genes involved in UV damaged DNA repair. ND=not detected.

1.2.6.1 Global genomic repair

1.2.6.1.1 Damage recognition

1.2.6.1.1.1 DDB1 & DDB2

In mammalian systems, damage detection in the GG-NER pathway involves UV-Damaged DNA Binding protein 1 and 2 (DDB1 and DDB2) followed by the XPC-HR23B-CEN2 complex. In humans DDB2 complements the *XPE* mutation and plays a role in recognition of the UV-induced lesion, while DDB1 is required for high affinity interaction of the DDB1-DDB2 complex (Groisman et al. 2003; Luijsterburg et al. 2007; Rapic-Otrin et al. 2002). *S. pombe* Ddb1 knockouts result in chromosomal segregation defects, UV sensitivity and slow S phase progression leading to defects in meiosis (Holmberg et al. 2005). DDB1 and DDB2 homologues have been identified in rice, where they are UV-induced in proliferating tissues (Ishibashi et al. 2003). *Arabidopsis thaliana* encodes two homologs of DDB1 – DDB1A and DDB1B. These proteins are 91% identical with redundant function. Although *ddb1b* null alleles appear lethal, a viable partial loss of function allele exhibits no developmental or UV sensitive phenotypes (Bernhardt et al. 2010; Schroeder et al. 2002). Overexpression of DDB1A in Arabidopsis confers enhanced UV resistance and *ddb1a* knockouts exhibit mild UV sensitivity (Al Khateeb and Schroeder 2009; Molinier et al. 2008). *AtDDB2* encodes a 48 kDa nuclear localized protein with upregulated expression upon UV-induction. *AtDDB2* loss of function results in UV sensitivity while overexpression increases UV tolerance (Biedermann and Hellmann 2010; Koga et al. 2006; Molinier et al. 2008).

1.2.6.1.1.2 Cullin based E3 ligases

The 127 kDa DDB1 homologs function as substrate adaptors for CULLIN4 based E3 ubiquitin (Ub) ligases (Groisman et al. 2003). E3 Ub ligases are multimeric complexes that add ubiquitin moieties to target proteins and contain CULLIN proteins as scaffolding subunits (Hua and Vierstra 2011). CUL4 based E3 ubiquitin ligases consist of three core subunits: CULLIN4 (CUL4), RING finger protein REGULATOR OF CULLINS1 (ROC1)/RING-BOX1 (RBX1), and DDB1. The CUL4 – RBX1 – DDB1 complex interacts with a large number of proteins containing WD40 motifs referred to as DCAF proteins (DDB1-CUL4 Associated Factor) or DWD proteins (DDB1 binding WD40 proteins) (Lee and Zhou 2007). DDB2 is an example of a WD40 domain containing DCAF protein. WD40 motifs are characterized by 40 amino acid repeats initiated by a glycine-histidine dipeptide and terminated by a tryptophan-aspartate (WD) dipeptide facilitating protein-protein interactions. DDB1 is composed of three β propeller domains (BPA, BPB and BPC) and DDB2, in addition to the WD40 domain, contains a helix loop helix (HLX) segment in the N terminal. While the clam shaped BPA-BPC of DDB1 mediates interaction with the HLX motif of DDB2 and other DCAF substrates, BPB exhibits exclusive interactions with CUL4 (Scrima et al. 2008). AtCUL4 is a 91 kDa protein with a conserved CH motif and an extended N terminal region of 65 amino acids that shares close sequence similarity to its human/mouse orthologs. *AtCUL4* loss of function results in abnormal plant development (Bernhardt et al. 2006; Chen et al. 2006) and UV sensitivity (Molinier et al. 2008). Examples of DCAF proteins interacting with the Arabidopsis CUL4–DDB1A/B complex include AtDDB2 (Bernhardt et al. 2006), AtCSA-1&2 (Biedermann and Hellmann 2010; Zhang et al. 2010), as well as the

negative regulator of photomorphogenesis DET1 (De-etiolated1) (Schroeder et al. 2002), and many other DWD proteins (Lee et al. 2008). Recent results have shed light on the cross talk between photomorphogenesis regulation and repair of UV damaged DNA. HY5, a positive regulator of photomorphogenesis, has been shown to regulate gene sets connected to UV tolerance, such as the *UVR3* and *PHR1* photolyases, as well as secondary metabolite transcriptional regulators (Oravecz et al. 2006; Ulm et al. 2004).

DET1, initially identified as a nuclear localized negative regulator of photomorphogenesis, exhibits a constitutively light grown phenotype in addition to increased levels of flavanoids (Pepper et al. 1994). Recent papers show that *det1* mutants exhibit enhanced UV tolerance through increased levels of secondary metabolites reflecting/absorbing UV irradiation as well as by upregulation of photolyase genes. Further it appears that DET1 protein dosage influences UV sensitivity of plants as DET1 overexpressing lines exhibit increased UV sensitivity (Castells et al. 2010, 2011).

1.2.6.1.1.3 Histone ubiquitination facilitates NER machinery entry

In mammals, in the absence of UV irradiation, DDB2-DDB1-CUL4-RBX1 (DDB2 complex) was found to be associated with the COP9 Signalosome complex (CSN). CSN shares significant structural homology with the 19S lid of 26S proteosome. The CSN deconjugates neddylation (Nedd8) from CULLINs, thereby regulating the activation, stability or the disassembly of CULLIN based E3 ligase activity (Parry and Estelle 2004; Schwechheimer and Deng 2001). The DDB2 - CSN complex show no ubiquitin ligase activity, but upon UV irradiation, these complexes disassociate in parallel

with increased neddylation and activation of CUL4 (Groisman et al. 2003). Core histone proteins have been identified as potential targets for DDB2-DDB1-CUL4-RBX1 mediated proteosomal degradation. Kapetanaki et al. (2006) and Wang et al. (2006) describe the ubiquitination of H2A, H3 and H4 histone proteins. Reduction of histone H3 and H4 ubiquitination by knockdown of *cul4* impairs recruitment of the repair protein XPC to the damaged foci and inhibits the repair process. Thus biochemical studies indicate that DDB-CUL4-RBX1-mediated histone ubiquitination weakens the interaction between histones and DNA to further facilitate the recruitment of repair proteins to damaged DNA (Guerrero-Santoro et al. 2008; Higa et al. 2006). The activated DDB2 complex recruits XPC via protein-protein interactions, followed by ubiquitination of XPC and DDB2. Polyubiquitinated DDB2 exhibits reduces affinity for damaged DNA and is subsequently displaced from the damaged foci, whereas polyubiquitinated XPC enhances its binding to DNA (Sugasawa et al. 2005). In Arabidopsis, DDB2 turnover is abrogated in *cul4, ddb1a, atr* and *det1* mutants suggesting that ATR and DET1 may co-operate with the CUL4-DDB1 E3 ligase complex in regulating NER (Castells et al. 2011; Molinier et al. 2008).

1.2.6.1.1.4 XPC-HR23B-CEN

The next step in GGR involves the homologous heterodimers hXPC-hHR23B (in Humans) and RAD4-RAD23 (in yeast). In addition to hXPC-hHR23B, Araki et al. (2001) identified hCEN2, a Ca^{2+} binding protein contributing to the stability of the hXPC-hHR23B complex. Hence in mammalian systems the identified protein recognition complex is hXPC-hHR23B-hCEN2, however neither hHR23B nor hCEN2 bind to

damaged DNA but enhance both the affinity and specificity of hXPC binding to damaged DNA (Fitch et al. 2003; Nishi et al. 2005). AtCEN2 shares 49% identity with hCEN2, *Atcen2* mutants are UV sensitive, and AtCEN2 overexpression resulted in enhanced repair. Upon UV irradiation, AtCEN2 level increases and it rapidly translocates to the nucleus. AtCEN2-AtXPC interaction in *Arabidopsis thaliana* has also been demonstrated (Liang et al. 2006; Molinier et al. 2004). Potential homologs of HR23B/RAD23 have been identified in *Arabidopsis thaliana*, *Oryza sativa* and *Daucus carota* (Schultz and Quatrano 1997; Sturm and Leinhard 1997). The Arabidopsis genome has 4 loci encoding RAD23 homologs (RAD23a, RAD23b, RAD23c, RAD23d), and although mutants exhibit multiple pleiotrophic developmental defects (Farmer et al. 2010), UV sensitivity or complex interactions with the Arabidopsis NER machinery have not yet been reported.

1.2.6.1.2 DNA unwinding complex assembly

Following recognition, the damaged region is unwound by the TFIIH transcription factor which joins the XPC-CEN2-HR23B complex. TFIIH is a complex of 10 proteins, seven of which are players in the NER pathway (helicases XPB and XPD, p62, p44, p34, p52, and p8). The last five proteins are encoded by GTF2H1, GTF2H2, GTF2H3, GTF2H4, GTF2H5 (Frit et al. 1999). TFIIH not only participates in NER of transcriptionally active and inactive DNA, but also in RNA POL II dependent transcription, cell cycle control and regulation of nuclear receptor activity (Chen and Suter 2003). ATP dependent 5'–>3' and 3'–>5' helicase activities associated with XPD/RAD3 and XPB/RAD25 respectively unwind the DNA encompassing the lesion

with the concomitant release of the recognition complex. Human XPB and the corresponding yeast RAD25 knockouts are lethal. *Arabidopsis thaliana,* unlike the sugarcane, rice or mammalian genomes, encodes two homologs of XPB – AtXPB1 and AtXPB2. These proteins are 95% identical with redundant functions and are expressed constitutively throughout plant development (Morgante et al. 2005; Ribeiro et al. 1998). Despite this redundancy, *xpb1* mutants exhibit delayed germination and flowering phenotypes but are not UV sensitive (Costa et al. 2001). Phenotypes of Arabidopsis *xpb2* or double mutants have not yet been reported. Arabidopsis XPD is 56% and 50% identical to human and yeast sequences. Arabidopsis *XPD/RAD3* null mutations are lethal, however viable point mutation alleles are UV sensitive and were identified as *uvh6* (*uv hypersensitive 6*) mutants (Jenkins et al. 1997; Liu et al. 2003). Another component of the of TFIIH complex, p44, was identified in Arabidopsis as ATGTF2H2 and was found to interact in vivo with AtXPD (Vonarx et al. 2006).

1.2.6.1.3 Endonuclease recruitment following unwinding

TFIIH further recruits additional factors such as XPA and heterotrimeric Replication Protein A (RPA), composed of 70, 32 and 14 kDa subunits, to promote and stabilize the formation of an open intermediate essential for the dual incision by XPG and XPF-ERCC1 (Excision Repair Complementing defective repair in Chinese hamster 1) (RAD1/RAD10) endonucleases at the 3’ and 5’ sites respectively (Tapais et al. 2004). The RPA-XPA complex exhibits interactions with both endonucleases (He et al. 1995; Wold 1997), however the specific function of XPA is still not evident. Initially it was thought to function as a lesion recognition complex in concert with XPC, but was later

determined to be recruited after TFIIH entry and facilitate XPC complex departure (Hey et al. 2002; Volker et al. 2001). In addition, XPA homologues do not exist in plants (Kunz et al. 2005). The dual incisions catalyzed by the endonucleases excise oligonucleotides of about 20-30 bases containing the lesion (Reidl et al. 2003).

Potential homologs of ERCC1, XPF, XPG and RPA have been identified in plants. Although ERCC1 was first cloned from male germ line cells of *Lilium longiforum*, southern blots confirmed the presence of ERCC1 across diverse plant species such as *A. thaliana*, *B. napus*, *Z. mays*, *L. esculentum*, *N. tobacum*, and *O. sativa* (Xu et al. 1998). Hefner et al. (2003) mapped the Arabidopsis *uvr7* mutant to *AtERCC1*. *Atercc1* knockouts are phenotypically normal in contrast to the lethal mammalian counterparts (Weeda et al. 1997). *Atercc1* mutants are extremely sensitive to DNA damaging chemicals such as mitomycin and ionizing agents such as UV and γ – radiation (Hefner et al. 2003). More recent studies in Arabidopsis indicate significant roles of *AtERCC1* in concert with *AtXPF* in homologous recombination and chromosomal stability (Dubest et al. 2002, 2004; Vannier et al. 2009). Gallego et al. (2000) and Liu et al. (2000) characterized the single copy AtXPF which is 37% and 29% identical to human XPF and *S. cerevisiae* RAD1 respectively. *AtXPF* is homogenously expressed, was mapped to the *uvh1* mutant in Arabidopsis, and partially complements the yeast *rad1* mutant (Gallego et al. 2000). *AtXPF* point mutations result in sensitivity to ionizing radiation and mitomycin C, and impaired removal of photoproducts (Fidanstef et al. 2000; Vonarx et al. 2002). *AtXPG* was mapped to the *UVH3* locus and knockouts result in UV sensitivity as well as premature senescence and reduced seed production (Liu et al. 2001). The *XPG* rice

homolog, *OsSEND-1*, exhibits upregulated mRNA levels in response to UV and DNA damaging agents (Furukawa et al. 2003a).

ssDNA binding RPA proteins in plants were first identified in rice (Ishibashi et al. 2001). Unlike most eukaryotic organisms, Arabidopsis and rice possess multiple copies of the RPA homologs. In addition to participating in DNA repair, RPA proteins play a role in homologous recombination and DNA replication in humans and yeast (Sakaguchi et al. 2009). The rice genome encodes three OsRPA70 (OsRPA70A, OsRPA70B, OsRPA70C), three OsRPA32 (OsRPA32-1, OsRPA32-2, OsRPA32-3) and one OsRPA14. *In vivo* interactions in rice confirms three different complex formations: OsRPA70A-OsRPA32-2-OsRPA14 (Type1); OsRPA70B-OsRPA32-1-OsRPA14 (Type2); and OsRPA70C-OsRPa32-3-OsRPA14 (Type3). Subcellular localization of all three OsRPA32 was detected in both the nucleus and chloroplasts. OsRPA70A was only in the chloroplast whereas OsRPA70B and OsRPA70C were exclusively to the nucleus. This data suggest that while the Type1 complex may participate in chloroplast DNA repair, Type2 and Type3 complexes concentrate on nuclear DNA repair (Ishibashi et al. 2006). OsRPA70A and OsRPa70B share only 33% sequence homology and exhibit differences in expression pattern (Ishibashi et al. 2001). A T-DNA insertion in *OsRPA70A* resulted in partial male sterility, complete female sterility and hypersensitivity to DNA mutagens (Chang et al. 2009). OsRPA32 protein abundance is regulated by both UV irradiation and cell cycle phase (Marwedal et al. 2003). Arabidopsis, on the other hand, encodes five putative RPA70 genes and two copies each of RPA32 and RPA14. Arabidopsis RPA70A interacts preferentially with AtRPA32A rather than AtRPA32B. Knockouts of both AtRPA70A and AtRPA70B exhibited UV

sensitivity when irradiated, but exhibited wildtype characteristics under normal conditions (Ishibashi et al. 2005; Takashi et al. 2009).

1.2.6.1.4 Repair synthesis and ligation

In mammals, the gap formed by the excision is filled via PCNA (Proliferating Cell Nuclear Antigen) and RFC dependant DNA synthesis by DNA POL δ/ε. These components are likely recruited by XPG and RPA as RFC exhibits interaction with RPA (Yuzhakov et al. 1999). RFC catalyzes the ATP dependant loading of PCNA on DNA at the 3' OH. PCNA is a homotrimeric protein which forms a sliding clamp-like complex (Gulbis et al. 1996) and interacts with the DNA POL to ensure replication occurs processively (Mocquet et al. 2008). The final phase of NER is completed by phosphodiester backbone rejoining of the repaired DNA strand by DNA Ligase I. Although PCNA and RFC homologues have been identified in plants, their specific role in nucleotide excision repair has not yet been elucidated (Furukawa et al. 2003b; Strzalka and Ziemienowicz 2011). Recently, Roy et al. (2011) cloned and characterized a homolog of mammalian DNA POLλ in Arabidopsis. AtPOLλ was upregulated upon UV induction under dark conditions, and *Atpolλ* mutants exhibited UV sensitivity and decreased DNA repair. Thus, this report suggests a role for DNA POLλ in plant NER.

1.2.4 Transcription coupled repair

The emerging picture of mammalian TC-NER is of a complex mechanism requiring two essential assembly factors (CSA and CSB), certain TC-NER specific

proteins (P300, HMGN1, XAB2 and TFIIS), as well as core NER proteins. UV induced DNA damage is initially recognised by RNA POL II, which stalls when it encounters helix-distorting lesions on the template strand during transcription. Stalled RNA POL II backtracks a few nucleotides and is recognised by the CSB protein which in turn co-ordinates the recruitment of the repair factors required to accomplish Transcription Coupled NER (Mellon 2005). Cloning and characterization of the mammalian CSB gene revealed a nuclear protein of 168 kDa with a region of homology to the SWI2/SNF2 family of helicases. CSB has been shown to interact with RNA POL II and this interaction is enhanced and prolonged by UV exposure (van den Boom et al. 2004). Studies propose that functional CSB in the absence of UV could also serve as a component of the transcriptional machinery promoting elongation (Fousteri and Mullenders 2008; Hanawalt and Spivak 2008). Further, CSB facilitates the entry of the core NER factors XPA, XPG and TFIIH through complex interactions (Laine and Egly 2006; Saxowsky and Doetsch 2006; Svejstrup 2002). Mammalian CSA on the other hand is a 46 kDa DWD protein containing seven WD40 repeats that associates with DDB1-CUL4 type E3 ligases and is recruited to the damaged site after CSB. CSA physically interacts with the CSB-RNA POL II complex in a UV dependent manner (Groisman et al. 2003; Fousteri et al. 2006). Interestingly, unlike the DDB2 complex, the CSA-CUL4 Ub ligase complex is active under normal conditions but is rapidly inactivated upon UV irradiation by CSN. Hence CSN plays a differential and dynamic role in regulating both pathways of Nucleotide Excision Repair. The stable CSN-CSA-CSB complex is required for the recruitment of the other NER factors. Following repair, CSN dissociates, reactivating CSA Ub ligase activity and resulting in CSB degradation. Clearance of CSB

is required for the reinitiation of transcription by RNA POL II (Groisman et al. 2003, 2006).

Several papers over the years propose the fate of RNA POL II during the coupling process: either ubiquitinated and degraded, translocated or bypassed from the lesion site, or simply stalled during the entire repair process, is still a matter of debate (Reviewed in Tornaletti 2009). XAB2 (XPA binding protein 2) is a RNA-binding protein with 15 tetratricopeptide repeats. In addition to interacting with XPA, XAB2 is capable of interacting with CSA, CSB and RNA POL II (Nakatsu et al. 2000). XAB2 is thought to stabilize protein assemblies by functioning as a scaffolding subunit. XAB2 knockouts in mammalian cells exhibit hypersensitivity and decreased recovery of mRNA synthesis post UV irradiation (Kuraoka et al. 2008). Increased amounts of histone acetyl transferase p300 and High Mobility Group Nucleosome binding domain containing protein 1 (HMGN1) interact with RNA POL II in a CSB-dependant manner upon UV irradiation but exhibit weak interactions under normal conditions (Fousteri et al. 2006). Both HMGN1 and p300 are nucleosome interacting proteins which remodel the chromatin structure behind the arrested polymerase and facilitating the backward translocation of RNA POL II (Hanawalt and Spivak 2008). TFIIS, functioning as a transcription elongation factor, stimulates the arrested RNA POL II to restart elongation. This TFIIS-RNA POL II interaction is significantly increased upon UV irradiation (Fousteri et al. 2006). Hence it is proposed that TFIIS facilitates resumption of transcription post DNA lesion removal in a CSA/B-dependent manner.

Elucidation of the TC-NER mechanism in plants is still at its infancy. While there is no plant homologue for HMGN1, the Arabidopsis genome encodes homologues of

XAB2, TFIIS, and five p300/CBP homologues, however the role of these genes in DNA repair has not been assessed (Grasser et al. 2009; Kunz et al. 2005; Pandey et al. 2002). Only recently was the homolog of human CSA cloned and characterized in Arabidopsis. In contrast to mammalian systems, the Arabidopsis genome encodes two homologs of CSA – AtCSA1A and AtCSA1B, 92% identical DWD proteins with overlapping subcellular localization and expression patterns. These proteins exist as heterotetramers in planta and are capable of interacting with the DDB1-CUL4 E3 complex. Knockouts of either gene result in UV sensitivity and decreased photoproduct removal (Zhang et al. 2010). Concurrently, another group overexpressed AtCSA1A, which surprisingly also resulted in increased UV sensitivity. This result is hypothesised to be due to competition between CSA and with other DWD proteins to interact with the DDB1-CUL4 complex. Interestingly AtCSA1A levels remained constant upon UV induction (Biedermann and Hellmann 2010). RNAi of a putative Arabidopsis CSB homolog resulted in a UV sensitive phenotype (Shaked et al. 2006). Hence, taken as a whole, these results confirm the role of the CUL4-DDB1-CSA and CSB pathway in plants.

1.2.5 Conclusions

Thus, despite the barrage of damage resulting from solar UV exposure plants face every day, they have a variety of mechanisms which allow them to survive. UV induced DNA damage is repaired by direct photoreactivation via photolyases, or by dark repair (NER) in both transcribed (TC-NER) and non-transcribed regions (GG-NER). The continued study of these pathways and the interplay between them in plants is sure to bring additional insight.

1.3 UBIQUITIN LIGASES IN *ARABIDOPSIS*

E3 ubiquitin ligases are key regulatory components of the ubiquitin proteasome pathway. They specifically target substrate proteins for degradation via the 26S proteasome, thereby regulating several aspects of plant development. The E3 based Ub ligase operates in conjugation with the E1 (Ub activating) and E2 (Ub conjugating) complexes. The E1 subunit serves as an ATP source for transferring ubiquitin moeities to the E2 complex which in turn interacts with conserved E3 ligases. The E3 ligase catalyzes protein turnover by ubiquitinating the Lys residue of the target protein for 26S mediated proteosomal degradation (Stone and Callis 2007). The 2 MDa 26S proteosome is composed of the 20S core cylindrical structure containing proteolytic sites and the 19S regulatory particle that caps both ends of the core cylindrical structure. E3 ligases are multimeric complexes that contain CULLIN based proteins as scaffolding subunits. The *Arabidopsis thaliana* genome encodes six cullin proteins – CUL1, CUL2, CUL3a, CUL3b, CUL4 and CUL5. *At* CUL4 is a 91 kDa protein with a conserved CH motif and an extended N terminal region of 65 amino acids. It shares a close sequence similarity to its human/mouse orthologs (Bernhardt et al. 2006) and is of particular interest in this study as it regulates both plant photomorphogenesis and UV induced DNA repair.

CUL4 antisense lines are dwarf in stature, with abnormal leaf and root development and defects in stomatal patterning, but do not alter *COP1*, *DET1* or *DDB1A* mRNA levels (Bernhardt et al. 2006). Similarly CUL4 co-suppressed lines (*cul4cs*) exhibited abnormal development such as dwarf stature, aberrant leaf patterning, abnormal flower and silique development, secondary inflorescence (Chen et al. 2006) and enhanced sensitivity to ABA and NaCl (Lee et al. 2010). Further *cul4cs* lines in the *cop1* and *det1*

backgrounds severely enhance photomorphogenic traits thus confirming strong genetic interactions in regulating photomorphogenesis (Chen et al. 2006a; 2010).

CUL4-based E3 ubiquitin ligases consist of four core subunits (Fig. 1.7):

1) CULLIN4 (CUL4)
2) RING finger protein REGULATOR OF CULLINS1 / RING-BOX1 (RBX1)
3) UV DAMAGED DNA BINDING PROTEIN 1 (DDB1)
4) DCAF (DDB1- CUL4 Associated Factor) – Eg: DDB2, COP1

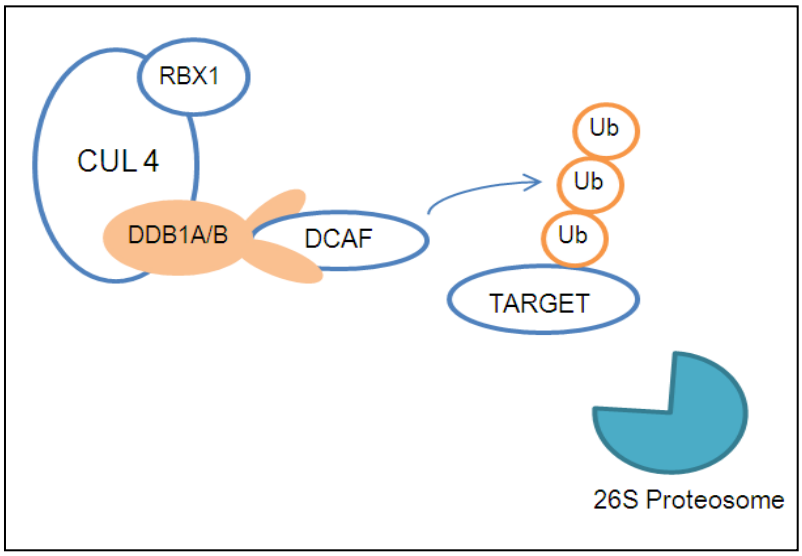

Fig. 1.7 CUL4-DDB1 complex formation. 26S proteosomal degradation following CUL4 mediated polyubiquitination of target proteins.

1.3.1 DDB1-DCAF interaction

DDB1 serves as a substrate adaptor for CUL4 based E3 Ubiquitin ligase. The homologs are composed of approximately 21 WD40 like repeats which facilitates

protein-protein interactions. WD40 motifs are characterized by 40 amino acids repeats initiated by glycine-histidine dipeptide and terminated by trptophan-aspartate (WD) dipeptide. The WD40 region of DDB1 folds into 3 unique β propeller structures – BPA, BPB and BPC. While BPB exclusively interacts with CUL4, BPA and BPC assemble as a clam shaped structure interacting with the DCAF (DDB1-CUL4 associated factor) proteins or DWD (DDB1-WD40) proteins (Fig 1.6).

The Arabidopsis genome encodes around 230 WD40 proteins but only a fraction of them (around 86 proteins) have one or more WDxR motifs within the WD40 domain capable of interacting with DDB1 (Lee et al. 2008). Examples of a few DDB1 interacting proteins of interest include: photomorphogenesis proteins COP1, SPA1-4 proteins (Chen et al. 2010a), COP10 (Yanagawa et al. 2004), DET1 (non-DWD protein) (Schroeder et al. 2002), DCAF1 (Zhang et al. 2008), UV induced DNA repair proteins DDB2 (Bernhardt et al. 2010) and CSA (Biedermann and Hellmann 2010), with negative regulators of ABA signaling (DWA1, DWA2 and DWA3) (Lee et al. 2010; 2011) and with other WD40 proteins namely MSI1, MSI2 and MSI3, Flowering Locus Y, etc. (Biedermann and Hellmann 2011).

1.4 DDB COMPLEXITIES

Damaged DNA Binding Protein was initially identified in mammalian systems. DDB1 and DDB2 are core components of the Damaged DNA Binding Protein complex. As the name suggests, homologs of the DDB complex are vital components of the UV damaged DNA repair machinery in addition to regulating photomorphogenesis and several other signaling cascades in plants. Arabidopsis, unlike any other organism

encodes two homologs of DDB1 – DDB1A and DDB1B which are 91% similar (Fig. 1.7) (Schroeder et al. 2002). Both DDB1A and DDB1B is localised to the cytosol and the nucleus (Molinier et al. 2008; Zhang et al. 2008).

1.4.1 Damaged DNA Binding Protein 1B

DDB1B is a ~120 kDa localized in both the cytoplasm and the nucleus (Zhang et al. 2008). It is mapped onto chromosome 4 with a genomic sequence of 6.7 Kb. *DDB1B* total loss of function mutants appear lethal (Schroeder et al. 2002). However we have recently identified a viable *ddb1b* allele (SALK_061944) with a T-DNA insertion in exon 19 which results in a partially truncated protein (deficient in the terminal 112 amino acids). This allele will henceforth be known *as ddb1b-2.* Null alleles of the other DDB1 homolog, *DDB1A* have a T-DNA insertion in exon 10 and were previously described in Al Khateeb and Schroeder (2007) and Schroeder et al. (2002). Note that *DDB1A* is expressed two fold or higher in comparison to *DDB1B* in all tissues throughout plant development except the pollen (Fig. 1.8) and during various stress responses across time frames (Fig 1.9).

Primary Goals of this thesis:

- Characterization of the viable *ddb1b-2* allele.
- Characterization of Arabidopsis *ddb1a ddb1b-2* double mutants.
- Effect of *ddb1b-2* in the *ddb2* background.
- Effect of *ddb1b-2* in the *det1* and *cop1* backgrounds

Colour: Physiochemical properties of Amino acids.
* (asterick) indicates positions which have a single, fully conserved residue.
: (colon) indicates conservation between groups of strongly similar properties.
. (period) indicates conservation between groups of weakly similar properties

Fig. 1.8 Amino acid sequence alignment of Arabidopsis DDB1A and DDB1B exhibiting 91% similarity.

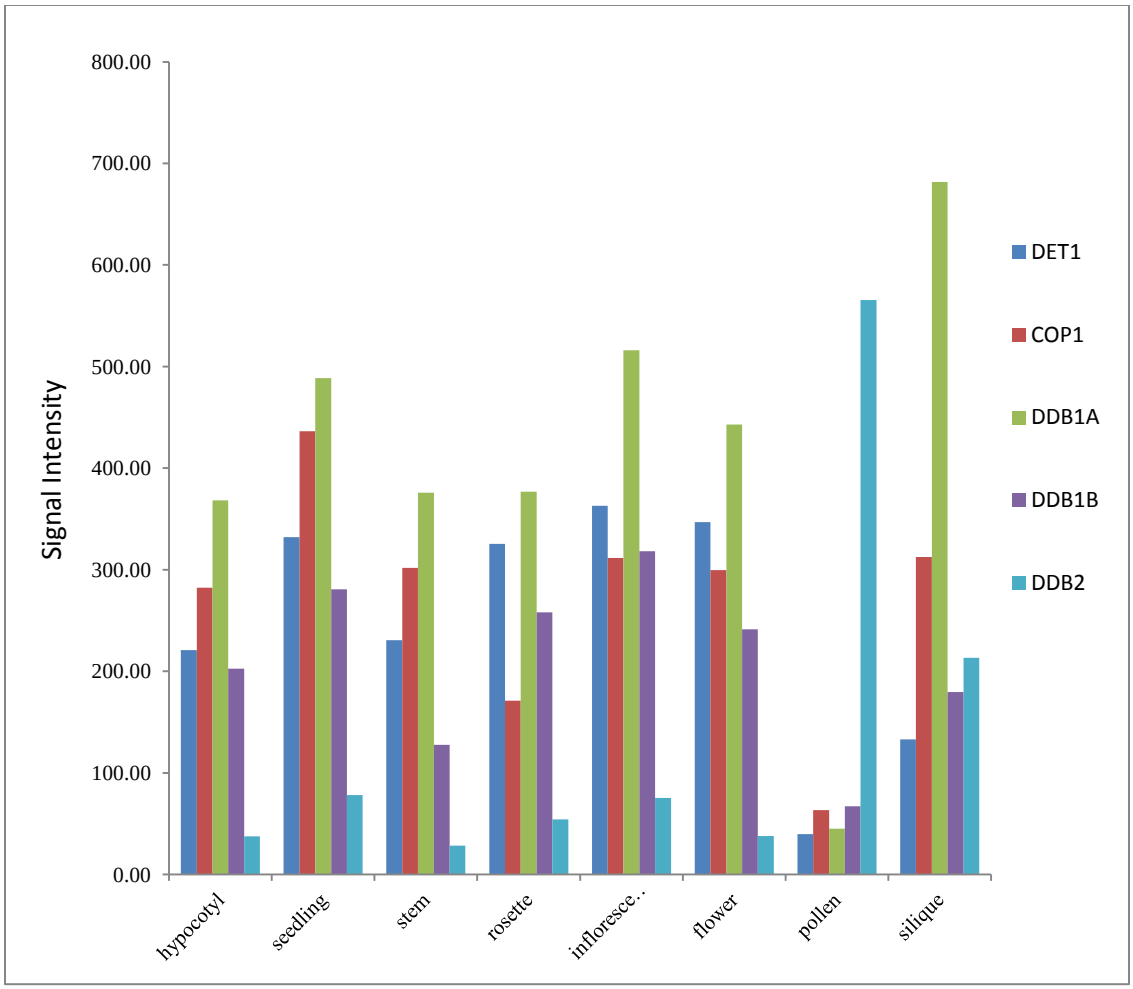

Fig. 1.9 Expression analysis (as derived from Salk Institute Genomic Analysis Laboratory - AtGenexpress) of *DET1*, *COP1*, *DDB2*, *DDB1A* and *DDB1B* in plant tissues across development.

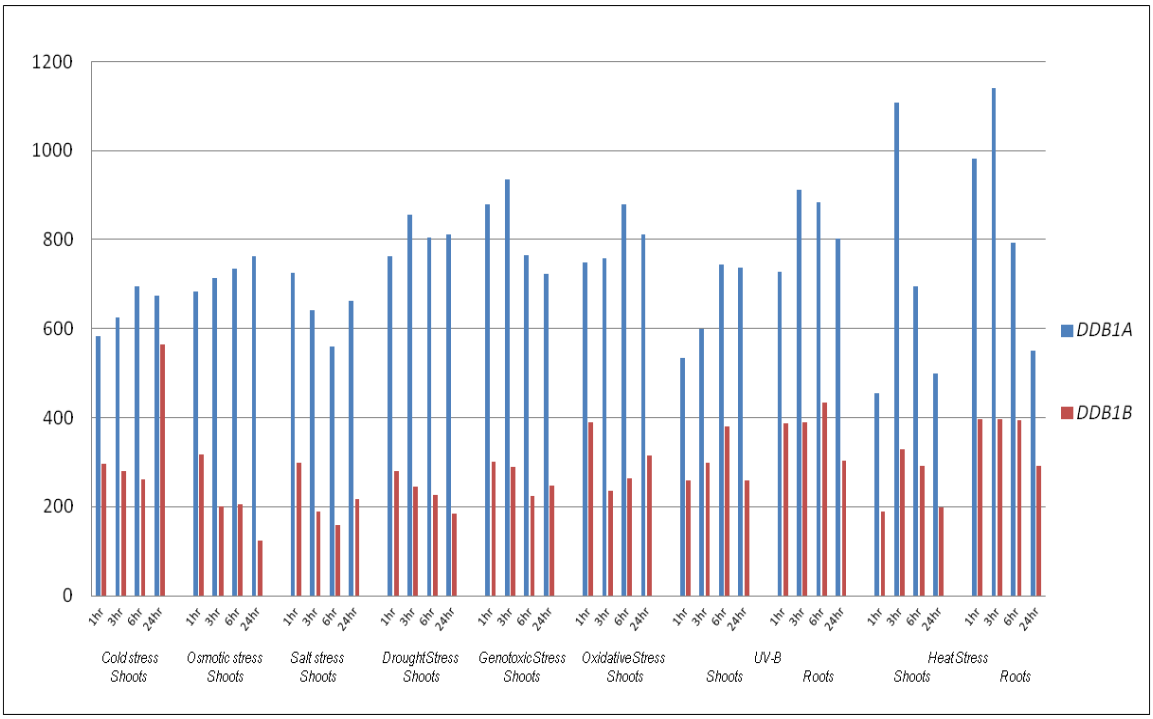

Fig. 1.10 Abiotic stress induced expression analysis (as derived from Salk Institute Genomic Analysis Laboratory - AtGenexpress) of *DDB1A* and *DDB1B* at various time points.

2. Genetic interactions of *Arabidopsis thaliana* Damaged DNA Binding protein 1B (*DDB1B)* with *DDB1A, DDB2, DET1* and *COP1*

Ashwin L Ganpudi and Dana F Schroeder

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2.1 INTRODUCTION

Light, an essential environmental cue, has profound effects on all stages of plant growth and development. Under dark conditions, seedlings follow a skotomorphogenic (or etiolated) growth pattern (elongated hypocotyls and closed unexpanded cotyledons protected by an apical hook). In contrast, upon perceiving light, seedlings switch to a photomorphogenic (or de-etiolated) growth pattern (short hypocotyls and open expanded cotyledons with active chloroplast differentiation). This transition from etiolation to de-etiolation is controlled by the *COP/DET/FUS* genes (Chen and Chory 2011). All of the pleiotrophic *Arabidopsis thaliana cop/det/fus* mutants display a de-etiolated (*det*) or constitutively photomorphogenic (*cop*) phenotype in the absence of light with increased anthocyanin accumulation, partial chloroplast development and differential expression of hundreds of light regulated genes (Ma et al. 2003; Schroeder et al. 2002).

The *COP/DET/FUS* genes have been cloned and shown to be involved in protein degradation. Six of *COP/DET/FUS* family genes encode components of the COP9 Signalosome (CSN). The CSN exhibits high homology to the 19S lid sub-complex of the 26S proteosome and regulates CULLIN based E3 ubiquitin ligases via deconjugating/conjugating RUB/NEDD8 (Wei et al. 2008). COP1 is a RING-finger protein with a zinc finger motif at the N terminus, followed by a coiled-coil domain and seven WD40 domains at the C terminus. Cellular localization of COP1 is light regulated. Several positive regulators of photomorphogenesis, such as HY5, HYH, LAF1 and HFR1, as well as the photoreceptors Phytochrome A and Cryptochrome 2, have been identified as targets of COP1-mediated degradation (Yi and Deng 2005). DET1, a 68 kDa nuclear localized protein, associates with non-acetylated core histones (Benvenuto et al. 2002) and exhibits biochemical and genetic interactions with DDB1A. Arabidopsis

encodes two homologs of DDB1 – DDB1A and DDB1B, which are 91% identical (Schroeder et al. 2002). DET1 and DDB1A interact with COP10 to form the CDD complex, which in turn interacts with CULLIN4 (Bernhardt et al. 2006; Chen et al. 2006; Yanagawa et al. 2004). Interestingly COP1 also interacts with DDB1 and CULLIN4 (Chen et al. 2010).

DDB1 and DDB2 are core components of the UV Damaged DNA Binding protein complex (DDB) initially identified in human cells. The primary UV-induced DNA lesions include Cyclobutane Pyrimidine Dimers (CPD) and 6-4 Pyrimidine-Pyrimidone Photoproducts (6-4 PP) (Kunz et al. 2006). In order to counteract this damage, plants employ specific mechanisms: photoreactivation, catalyzed by the blue light dependent photolyase class of enzymes, and the light independent Nucleotide Excision Repair (NER) pathway. The *UVR3* and *UVR2/PHR1* genes in Arabidopsis encode 6-4 PP and CPD specific photolyases, respectively, which catalyze dimer monomerization. The dark repair pathway, NER, has specific repair subpathways for transcriptionally active (Transcription Coupled Repair (TC-NER)) or silent (Global Genomic Repair (GG-NER)) DNA regions. Both TC-NER and GG-NER exhibit different damage recognition strategies followed by a common repair pathway. In GG-NER, lesion recognition is mediated by the CUL4-DDB1^{-DDB2} complex followed by XPC-HR23B-CEN2 recruitment. In TC-NER, CUL4-DDB1^{-CSA} recognizes the stalled RNA POL II bound to CSB. Thus both sub-pathways of NER are regulated by the CUL4-DDB1 complex interacting with specific recognition substrates: DDB2 (in GG-NER) and CSA (Cockayne Syndrome A) (in TC-NER). Following recognition, both mechanisms employ a common repair pathway (Ganpudi and Schroeder 2011).

DDB1 is 127 kDa and composed of three β propeller domains (BPA, BPB and BPC). While BPB interacts with CUL4, the clam shaped BPA-BPC pocket mediates interactions with a large number of proteins containing WD40 domains, referred to as DCAF proteins (DDB1-CUL4 Associated Factor) or DWD proteins (DDB1 binding WD40 proteins) (Biedermann and Hellmann 2011; Lee and Zhou 2007). The Arabidopsis genome encodes around 230 WD40 proteins but only a fraction of them (around 86 proteins) have one or more WDxR motifs within the WD40 domain capable of interacting with DDB1 (Lee et al. 2008). Examples of DDB1-interacting WD40 proteins include the UV induced damage recognition factors DDB2 and CSA, the negative regulator of photomorphogenesis COP1, as well as the negative regulators of ABA signaling DWA1, DWA2 and DWA3 (Biedermann and Hellman 2011).

Unique among model systems, Arabidopsis encodes two functional homologs of DDB1, DDB1A and DDB1B (Schroeder et al. 2002). Both homologs are localized to the cytosol and nucleus (Molinier et al. 2008; Zhang et al. 2008). *DDB1A* is expressed at least two fold higher than *DDB1B* throughout development (Al Khateeb and Schroeder 2007; Bernhardt et al. 2010). Null alleles of *DDB1A* do not exhibit obvious developmental phenotypes while null alleles of *DDB1B* appear lethal. *DDB1A* exhibits genetic interactions with *DET1* (Schroeder et al. 2002). Upregulated levels of both *DDB1A* and *DDB1B* mRNA are observed following UV irradiation, while mild to severe UV sensitivity was observed in *ddb1a* and *ddb2* mutants and over-expression of *DDB1A* and *DDB2* confers increased UV resistance (Al Khateeb and Schroeder 2009; Koga et al. 2006; Molinier et al. 2008). In this study we examine the role of *DDB1B* by analyzing the

genetic interactions of a partial loss of function *DDB1B* allele with *DDB1A*, *DDB2*, *DET1*, and *COP1*.

2.2 MATERIALS AND METHODS

2.2.1 Plant materials and growth conditions

All lines in this study were in the Col background of *Arabidopsis thaliana*. *det1-1*, *ddb2*, *ddb1a* and *det1 ddb1a* were as previously described (Al Khateeb and Schroeder 2007; Chory at al. 1989, Schroeder et al. 2002). Strong and weak alleles of *cop1*, *cop1-1* and *cop1-4* respectively, were kindly provided by XW Deng (Yale University). The *ddb1b-2* allele (SALK_061944) was obtained from the Arabidopsis Stock Center (Alonso et al. 2003). Various double mutant combinations were generated using standard protocols (Weigel and Glazebrook 2002). *ddb1a* and *ddb2* genotyping was as described in Al Khateeb and Schroeder (2007). For *ddb1b-2*, the T-DNA insertion was detected using LB2 (TTGGGTGATGGTTCACGTAGTGGGCCATCC) and UV2.21 (CAGAGAAGGAAACCAAGGGAGC) while wildtype *DDB1B* was detected using UV2.21 and *DDB1B* 3'UTR (AGGGGAAGAGGAGAGCTTGGA). Since *ddb1a ddb1b-2* is embryonic lethal, these lines were maintained as *ddb1b-2 ddb1a/+* and *ddb1a ddb1b-2/+*. Seeds were sterilized and plated on Linsmaier and Skoog media (Caisson) supplemented with either 2% sucrose or 0.6% sucrose (depending on genotype as photomorphogenic mutants exhibit more obvious phenotypes on 2% sucrose) and 0.8% Phytoblend (Caisson). Following 2 days of stratification at 4°, plates are transferred to a growth chamber (20° with 50% R.H.). Light was provided by fluorescent bulbs (100 µM photons m^{-2} sec^{-1}). Short day conditions correspond to 10hr light: 14hr dark relative to

long day conditions which correspond to 16hr light: 8hr dark. For adult growth, 14 day old seedlings were transplanted to Sunshine Mix Number 1 (SunGro, WA).

2.2.2 RNA extraction and RT-PCR

Total RNA was extracted from 7 day old seedlings using a RNeasy plant minikit (Qiagen) according to the manufacturer's instructions. Quality of extracted RNA was measured by spectroscopic analysis based on UV absorbance. cDNA synthesis and amplification was a one step process using an Access RT-PCR kit (Promega). *Actin* was used as the loading control. PCR products were separated on 1% (w/v) agarose gels and band intensities were analyzed using ImageJ software (1.36b NIH, USA). The following primers were used: for *DDB1B* 2.W1 (P1) CACGAAACCAACAATTGCAG, 2.15(P2) CATTGTCCAGATACGAGATGGAA, 2.21 (P3) CAGAGAAGGAAACCAAGGGAG, 2.27 (P4) CACACAATGAAACTCTTATTAA, for *DDB1A* 10XL (P5) TAAAGAAGTT AGTCATATGTGCCT, 1.4 (P6) GCAACCTCCCATCACTATAAATACTA.

2.2.3 Seedling analysis

For hypocotyl length and cotyledon width assays, 7 day old seedlings, grown in either long day or dark conditions (after an initial 6 hr light treatment) were scanned and analyzed using NIH Image software. For chlorophyll content analysis 7 day old seedlings were extracted with 80% acetone overnight, A_{645} and A_{663} was determined in a spectrophotometer (model 2100 pro Ultrospec) and chlorophyll content calculated according to the MacKinney method (Mackinney 1941). Anthocyanin content was determined using standard protocol as described in Fankhauser and Casal (2004).

Pigment analysis experiments were repeated at least three times with two replicates per line in each experiment.

2.2.4 Seedling abiotic stress experiments

For germination assays (horizontally aligned) and for root length assays (vertically aligned) plates supplemented with either 100 mM NaCl or 200 mM Mannitol were used. Germination was scored three days after transfer to long day conditions and root growth was measured 7 days after transfer to long day conditions.

For heat assays, seedlings plated on equal volumes of growth medium were dark grown for 4 days, followed by heat treatment (45°) for 4 hours. The hypocotyl length was measured after an additional 4 days of dark growth post treatment.

2.2.5 Adult growth parameters

14 day old seedlings were transplanted to soil. General growth parameters such as flowering time (number of days until bud emergence and number of rosette and cauline leaves), rosette diameter (at 4 weeks) and plant height, # of stems and silique length (at approximately 6 weeks) were determined.

2.2.6 Embryo preparation and analysis

Plump white underdeveloped ovules and mature ovules from the same siliques at different developmental stages were cleared in Hoyer's solution (Liu and Meinke 1998) for 4-5 hours. The cleared ovules were visualized with a Zeiss AXIO Imager ZI

Microscope equipped with differential interface contrast optics and Axio vision 4.6 software.

2.2.7 UV tolerance assays

Shoot assays: Twenty one day old plants were irradiated with 450 J m^{-2} UV C light (254 nm) using a UV lamp (Model XX-15S, UVP, Upland, CA) with a flux rate of 1.6 mW cm^{-2}. Post irradiation, plants were dark incubated for 3 days to avoid photoreactivation based DNA repair. Plants were then transferred to standard growth conditions where percentage sensitivity was assessed by leaf yellowing and necrosis.

Root assays: Seeds were grown on vertically oriented plates for 3 days under the same long day growth conditions as mentioned above. For light assays, plates were irradiated with 600 Jm^{-2} UV-C, rotated 90° and incubated vertically under long day conditions for one day. Fresh root growth (starting from the bending point) was measured using NIH Image J software. For dark assays, plates were UV-C irradiated with 1500 J m^{-2}, similarly rotated and incubated under dark conditions for 3 days. New growth was detected by bending assay and measured using NIH Image J software .

2.2.8 Statistical analysis

All experiments were repeated at least three times. Data was compared by student's *t* test and P values of 0.05 or less considered statistically significant.

2.3 RESULTS

2.3.1 Interactions between *ddb1b* and *ddb1a*

Arabidopsis DDB1A and DDB1B are 91% identical at the amino acid level and both are expressed throughout plant development. *ddb1a* null alleles exhibit no obvious developmental defects, suggesting that *DDB1B i*s acting redundantly. However *ddb1b* null alleles appear lethal as viable homozygotes cannot be obtained (Al Khateeb and Schroeder 2007; Schroeder et al. 2002). In this study we utilize a T-DNA allele in the *DDB1B* gene (Salk _61944) (Fig. 2.1A), which although predicted to result in deletion of the last 112 aa of the protein (Fig. 2.1B), does not affect transcript level of the rest of the *DDB1B* gene (Fig. 2.1B), thus results in a viable partial loss of function allele, referred henceforth to as *ddb1b-2*. *DDB1A* transcript levels are unchanged in the *ddb1b-2* background (Fig. 2.1B) and *DDB1B* levels unaffected in the *ddb1a* null mutant (Fig. 2.1B). The *ddb1b-2* allele was also described in Bernhardt et al. (2010).

Single *ddb1a* and *ddb1b-2* mutants exhibit no obvious developmental phenotypes, however after crossing the single mutants, no doubly homozygous lines could be detected. Among the segregating F2 population however, plants that were homozygous mutant for one gene and heterozygous for the other (*ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+*) were identified. Siliques of these lines exhibited approximately ~22% deformed seeds (Table 2.1, Fig. 2.2A). Further examination of these defective ovules revealed that the embryos had ceased to develop beyond the globular stage while their wildtype siblings developed normally (Fig. 2.2B). Hence, *ddb1a ddb1b-2* double mutants appear to be zygotic lethal, preventing analysis of traits later in development. Therefore we utilized the two single mutants and the two segregating heterozygotes (*ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+*) to examine the effect of *DDB1* dose on development and abiotic stress responses.

DDB1 complexes have been implicated in photomorphogenesis and other light regulated processes, so the phenotypes of *ddb1a, ddb1b-2,* and the *ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+* heterozygotes in dark grown seedlings, light grown seedlings, and adults were examined. In dark grown seedlings, no hypocotyl length or apical hook phenotypes were observed (Fig. 2.3A-C). Similarly, in light grown conditions, no phenotypes with respect to hypocotyl length, cotyledon width, anthocyanin or chlorophyll content were observed (Fig. 2.4A-E). In adults, no effects on flowering time (days and leaves), rosette diameter, height, apical dominance or silique length were detected (Fig. 2.5A-G). Thus a single wildtype copy of either *DDB1A* or *DDB1B* appears to be sufficient for normal development.

In several systems, DDB1 has been shown to interact with the WD40 proteins DDB2 and CSA during GG-NER and TC-NER respectively to repair UV damaged DNA (Ganpudi and Schroeder 2011). Here we examine UV sensitivity in *ddb1a, ddb1b-2,* and the *ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+* heterozygotes to determine the roles of *DDB1A* and *DDB1B* in Arabidopsis UV tolerance. Adult plants were exposed to UV-C and leaf damage scored (Fig. 2.6A,B). There was no significant difference in percentage dead leaves between the single mutants *ddb1a* and *ddb1b-2* relative to Col. Similarly, no differences between *ddb1b-2 ddb1a/+* relative to *ddb1b-2* was observed, but *ddb1a ddb1b-2/+* exhibited higher levels of tissue death relative to *ddb1a*. Thus in adults either a single wildtype allele of *DDB1A* (in *ddb1b-2 ddb1a/+)* or two wildtype alleles of *DDB1B* (in *ddb1a*) appear sufficient for UV tolerance. However a single wildtype allele of *DDB1B* (in *ddb1a ddb1b-2/+)* is not sufficient. Hence, in adult UV tolerance *DDB1A* and *DDB1B* appear to act redundantly, with *DDB1A* apparently the stronger player. In

seedlings, as in adult plants, the single mutants and *ddb1b-2 ddb1a/+* did not exhibit sensitive phenotypes, however *ddb1a ddb1b-2/+* again exhibited a root UV sensitive phenotype one day after UV irradiation when incubated in long day conditions (Fig. 2.6C). Interestingly, increased UV sensitivity in both *ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+* was observed following treatment with a higher dose of UV and three days dark incubation (Fig. 2.6D), suggesting that in these conditions neither a single wildtype version of *DDB1A* nor *DDB1B* is sufficient for full UV tolerance.

The DCAF proteins DWA1, DWA2 and DWA3 have recently been implicated in ABA signaling and NaCl tolerance (Lee et al. 2010; 2011), thus we examined the contributions of *DDB1A* and *DDB1B* to salt and osmotic stress tolerance using germination assays. While *ddb1b-2* and *ddb1b-2 ddb1a/+* exhibited normal germination rates on both 100 mM NaCl and 200 mM Mannitol, *ddb1a* and *ddb1a ddb1b-2/+* exhibited reduced germination rates in both these conditions (Fig. 2.6E). Thus *DDB1A* appears to have a critical role in regulation of germination during stress conditions, while no effect of *DDB1B* mutation could be detected in either the wildtype or *ddb1a* background. Although *ddb1a* and *ddb1a ddb1b-2/+* exhibited delayed germination, they did not exhibit any root growth phenotypes after 7 days in these conditions (Fig. 2.6F). In fact, root growth in *ddb1b-2* was found be slightly resistant to mannitol. Finally, we examined the role of *DDB1A* and *DDB1B* in heat sensitivity by analyzing the effect of heat on dark grown hypocotyl length (Fig. 2.6G). *ddb1b-2* exhibited mild heat sensitivity while *ddb1b-2 ddb1a/+*, *ddb1a* and *ddb1a ddb1b-2/+* all exhibited similar strong sensitivity. Thus both *DDB1A* and *DDB1B* contribute to heat tolerance.

2.3.2 Interactions between *ddb1b* and *ddb2*

DDB2 is a WD40 protein that serves as a lesion recognition factor by interacting with DDB1-CUL4 during GG-NER. Arabidopsis *DDB2* exhibits complex genetic interactions with *DDB1A* and *DET1* during photomorphogenesis and DNA repair (Al Khateeb and Schroeder 2007; Castells et al. 2011)**.** However *ddb1b-2 ddb2* double mutants did not exhibit any developmental phenotypes in dark grown seedlings, light grown seedlings, or adults (Fig. 2.7-2.9). In addition, no UV sensitive phenotypes were observed in shoots or roots of the *ddb1b-2 ddb2* mutants (Fig. 2.10). Thus *DDB1A* plays a compensatory role in this pathway.

2.3.3 Interactions between *ddb1b* and *det1*

DET1, a master repressor of photomorphogenesis, interacts both biochemically and genetically with DDB1A (Schroeder et al. 2002). Here we examine genetic interactions between *det1* and *ddb1b-2* in dark grown seedlings, light grown seedlings and adults.

In dark grown seedlings, *det1* mutants exhibit a constitutively de-etiolated phenotype with short hypocotyls, open cotyledons and increased anthocyanin content (Chory et al. 1989). As described previously (Schroeder et al. 2002), in the dark *ddb1a det1* mutants exhibit decreased hypocotyl length and cotyledon width as well as increased anthocyanin content relative to *det1* single mutants (Fig. 2.11). However the *ddb1b-2 det1* double mutants did not significantly differ from *det1* with respect to any of these phenotypes. This data suggests that *DDB1A* is more critical than *DDB1B* for *DET1* function in dark grown seedlings.

In light grown seedlings, *det1* mutants are small with decreased chlorophyll and increased anthocyanin levels. In the light as in the dark, the *ddb1a det1* mutants exhibit decreased cotyledon width and increased anthocyanin levels relative to *det1* (Fig. 2.12). While the *ddb1b-2 det1* mutants did not differ from *det1* with respect to hypocotyl length or cotyledon width, they did however exhibit enhanced anthocyanin levels, intermediate between those of *det1* and *ddb1a det1* (Fig 2.12D). Interestingly, *ddb1b-2 det1* mutants exhibited higher chlorophyll levels than *det1*, thus *ddb1b-2* partially suppresses the *det1* pale phenotype (Fig. 2.12E).

We also compared the effect of *ddb1a* and *ddb1b-2* on *det1* phenotypes in adult plants (Fig. 2.13). *det1* mutants exhibit early flowering time (Pepper and Chory 1997). Flower bud emergence in *det1* occurs at approximately 18 days in long day conditions in contrast to wildtype plants, where bud emergence occurs at approximately 24 days. Like *ddb1a det1* (bud emergence at approx 22 days), *ddb1b-2 det1* double mutants partially suppress early flowering in *det1*, with bud emergence at approximately 20 days under long day conditions (Fig. 2.13B). *ddb1b-2* also partially suppressed *det1* early flowering in short day conditions (Fig. 2.14A). In terms of leaf number at flowering, *ddb1b-2 det1* double mutants flowered at significantly increased leaf number relative to *det1* in long day (Fig. 2.13C), however no effect was observed in short day conditions (Fig. 2.14B).

det1 adults are dwarf in stature, with reduced rosette diameter, height, and silique length relative to wildtype (Fig. 2.13D-F). All three of these parameters are further decreased in *ddb1a det1* double mutants, thus *ddb1a* enhances the *det1* dwarf phenotype. *ddb1b-2* also enhanced these three *det1* phenotypes, but to a lesser extent than

ddb1a. In addition, *ddb1b-2* enhanced these three *det1* phenotypes in short day (Fig. 2.14 C-E). *det1* also has decreased apical dominance resulting in increased inflorescence number. Neither *ddb1a* nor *ddb1b-2* effect this phenotype in either long day or short day (Fig. 2.13G, 2.14F).

2.3.4 Interaction between *ddb1b* and *cop1*

Photomorphogenic protein COP1 has also recently been shown to form a CUL4-DDB1 complex (Chen et al. 2010). Here we examine *ddb1b-2 cop1* interactions during development. *ddb1b-2* double mutants were generated with two *cop1* alleles: a strong allele (*cop1-1*) and a relatively weak allele (*cop1-4*) (McNellis et al. 1994). *cop1* mutants, like *det1,* exhibit a constitutively photomorphogenic phenotype in the dark (Fig. 2.15). In dark grown seedlings, *ddb1b-2* enhanced the short hypocotyl phenotype in *cop1-4* but not *cop1-1* (Fig. 2.15B). No differences in cotyledon width or anthocyanin content were observed in either *ddb1b-2 cop1-4* and *ddb1b-2 cop1-1* double mutants relative to their respective single mutants (Fig. 2.15C,D). In light grown seedlings (Fig. 2.16), *ddb1b-2* had no effect on hypocotyl length in *cop1-4*, but suppressed the short hypocotyl phenotype in *cop1-1* (Fig. 2.16B). *ddb1b-2* had no significant effect on cotyledon width or chlorophyll content in either *cop1* allele (Fig. 2.16C,D). With respect to anthocyanin content, *ddb1b-2* had no effect on *cop1-4* but enhanced anthocyanin concentrations in *cop1-1* (Fig. 2.16E). In adults, *ddb1b-2* did not significantly alter any *cop1* phenotypes in either long day or short day conditions (Fig. 2.17, 2.14). Thus genetic interactions between *ddb1b-2* and *cop1* appear to be developmentally regulated.

2.4 DISCUSSION

The primary purpose of this study was to highlight differences between *DDB1A* and *DDB1B* in terms of redundant and distinct functions, and examine genetic interactions with specific DDB1 interactors. Firstly, the lethality the *ddb1a ddb1b-2* double mutants indicates the importance of the redundantly acting *DDB1s* in embryogenesis (Bernhardt et al. 2010). *CUL4* null alleles also result in embryo lethality (Dumbliauskas et al. 2011). Interestingly, while we did not detect any growth defects in the *ddb1a* and *ddb1b-2* single mutants and segregating heterozygotes, using the same viable *ddb1b-2* allele (SALK_069144), Bernhardt et al. (2010) identified seedling and adult phenotypes in these lines. These phenotypes included increased dark hypocotyl length in *ddb1a* and *ddb1a ddb1b-2/+*, accelerated flowering in long day in terms of leaf number in all lines, late flowering in long day in terms of days in *ddb1b-2 ddb1a/+*, decreased height in *ddb1a ddb1b-2/+*, and decreased silique length in both *ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+*. While the *ddb1b* alleles were the same for both these studies, there are differences in the *ddb1a* allele used. Although the T-DNA insertion sites are close to each other, in their allele in the middle of the ninth exon, in ours in the beginning of the tenth exon, their allele appears to result in partial loss of function, with transcript still detected 5' of the T-DNA, while ours is an apparent null. If their partial loss of function allele actually results in more severe phenotypes than the null, it may have some inhibitory activity. Alternatively, the differences in our results could be due to differences in experimental conditions.

In adult UV tolerance assays, the shoots of *ddb1a ddb1b-2/+* exhibited increased tissue sensitivity, a phenomenon not observed in the shoots of *ddb1b-2*

ddb1a/+, *ddb1a* or *ddb1b-2*. This implies dosage requirements for DDB1. While a single copy of *DDB1A* in the absence of *DDB1B* is enough for the plants to initiate the repair process, plants with a single copy of *DDB1B* in the absence of *DDB1A* appear more sensitive. Despite high identity between the DDB1 homologs, *DDB1A* is expressed almost two fold higher throughout development (Bernhardt et al. 2010; Al Khateeb and Schroeder 2007). Thus the presence of a single wildtype copy of *DDB1A* would be expected to result in higher overall *DDB1* levels than a single wildtype copy of *DDB1B.*

In seedling UV tolerance experiments, light incubation versus dark incubation influenced UV tolerance. Similar to the shoot assays, with light incubation post irradiation, sensitivity was observed significantly only in *ddb1a ddb1b-2/+*. Thus in light conditions, as in adults, a single copy of *DDB1B* is not sufficient for wildtype levels of UV tolerance, but a single copy of *DDB1A* is sufficient. In light conditions, both photolyase enzymes and Nucleotide Excision Repair contribute to repair of UV-damaged DNA. In dark conditions however plants are dependent on NER for repair. In our experiments, with dark incubation post irradiation both *ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+* are sensitive to UV treatment. These experiments also used a stronger UV dose than the light assay, 1500 Jm^{-2} as compared to 600 Jm^{-2}. Thus when the demand for NER is amplified by increasing the amount of UV damage and by removing the contribution of photolyases, neither a single wildtype copy of *DDB1B* nor *DDB1A* is sufficient for wildtype levels of UV tolerance. However two wildtype alleles of either gene is sufficient.

Germination in *ddb1a* is more sensitive to 100 mM NaCl and 200 mM Mannitol than germination in *ddb1b-2* or wildtype. Recently, Lee et al. (2010; 2011)

characterized WD40 proteins involved in ABA signaling, DWA1, DWA2 and DWA3. The single and double *dwa* mutants are sensitive to ABA and NaCl, as are *CUL4* co-suppression lines. While DWA1, 2 and 3 all interact with both DDB1A and DDB1B in vitro, our data suggests that *DDB1A* is more critical for germination in stress conditions. Interestingly, the sensitivity of *ddb1a* in germination had no effects on root growth, suggesting that *DDB1A* and *DDB1B* act redundantly to regulate this phenotype.

Heat sensitivity was observed in *ddb1b-2, ddb1a, ddb1b-2 ddb1a/+* and *ddb1a ddb1b-2/+* with plants lacking one or both copies of *DDB1A* the most sensitive. Other recent studies in our lab also implicate *DDB1A* in heat response (V. Ly, A. Hatherell, E. Kim, and D. Schroeder, unpublished).

We did not detect any phenotypes in *ddb1b-2 ddb2* double mutants in either normal development or response to UV. These are both partial loss of function alleles and we did not previously detect any growth defects in *ddb1a ddb2* double mutants (Al Khateeb and Schroeder 2007). The T-DNA insertion in the *ddb1b-2* allele is predicted to result in a truncated protein lacking the last 112 aa. This truncated protein failed to interact with DDB2 in yeast two hybrid assays (Bernhardt et al. 2010).

DET1 interacts biochemically with CUL4-DDB1 and exhibits genetic interactions with both *CUL4* and *DDB1A* (Chen et al. 2006; Schroeder et al. 2002). While *ddb1a* enhances *det1* phenotypes in dark grown seedlings, *ddb1b-2* has no effect. This data suggests that in dark grown seedlings DDB1A plays a more prominent role in DET1 function than DDB1B. In contrast, in light grown seedlings *ddb1b-2* enhanced the *det1* high anthocyanin phenotype and suppressed the *det1* low chlorophyll phenotype, suggesting that DDB1B is involved in DET1 regulation of pigment levels in the light. In

previous studies, *ddb2* was also found to suppress the *det1* low chlorophyll phenotype and this suppression was found to be *DDB1A* independent (Al Khateeb and Schroeder 2007). Thus both studies support the role of DDB1B in DET1 regulation of chlorophyll level.

In adult plants, *ddb1a* enhances the *det1* small phenotype, resulting in decreased rosette diameter, height and silique length, and partially suppresses early flowering in *det1* in terms of days. For all these phenotypes *ddb1b-2* has a similar effect on *det1* as *ddb1a,* but to a lesser extent. Given that the *ddb1a* null allele is stronger than the *ddb1b-2* partial loss of function allele, and that *DDB1A* is expressed at higher levels than *DDB1B* throughout development (Al Khateeb and Schroeder 2007), these results are consistent with both DDB1A and DDB1B contributing to DET1 regulation of adult growth.

Only a few effects of *ddb1b-2* on *cop1* phenotypes were observed. Interestingly, *ddb1b-2* enhanced the short hypocotyl phenotype in dark grown *cop1-4.* Since *ddb1b-2* had no effect on dark-grown *det1*, DDB1B appears to be more critical for COP1 function than for DET1 function in the dark. In light grown seedlings, *ddb1b-2* suppressed the short hypocotyl phenotype and enhanced anthocyanin levels in *cop1-1.* In light-grown *det1*, *ddb1b-2* also enhanced the high anthocyanin phenotype, suggesting that DDB1B has a common role in regulation of anthocyanin levels. In adults, *ddb1b-2* had no effect on any phenotypes on either *cop1* allele. In contrast, *ddb1b-2* modified the majority of *det1* adult phenotypes, indicating that in adults DDB1B is more critical for DET1 function than for COP1 function. Thus the requirement for DDB1B seems to vary in the course of development, from COP1-specific interactions in the dark to DET1-specific in adults. Whether this specificity is due to differential levels, cellular localization, or biochemical

interactions of DDB1B versus DDB1A is unknown. Interestingly, the *cop1* alleles used in this study, *cop1-4* (truncated protein predicted to lack the WD40 domain) and *cop1-1* (internal deletion potentially altering the conformation of the WD40 domain) (McNellis et al. 1994), would be predicted to be comprised in their ability to interact with DDB1 proteins. Thus any *ddb1b-2 cop1* genetic interactions observed may be indirect.

Thus in this study we have examined the relative contributions of DDB1B and DDB1A to stress response, as well as DET1 and COP1 function, and find that there appears to be developmental regulation of DDB1 interactions.

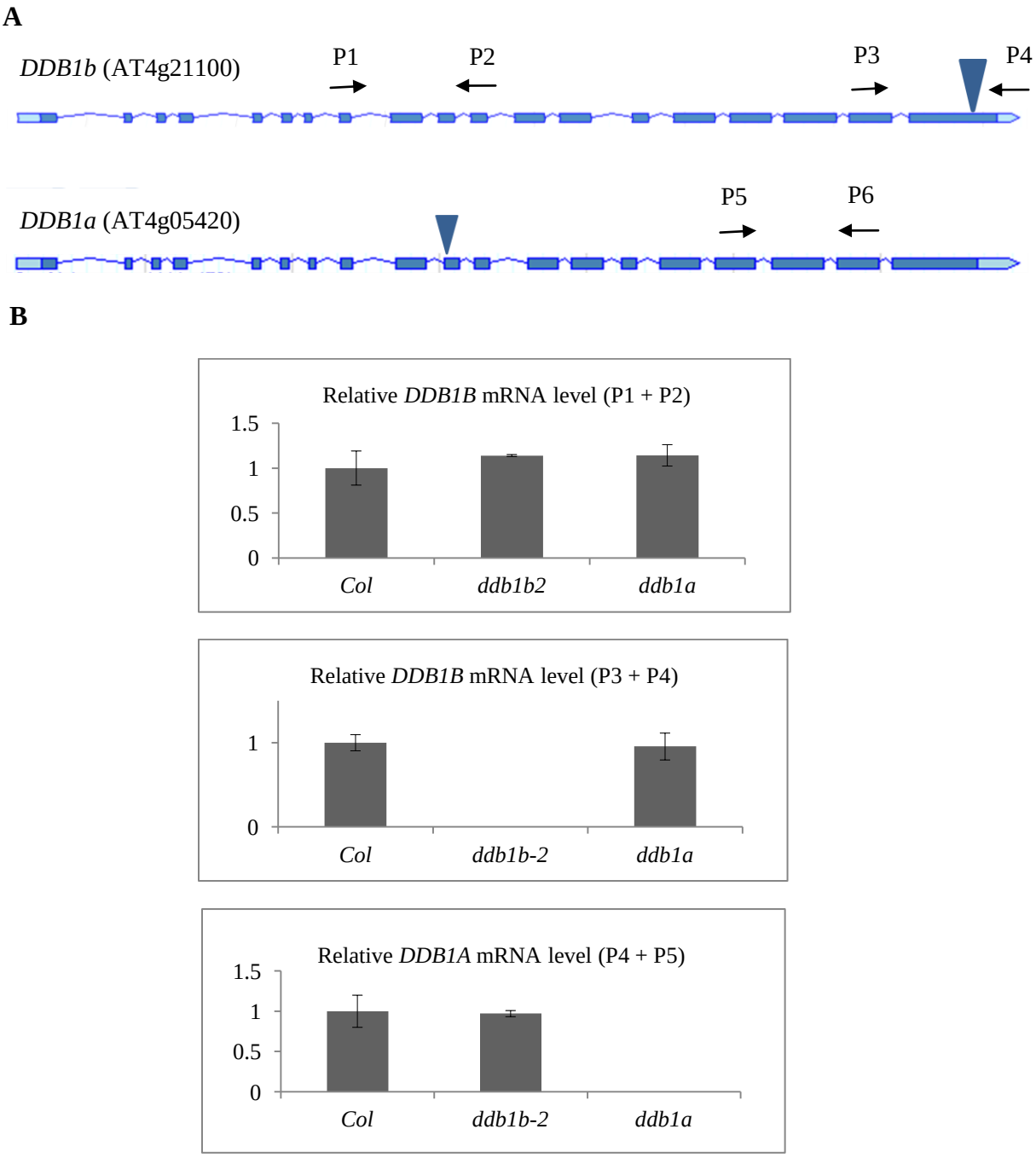


Figure 2.1 Arabidopsis *DDB1* allele characterization: A) Schematic representation of Arabidopsis *DDB1B* and *DDB1A* genes. Exons are represented as blue rectangles, introns as lines t-DNA insertion as inverted triangle and arrow heads indicate primer flanking sites. **B)** mRNA expression levels of *DDB1A* and *DDB1B* in control conditions in Col, *ddb1b-2* and *ddb1a* backgrounds. Data is relative to Col levels and normalized with *Actin*. Error bars = ± SE (n=2).

Genotypes	Wt. green	Med. white	Med. brown	Small brown	Total
Col	991 (100%)	0	0	0	991
ddb1a	998 (99.8%)	0	0	2 (0.2%)	1000
ddb1a ddb1b-2/+	1047 (78.5%)	154 (11.5%)	73 (5.5%)	60 (4.5%)	1334
ddb1b-2	888 (99.9%)	0	0	1 (0.1%)	889
ddb1b-2 ddb1a/+	577 (78.2%)	57 (7.8%)	37 (5%)	67 (9%)	738

Table 2.1: Seed abortion rates based on physical appearance

A

Figure 2.2 Silique and embryo phenotypes: (A) Images of siliques obtained from self pollinated wild type, *ddb1a*, *ddb1a ddb1b2/+*, *ddb1b-2*, and *ddb1b-2 ddb1a/+*. Abnormal ovules are visible in the heterozygous lines.

B

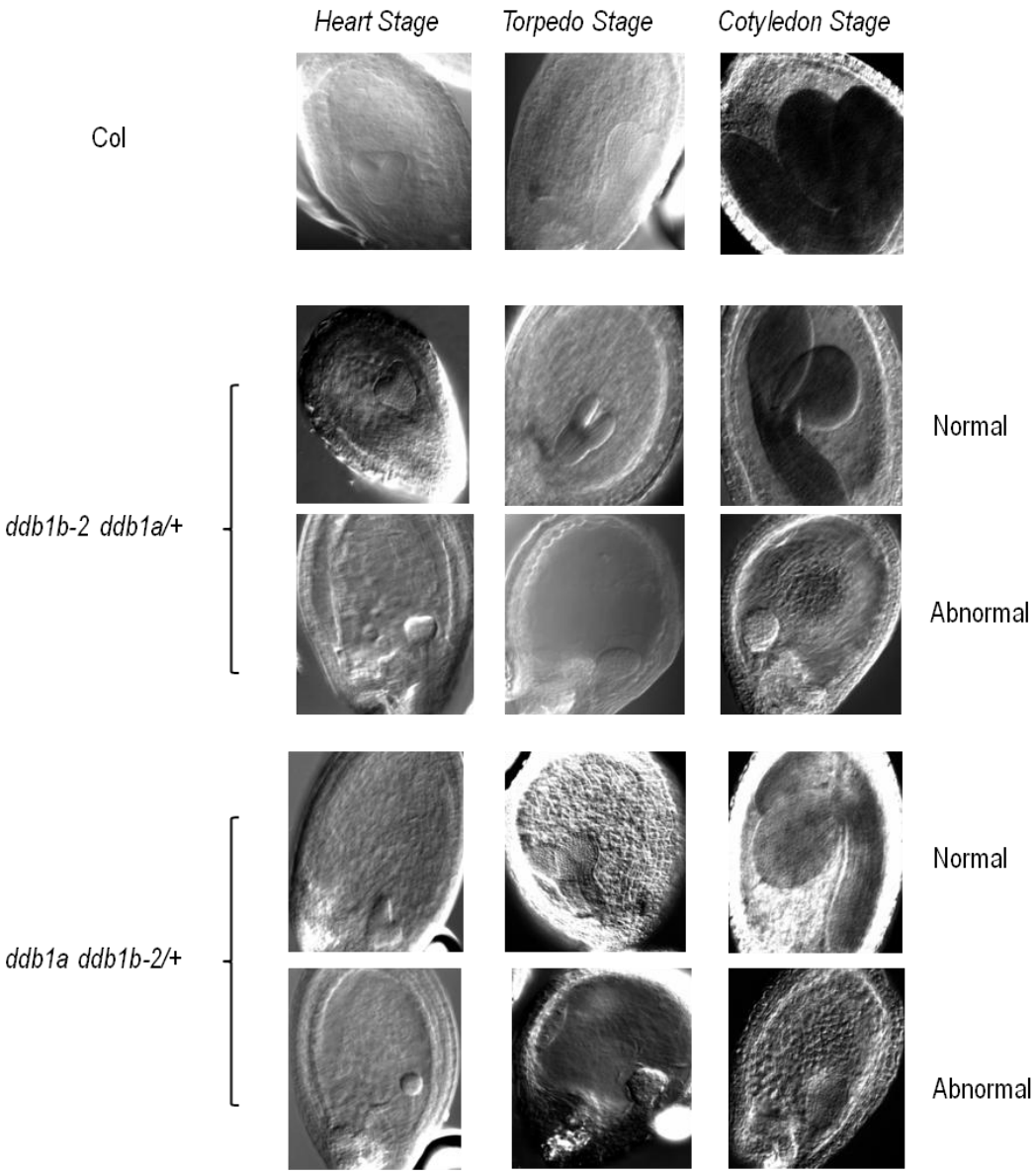
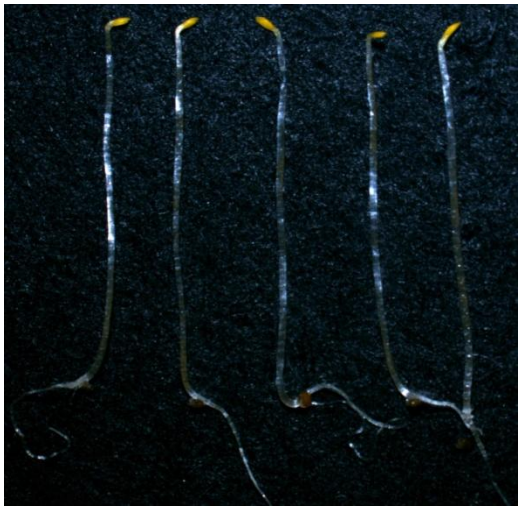


Figure 2.2 Silique and embryo phenotypes (B) DIC Images of cleared ovules, derived from: from top, self-pollinated Columbia, *ddb1a ddb1b-2/+*, and *ddb1b-2 ddb1a/+* siliques. For the heterozygotes, for each developmental stage (Heart – Torpedo – Cotyledon), normal (top) and abnormal (bottom) ovules from same silique are shown.

A



B

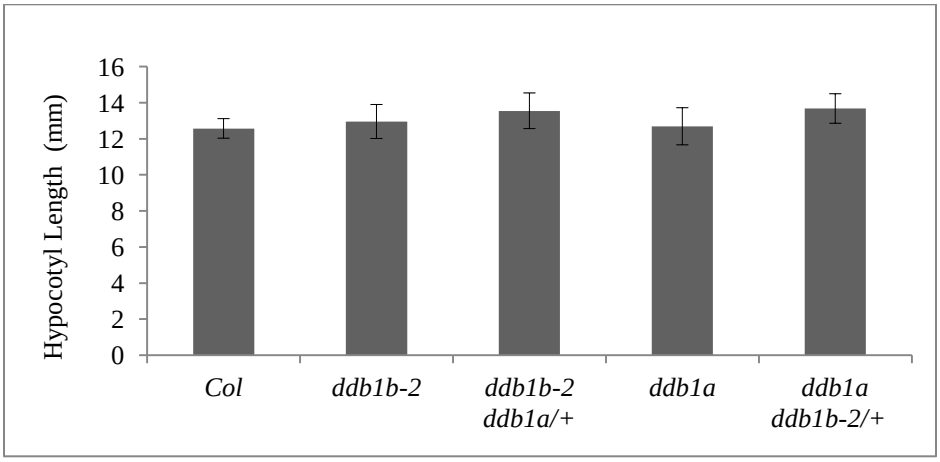


Figure 2.3 Phenotypic characterization of 7 day dark grown seedlings.

A: From left: *Col-0, ddb1b-2, ddb1a, ddb1b-2 ddb1a/+*, and *ddb1a ddb1b-2/+*

B: Hypocotyl Length (in mm)

Error bars indicate 95% C.I. (n=15). No significant differences were observed between *ddb1b-2 ddb1a/+* relative to *ddb1b-2*, *ddb1a ddb1b-2/+* relative to *ddb1a*, or *ddb1a* and *ddb1b-2* relative to *Col*.

C

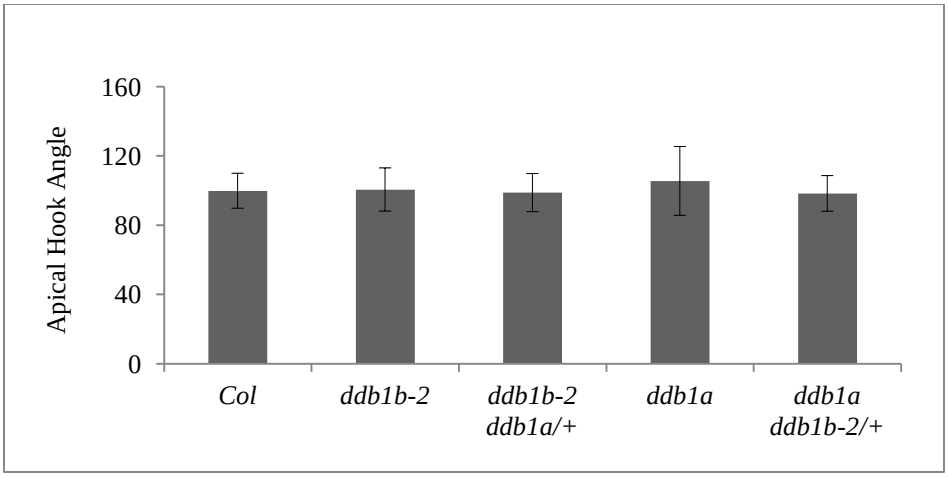


Figure 2.3 Phenotypic characterization of 7 day dark grown seedlings (Cont.)

C: Apical Hook Angle

Error bars indicate 95% C.I. (n=15). No significant differences were observed between *ddb1b-2 ddb1a/+* relative to *ddb1b-2*, *ddb1a ddb1b-2/+* relative to *ddb1a*, or *ddb1a* and *ddb1b-2* relative to *Col*.

A

B

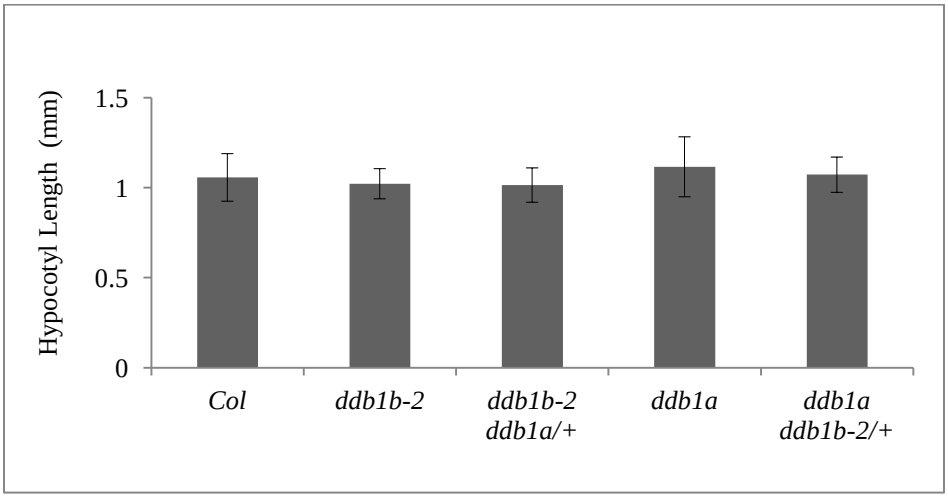


Figure 2.4 Phenotypic characterization of 7 day light grown seedlings

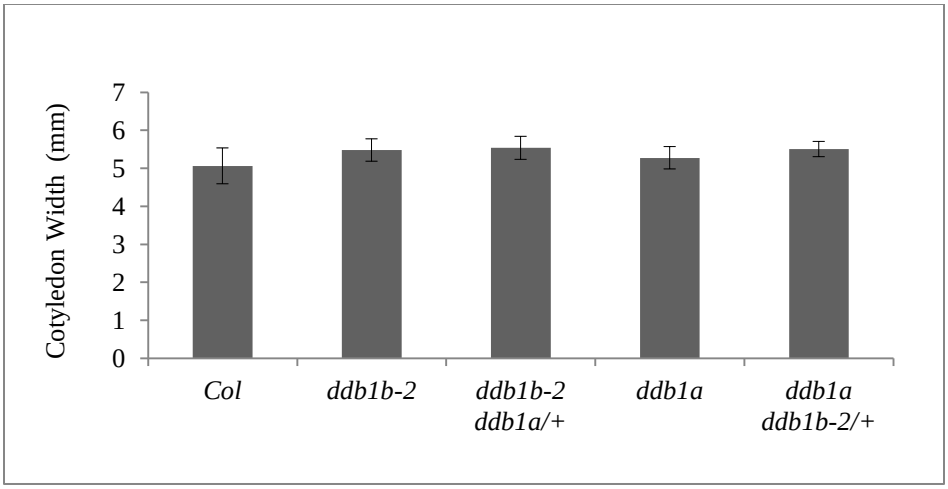
A: From left: *Col-0, ddb1b-2, ddb1a, ddb1b-2 ddb1a/+*, and *ddb1a ddb1b-2/+*

B: Hypocotyl Length (in mm)

Error bars indicate 95% C.I. (n=15). No significant differences were observed between *ddb1b-2 ddb1a/+* relative to *ddb1b-2*, *ddb1a ddb1b-2/+* relative to *ddb1a*, or *ddb1a* and *ddb1b-2* relative to *Col*.

.

C



D

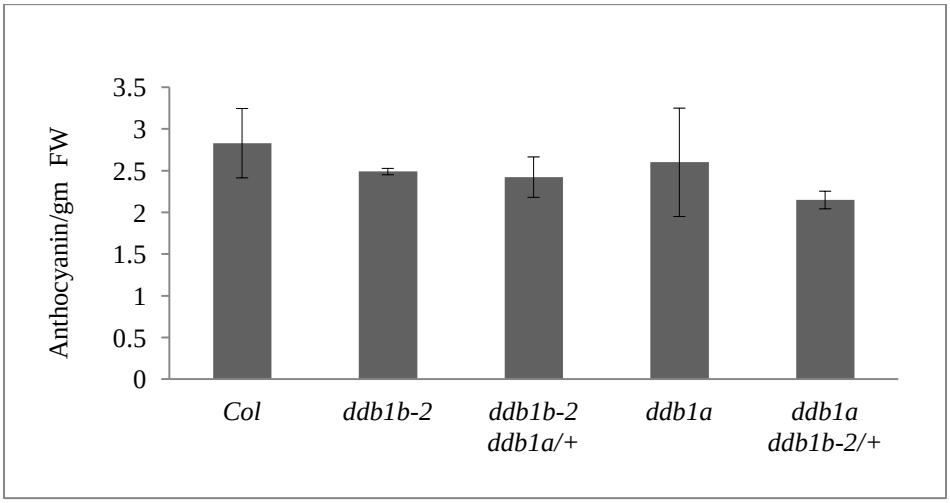


Figure 2.4 Phenotypic characterization of 7 day light grown seedlings (Cont.)

C: Cotyledon Width (in mm); **D:** Anthocyanin Content / gm FW (n=2)

Error bars indicate 95% C.I. No significant differences were observed between *ddb1b-2 ddb1a/+* relative to *ddb1b-2*, *ddb1a ddb1b-2/+* relative to *ddb1a*, or *ddb1a* and *ddb1b-2* relative to *Col*.

E

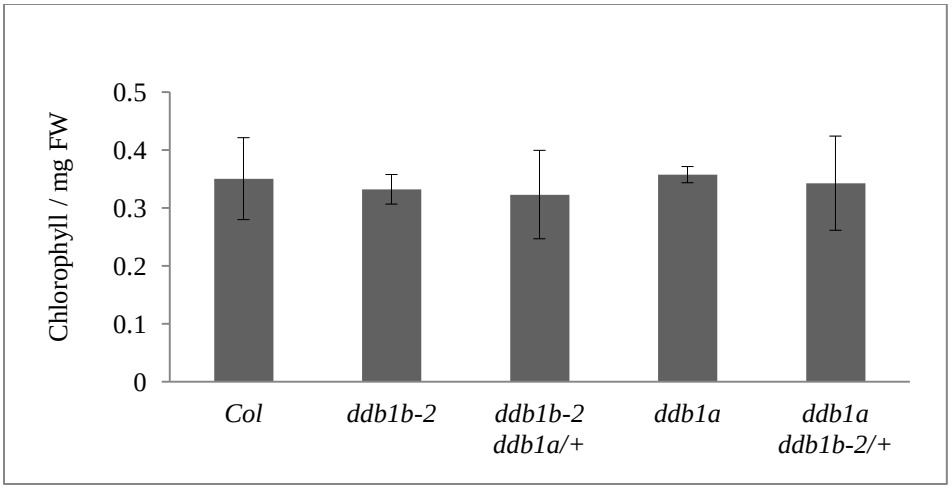


Figure 2.4 Phenotypic characterization of 7 day light grown seedlings (Cont.)

E: Chlorophyll content (µg chlorophyll / mg FW)

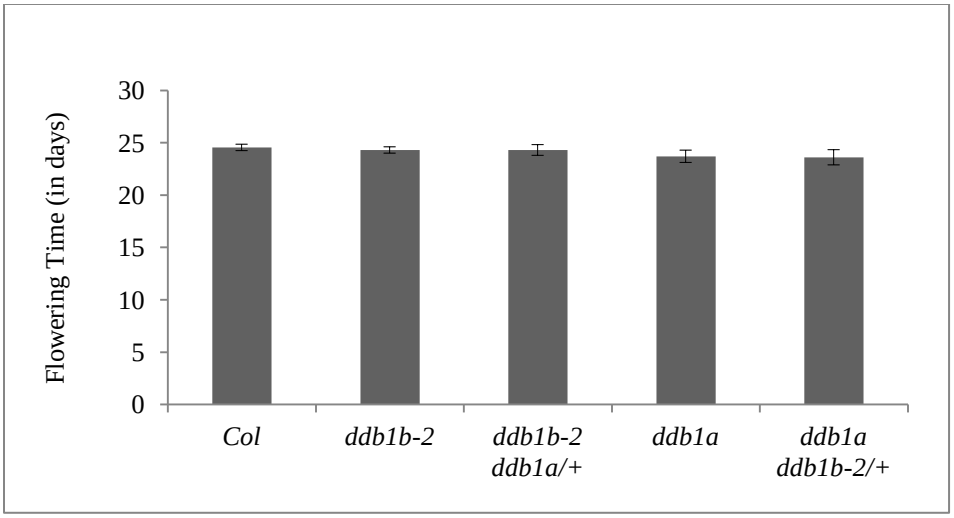
Error bars indicate 95% C.I., (n=2). No significant differences were observed between *ddb1b-2 ddb1a/+* relative to *ddb1b-2*, *ddb1a ddb1b-2/+* relative to *ddb1a*, or *ddb1a* and *ddb1b-2* relative to *Col*.

A

Figure 2.5 Adult Phenotypes

A: From left to right: *Col-0, ddb1b-2, ddb1a, ddb1b-2 ddb1a/+*, and *ddb1a ddb1b-2/+*

B



C

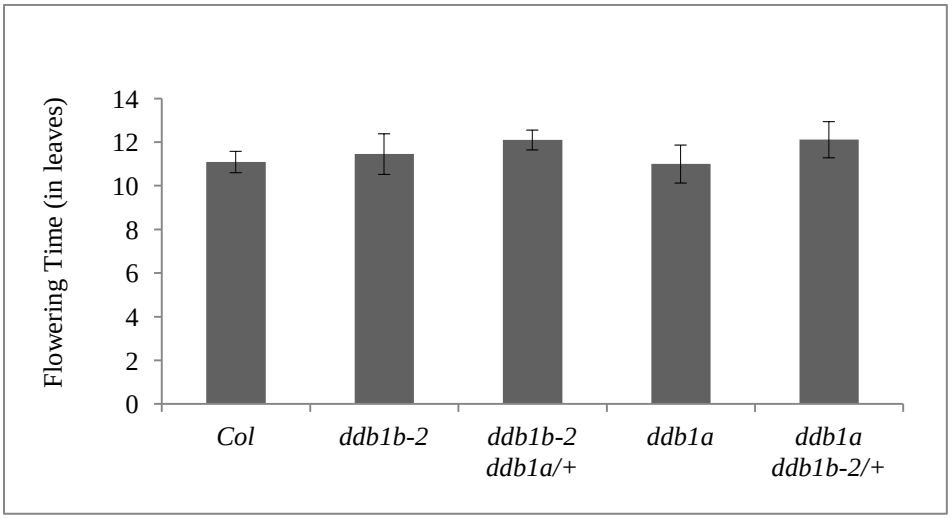
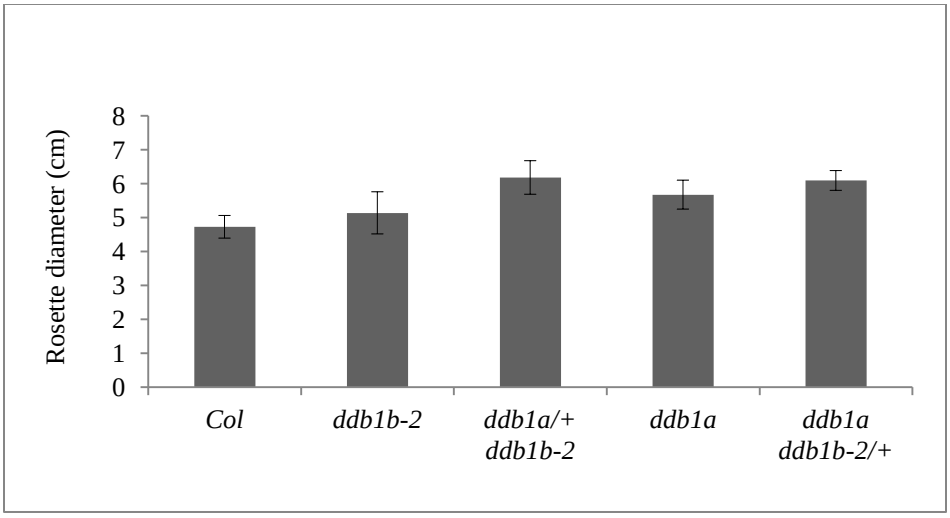


Figure 2.5 Adult Phenotypes (Cont.)

B: Flowering Time (in days); **C:** Flowering Time (in leaves)

Error bars indicate 95% C.I., (n=18). No significant differences were observed between *ddb1b-2 ddb1a/+* relative to *ddb1b-2*, *ddb1a ddb1b-2/+* relative to *ddb1a*, or *ddb1a* and *ddb1b-2* relative to *Col*.

D



E

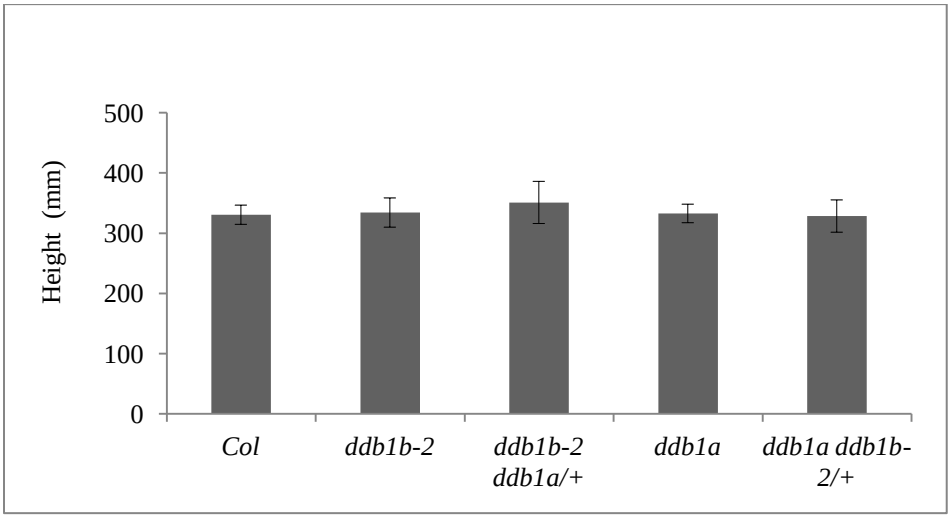
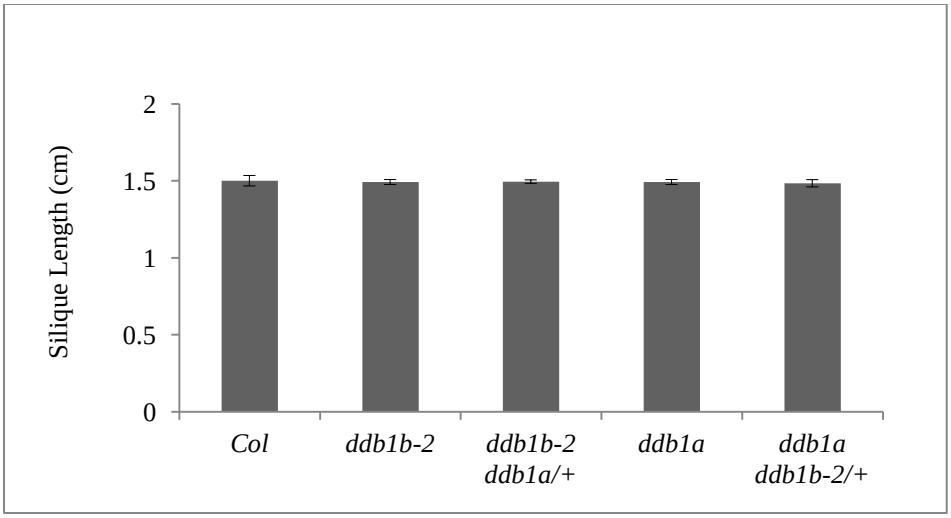


Figure 2.5 Adult Phenotypes (Cont.)

D: Rosette Diameter (in cm); **E:** Height (in mm)

Error bars indicate 95% C.I., (n=18). No significant differences were observed between *ddb1b-2 ddb1a/+* relative to *ddb1b-2*, *ddb1a ddb1b-2/+* relative to *ddb1a*, or *ddb1a* and *ddb1b-2* relative to *Col*.

F



G

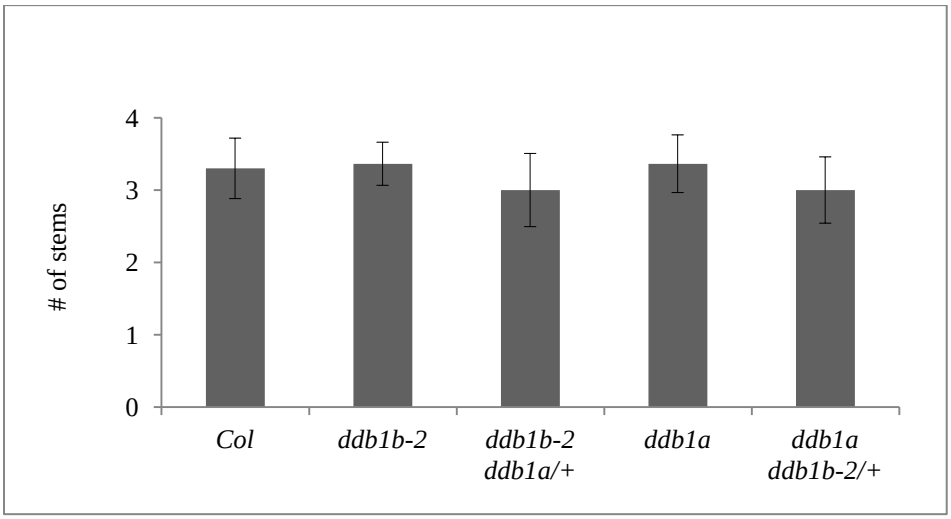


Figure 2.5 Adult Phenotypes (Cont.)

F: Silique length (in cm); **G:** # of stems

Error bars indicate 95% C.I., (n=18). No significant differences were observed between *ddb1b-2 ddb1a/+* relative to *ddb1b-2*, *ddb1a ddb1b-2/+* relative to *ddb1a*, or *ddb1a* and *ddb1b-2* relative to *Col*.

A

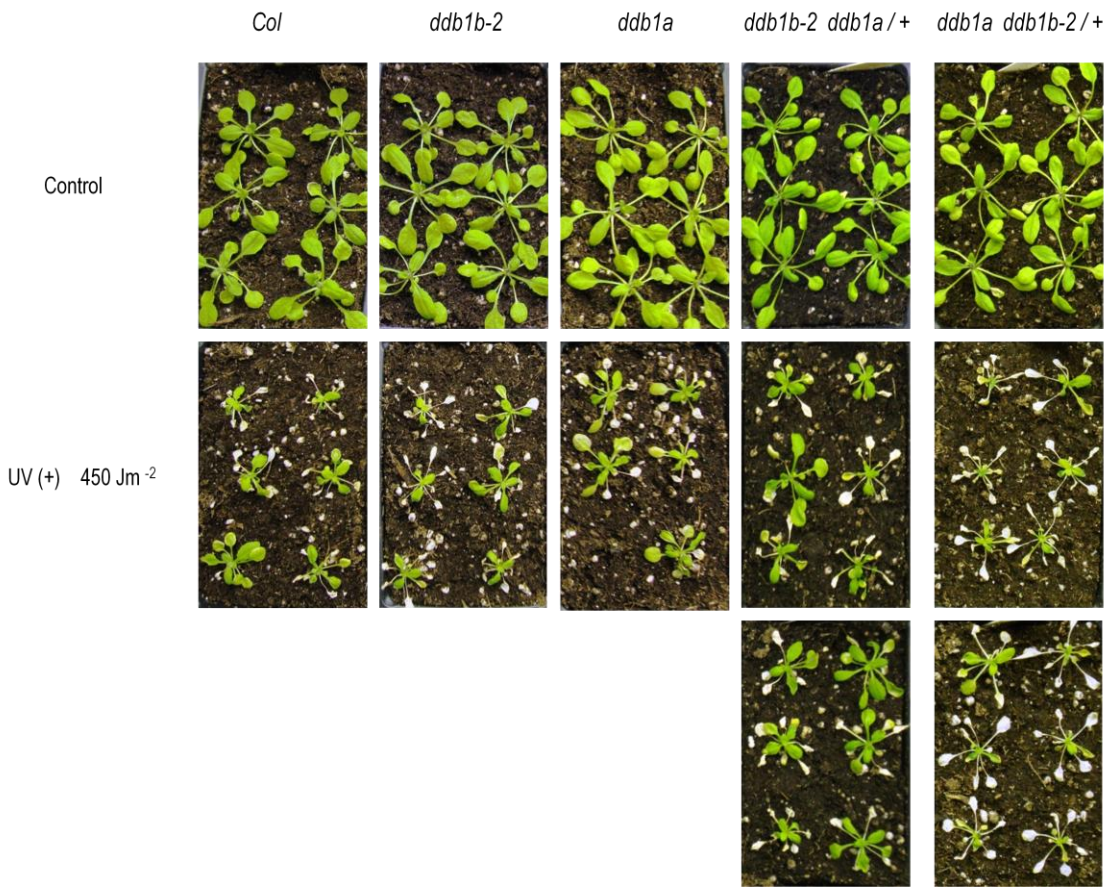


Figure 2.6 Abiotic Stress Response

A: UV Shoot Assay. Phenotypes of control and UV irradiated 21 day old *Col, ddb1b-2, ddb1a, ddb1b-2 ddb1a/+* and *ddb1a ddb1b-2/+* plants

B

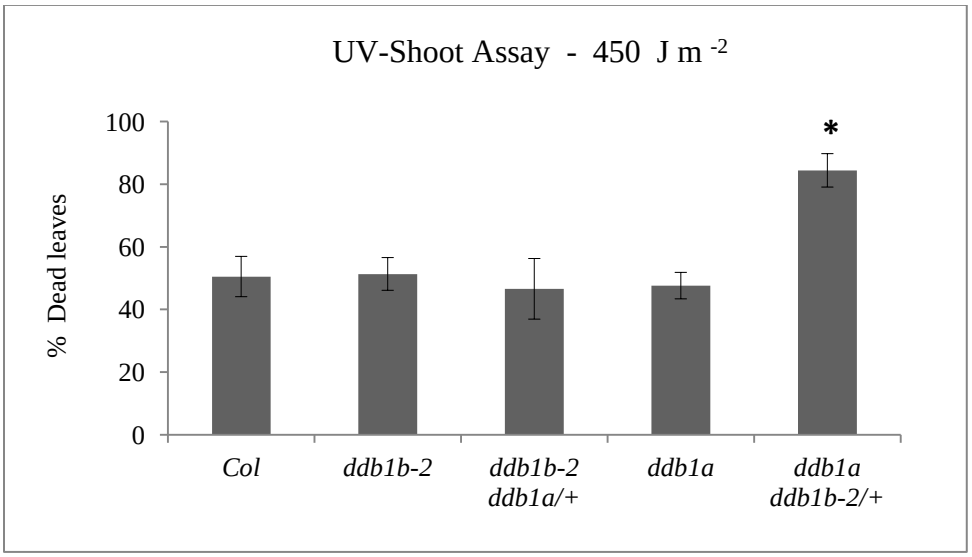


Figure 2.6 Abiotic Stress Response

B: Statistical representation of % leaf damage.

Error bars indicate 95% C.I., (n= 12), * = P≤0.05, *ddb1b-2 ddb1a/+* relative to *ddb1b-2* and *ddb1a ddb1b-2/+* relative to *ddb1a*, *ddb1a* and *ddb1b-2* relative to *Col*.

C

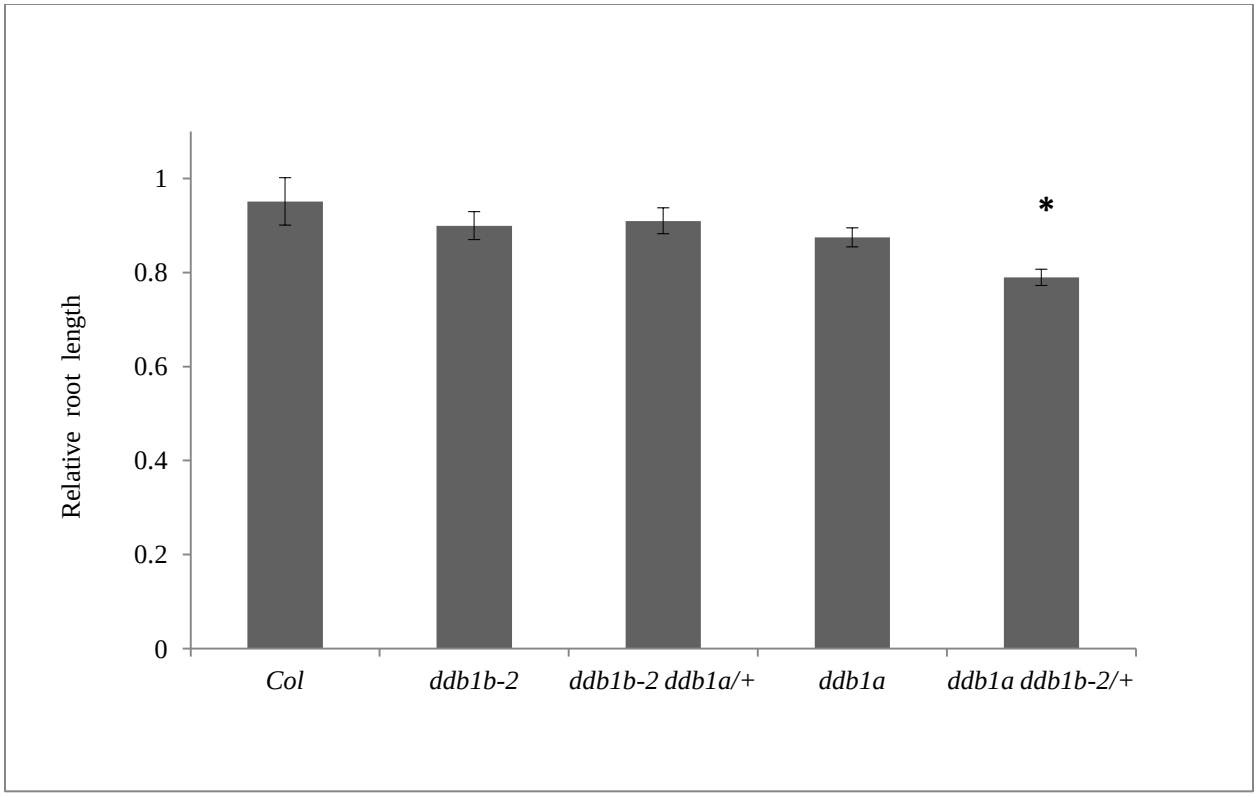


Figure 2.6 Abiotic Stress Response (Cont.)

C: UV Root Assay – Light Treatment. Relative root length of *Col*, *ddb1b-2*, *ddb1a*, *ddb1b-2 ddb1a/+* and *ddb1a ddb1b/+* irradiated with 600 J m^{-2} UV-C and then grown under long day conditions. Measurements were taken 24 hours post irradiation.

Data is expressed as root length relative to unirradiated control of the corresponding genotype. Error bars indicate ±SE, (n=25)

* = P≤0.05, *ddb1b-2 ddb1a/+* relative to *ddb1b-2* and *ddb1a ddb1b-2/+* relative to *ddb1a*, *ddb1a* and *ddb1b-2* relative to *Col*.

D

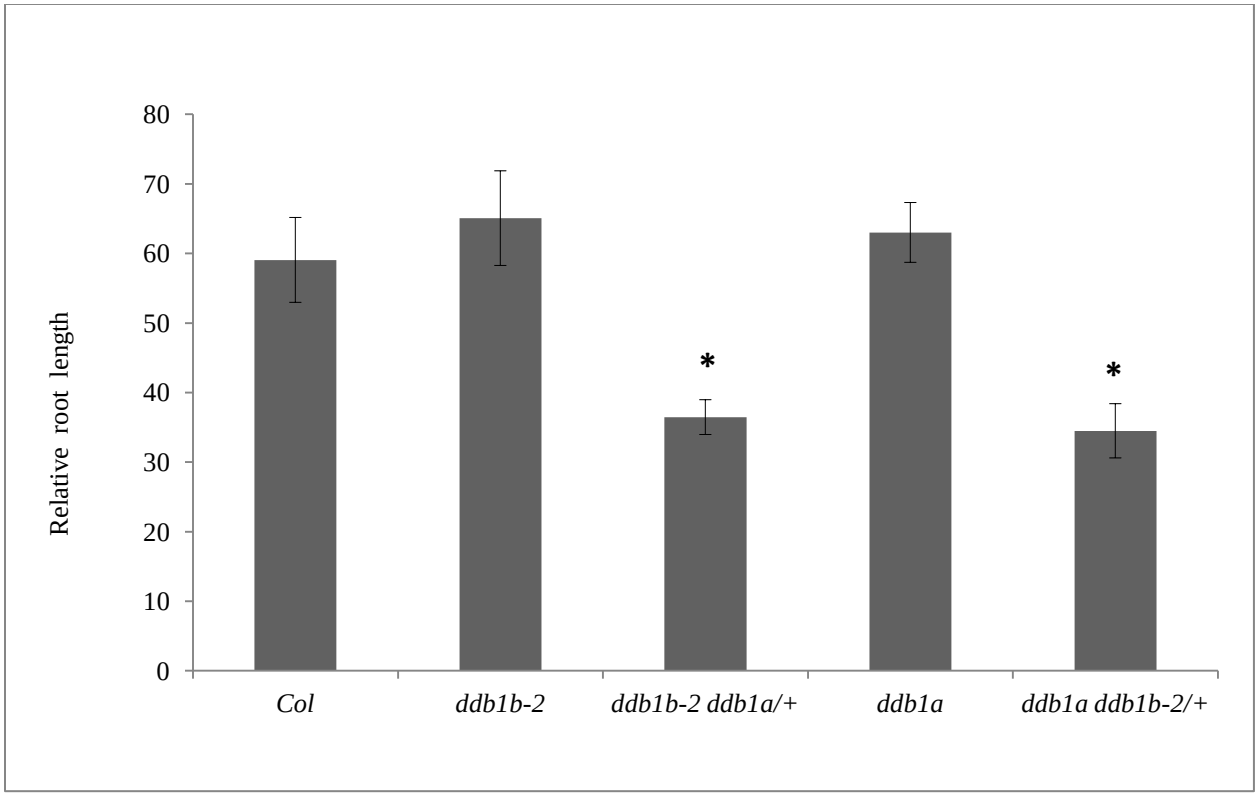


Figure 2.6 Abiotic Stress Response (Cont.)

D: UV Root Assay – Dark Treatment. Relative root length of *Col*, *ddb1b-2*, *ddb1a*, *ddb1b-2 ddb1a/+* and *ddb1a ddb1b/+* lines irradiated with UV-C (1500 $J.m^{-2}$).

Measurements were taken 3 days after dark incubation. Error bars indicate ±SE, (n=25)

* = P≤0.05, *ddb1b-2 ddb1a/+* relative to *ddb1b-2* and *ddb1a ddb1b-2/+* relative to *ddb1a*, *ddb1a* and *ddb1b-2* relative to *Col*.

E

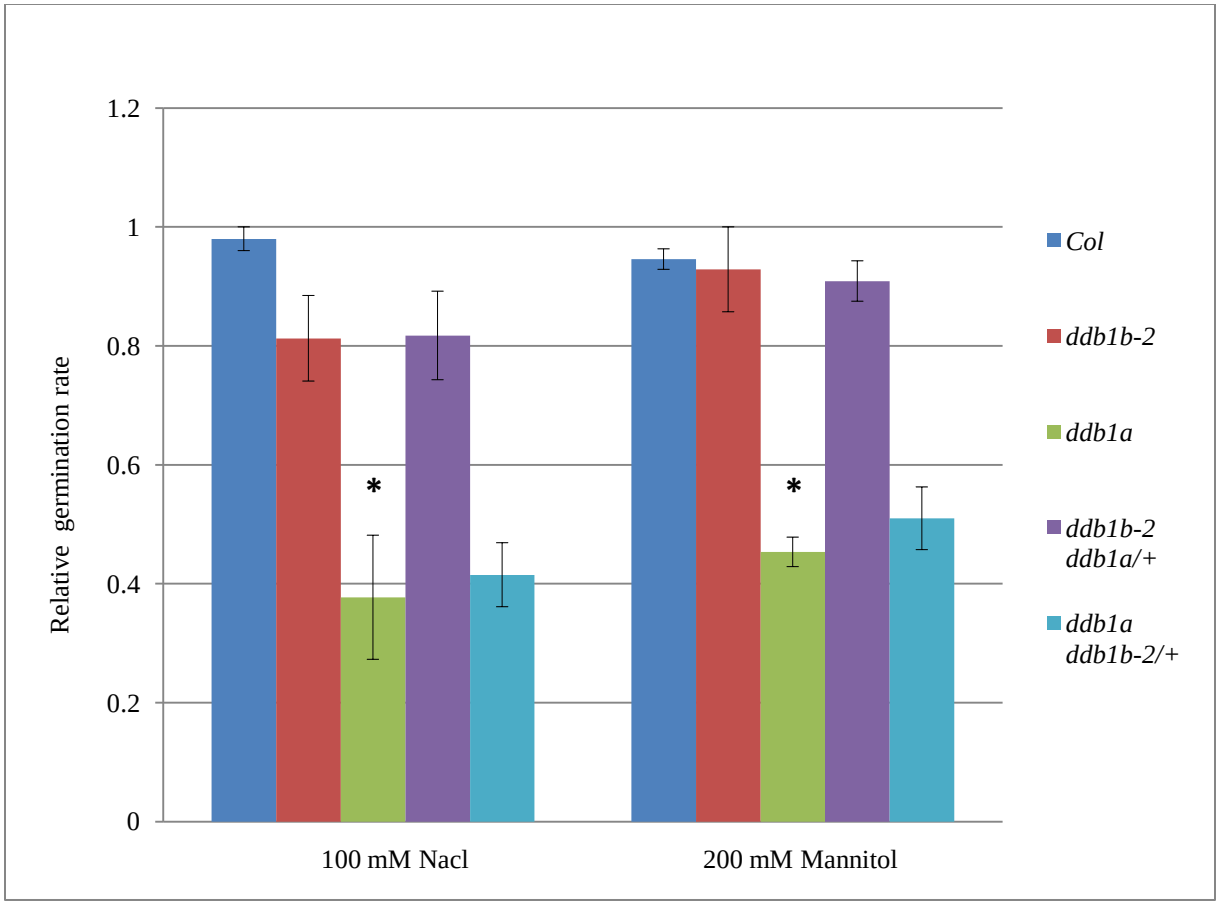


Figure 2.6 Abiotic Stress Response (Cont.)

E: Germination Assay**.** All seedlings were grown horizontally on germination medium plates supplemented with or without 100mM NaCl and 200mM Mannitol. Germination was scored 3 days after stratification and results represented as relative germination rate. Error bars indicate ±SE, (n=2, experimental repeats of 30-50 seedling each)

* = P≤0.05, *ddb1b-2 ddb1a/+* relative to *ddb1b-2* and *ddb1a ddb1b-2/+* relative to *ddb1a*, *ddb1a* and *ddb1b-2* relative to *Col*.

F

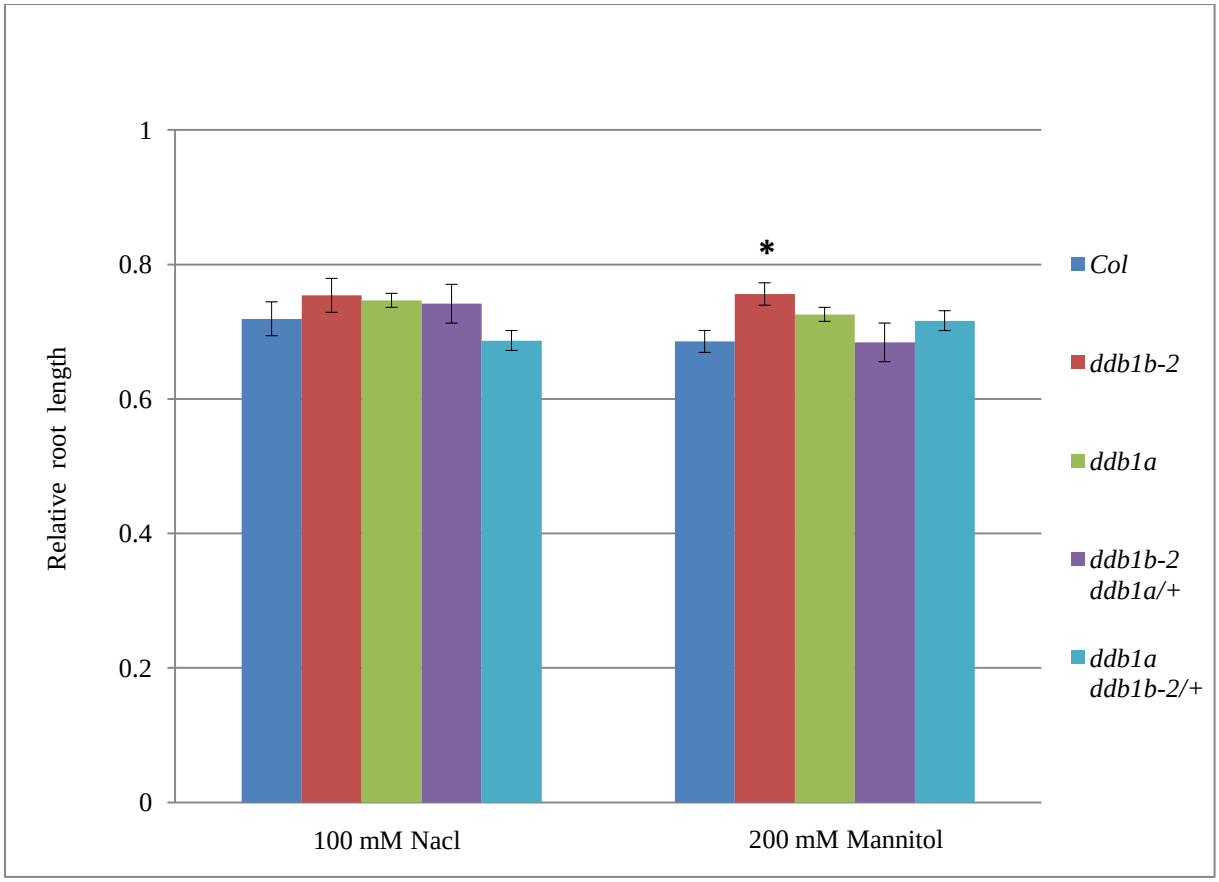


Figure 2.6 Abiotic Stress Response (Cont.)

F: Root Sensitivity Assay**.** All seedlings were grown vertically on germination medium plates supplemented with or without 100mM NaCl and 200mM Mannitol. Measurements were taken 5 days after stratification and results represented as relative root length. Error bars indicate ±SE, (n=10)

* = P≤0.05, *ddb1b-2 ddb1a/+* relative to *ddb1b-2* and *ddb1a ddb1b-2/+* relative to *ddb1a*, *ddb1a* and *ddb1b-2* relative to *Col.*

G

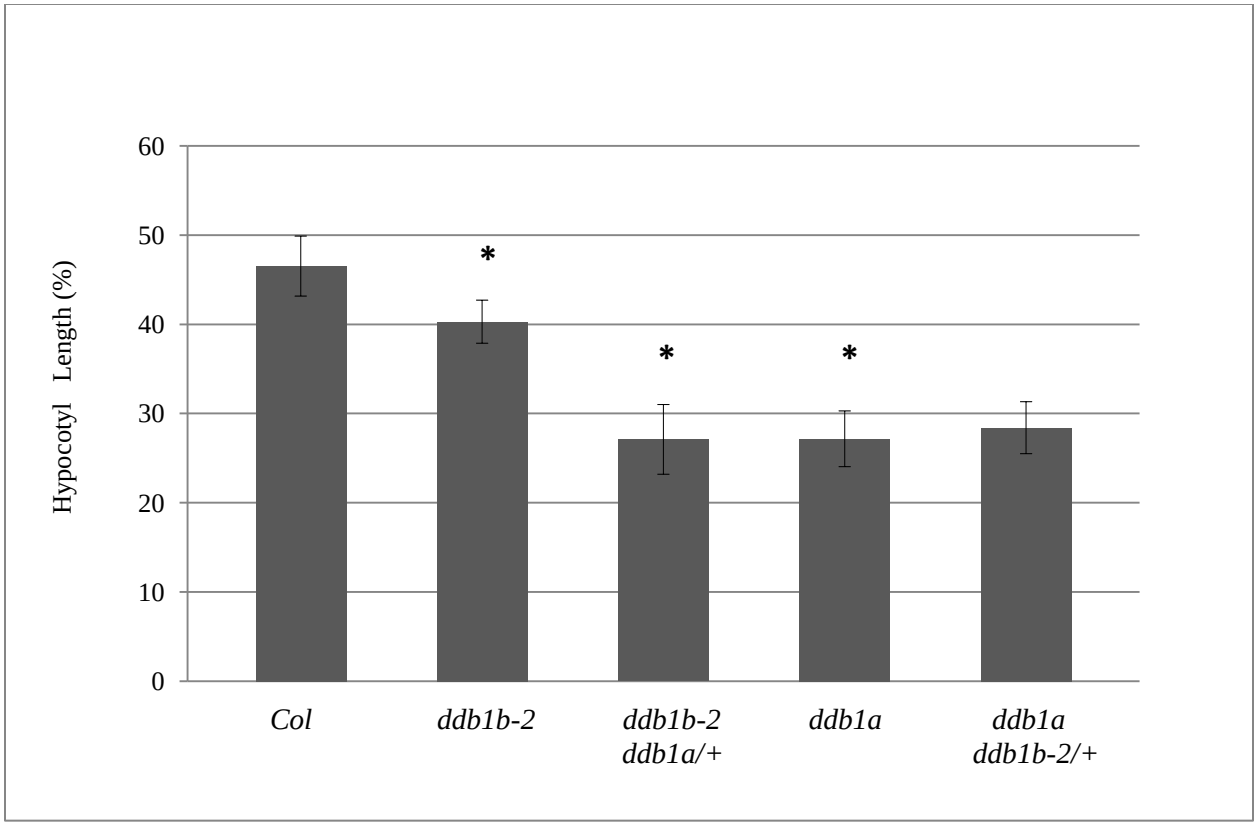


Figure 2.6 Abiotic Stress Response

G: Heat Sensitivity Assay. Relative hypocotyl length (%) of 4 day old dark grown seedlings treated with 45^{o} C for 4 hours, then dark grown at 20^{o} C for an additional 4 days. Hypocotyl length is relative to untreated controls of the same genotype. Error bars indicate ±SE, (n=15)

* = P≤0.05, *ddb1b-2 ddb1a/+* relative to *ddb1b-2* and *ddb1a ddb1b-2/+* relative to *ddb1a*, *ddb1a* and *ddb1b-2* relative to *Col-0*.

A

B

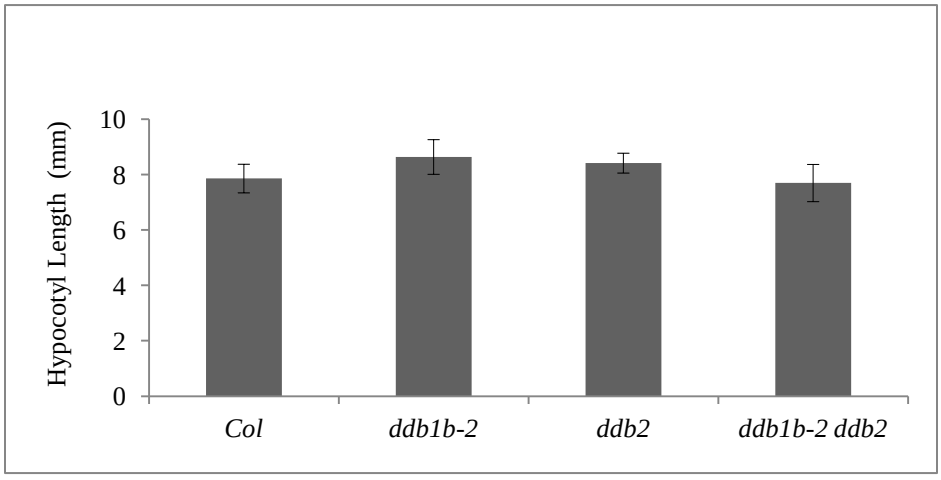


Figure 2.7 Phenotypic characterization of 7 day dark grown seedlings.

A: From left: *Col-0, ddb1b-2, ddb2, ddb1b-2 ddb2*

B: Hypocotyl Length (in mm)

Error bars indicate 95% C.I., (n=15), No significant differences were observed between *ddb1b-2 ddb2* relative to *ddb1b-2*, singles relative to *Col-0*

C

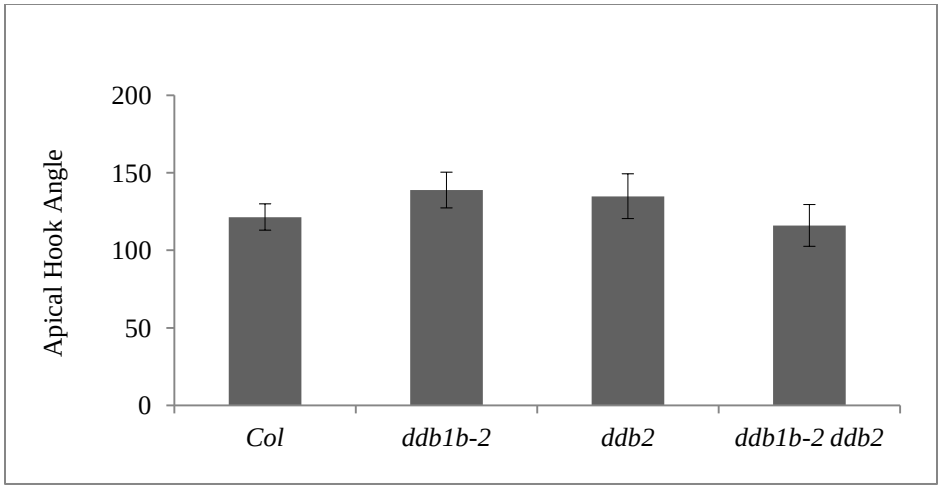


Figure 2.7 Phenotypic characterization of 7 day dark grown seedlings (Cont.)

C: Apical Hook Angle (in degrees)

Error bars indicate 95% C.I., (n=15). No significant differences were observed between *ddb1b-2 ddb2* relative to *ddb1b-2*, singles relative to *Col-0*

A

B

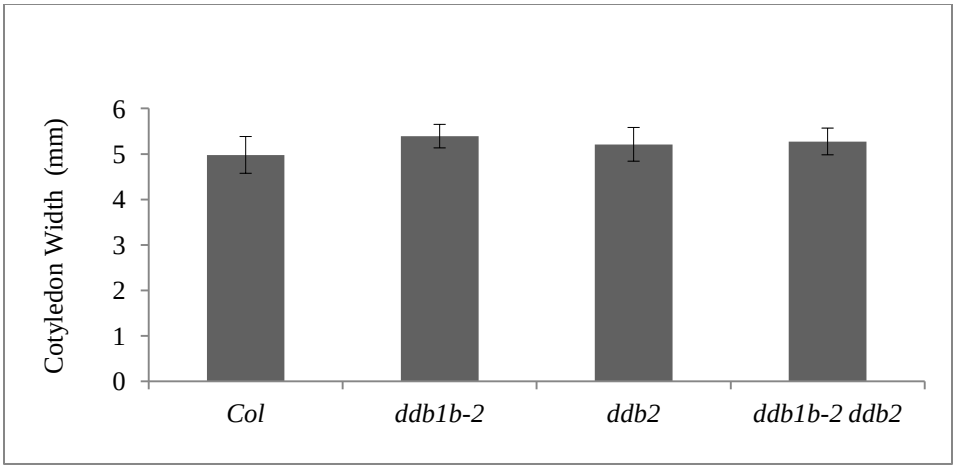


Figure 2.8 Phenotypic characterization of 7 day light grown seedlings.

A: From left: *Col-0, ddb1b-2, ddb2, ddb1b-2 ddb2*

B: Cotyledon Width (in mm)

Error bars indicate 95% C.I., (n=15). No significant differences were observed between *ddb1b-2 ddb2* relative to *ddb1b-2*, singles relative to *Col-0*

C.

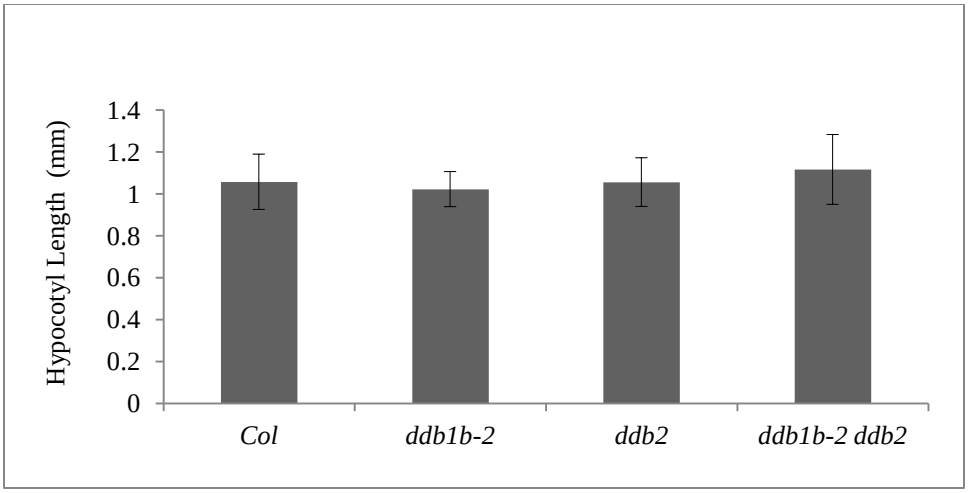


Figure 2.8 Phenotypic characterization of 7 day light grown seedlings (Cont.)

C: Hypocotyl length (mm)

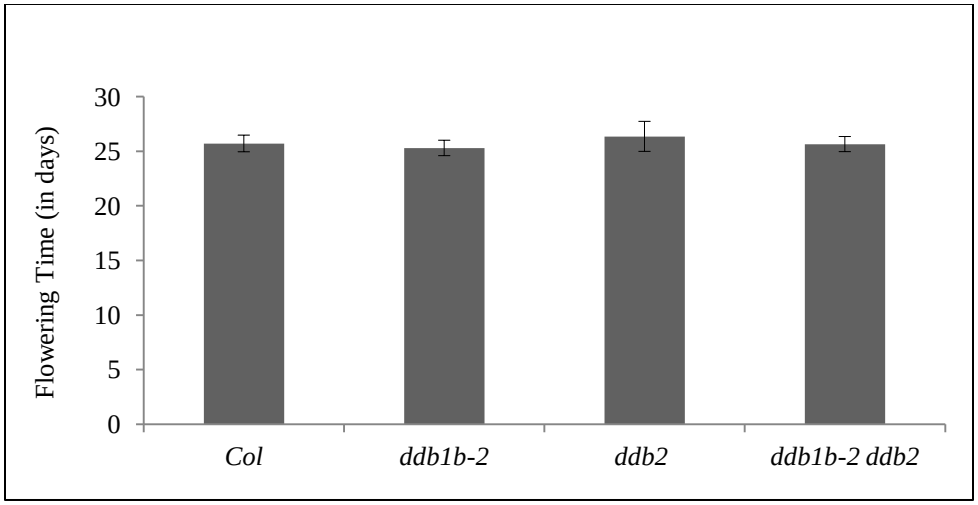
Error bars indicate 95% C.I., (n=15). No significant differences were observed between *ddb1b-2 ddb2* relative to *ddb1b-2*, singles relative to *Col-0*

A

Figure 2.9 Phenotypic characterization of Adult plants.

A: From left: *Col-0, ddb1b-2*, *ddb2* and *ddb1b-2 ddb2*

B



C

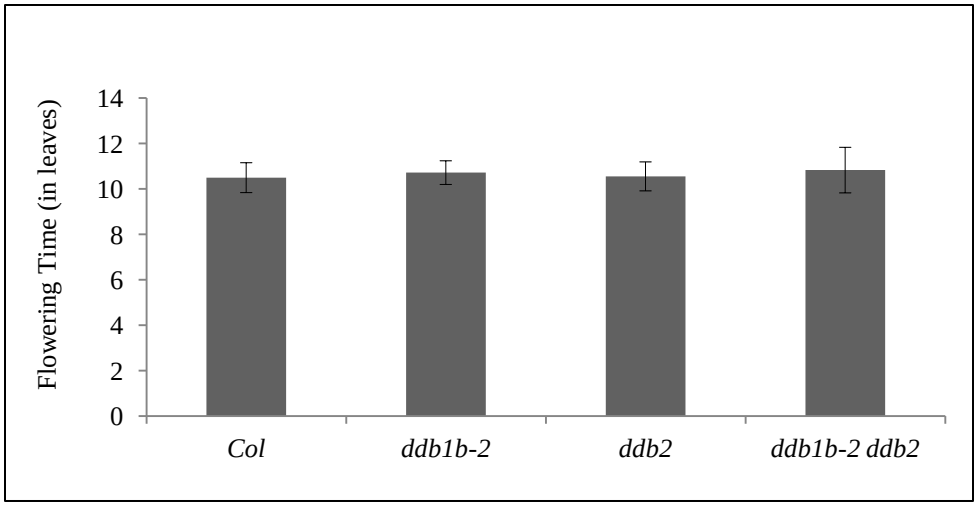
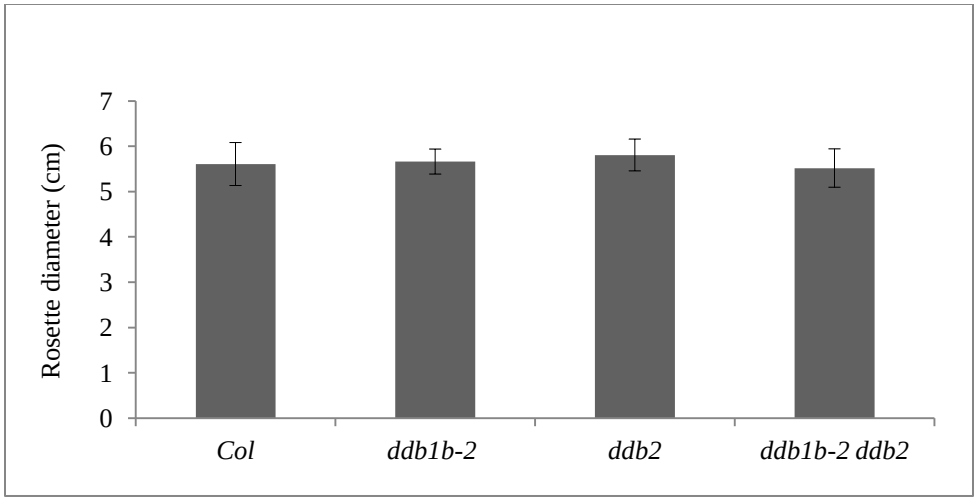


Figure 2.9 Adult Phenotypes (Cont.)

B: Flowering Time (in days); **C:** Flowering Time (in leaves)

Error bars indicate 95% C.I., (n=12). No significant differences were observed between *ddb1b-2 ddb2* relative to *ddb1b-2*, singles relative to *Col-0*

D



E

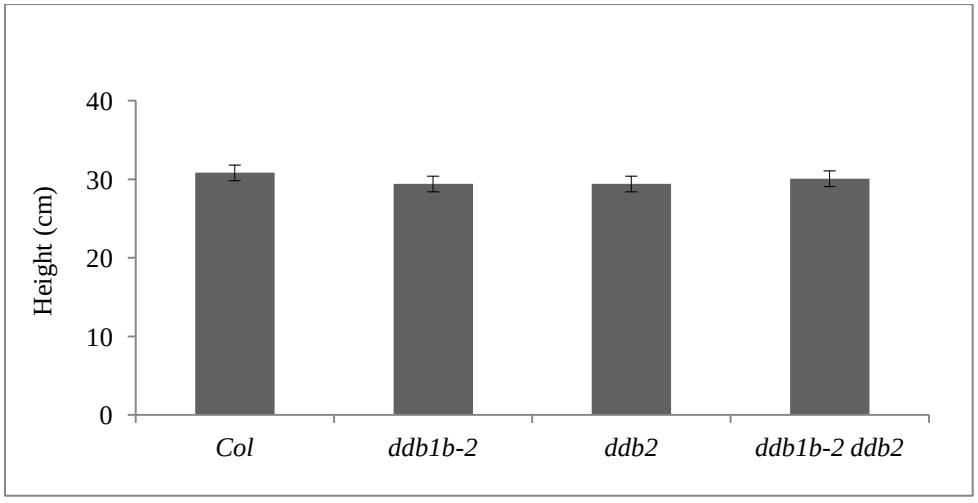
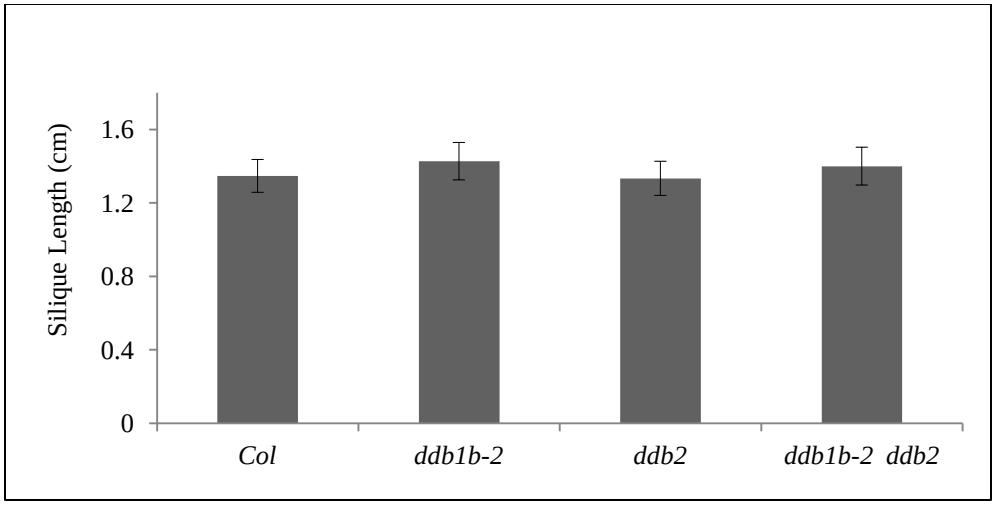


Figure 2.9 Adult Phenotypes (Cont.)

D: Rosette Diameter (in cm); **E:** Height (in mm)

Error bars indicate 95% C.I., (n=12). No significant differences were observed between *ddb1b-2 ddb2* relative to *ddb1b-2*, singles relative to *Col-0*

F



G

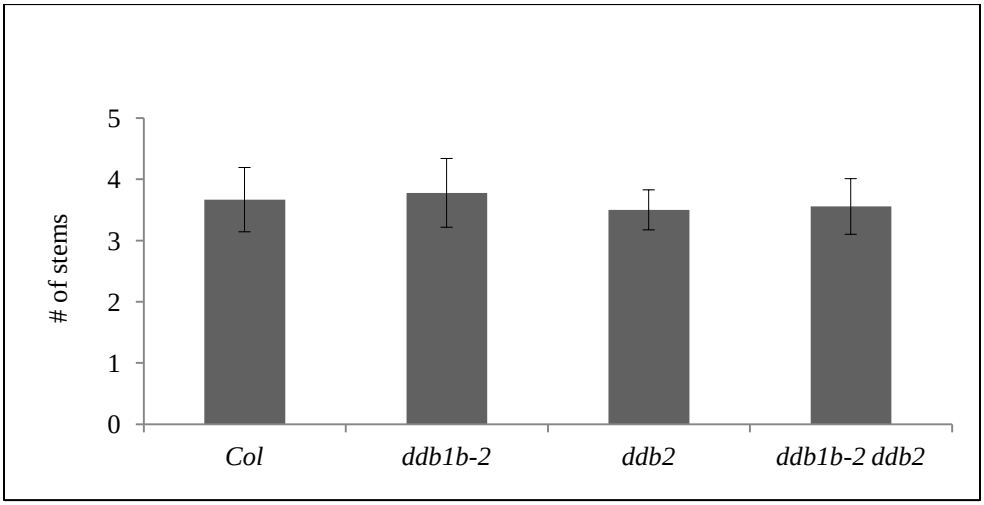
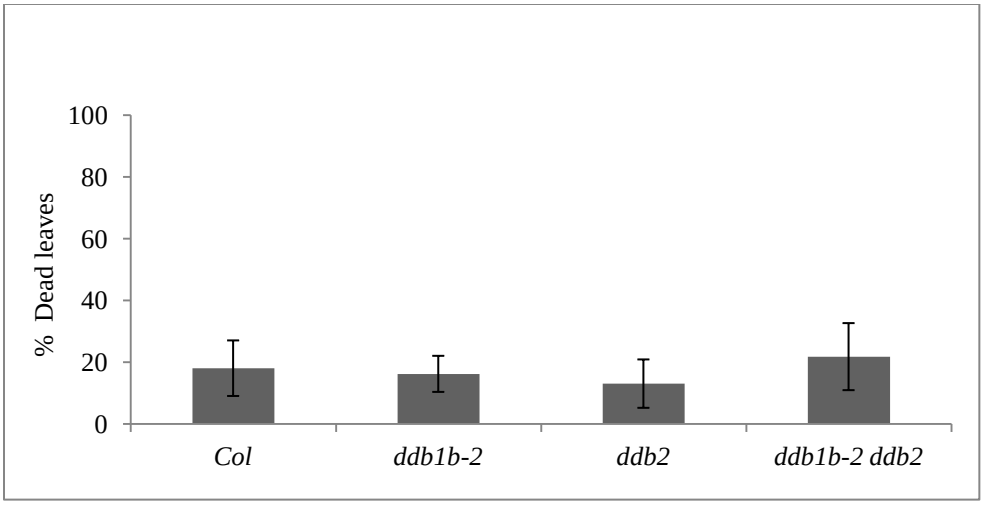


Figure 2.9 Adult Phenotypes (cont.)

F: Silique length (in cm); **G:** # of stems

Error bars indicate 95% C.I., (n=12). No significant differences were observed between *ddb1b-2 ddb2* relative to *ddb1b-2*, singles relative to *Col-0*

A



B

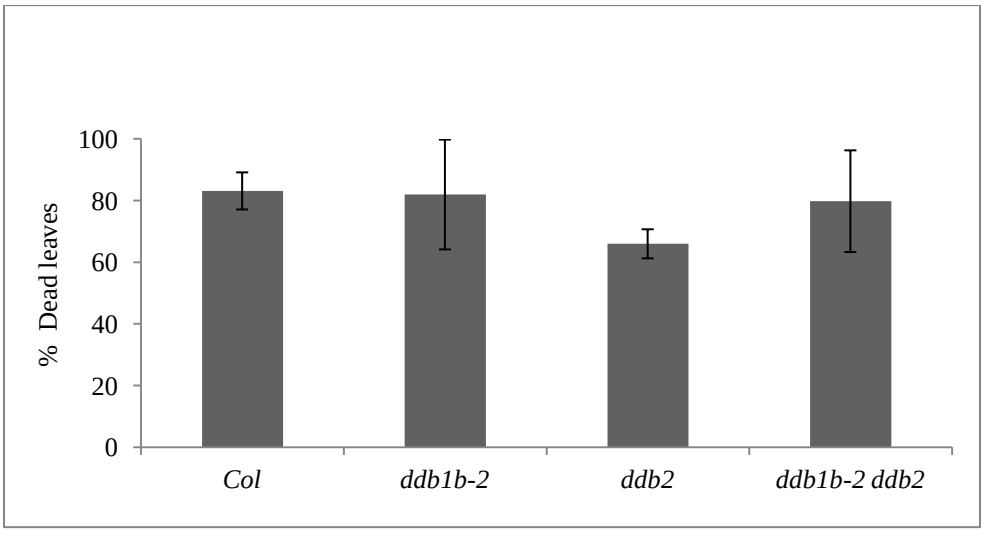


Figure 2.10 Abiotic Stress UV Shoot Assay

A: Statistical representation of % leaf damage at UV-C irradiation of 225 Jm $^{-2}$.

B: Statistical representation of % leaf damage at UV-C irradiation of 450 Jm $^{-2}$.

Error bars indicate 95% C.I., (n=12). No significant differences were observed between *ddb1b-2 ddb2* relative to *ddb1b-2*, singles relative to *Col-0*

C

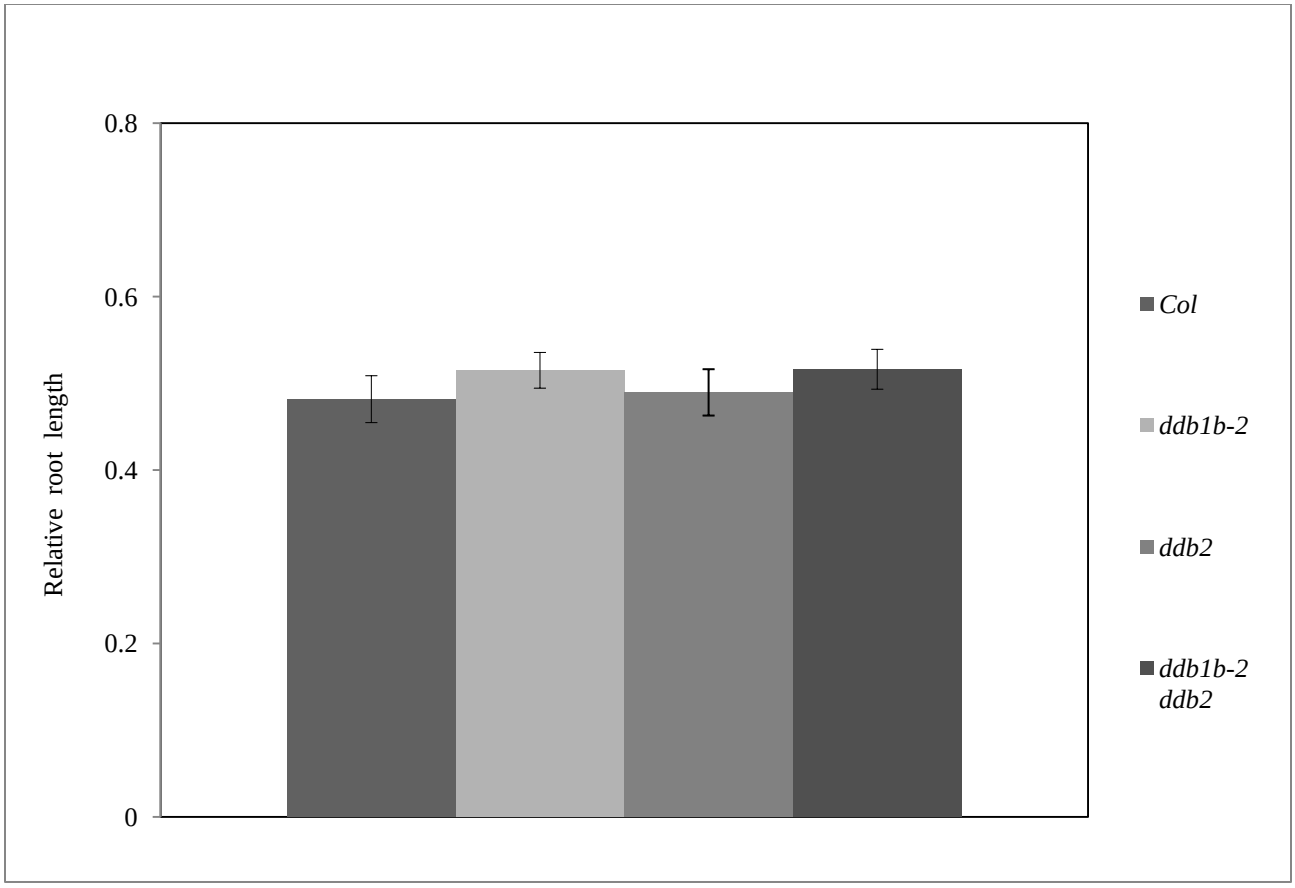


Fig 2.10 Abiotic Stress UV Root Assay – Dark Treatment

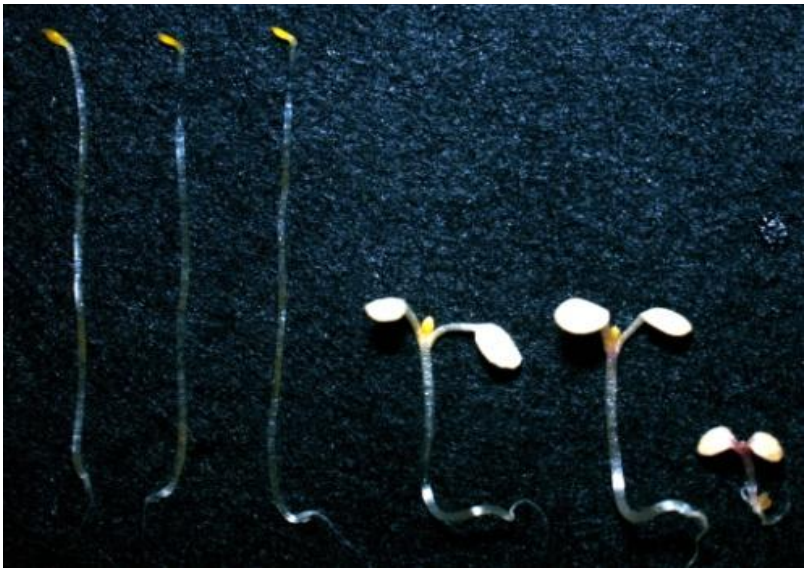
C: Relative root length of *Col*, *ddb1b-2*, *ddb2* and *ddb1b-2 ddb2* irradiated with 600 J m^{-2} UV-C and then grown under dark conditions. Measurements were taken 24 hours post irradiation.

Data is expressed as root length relative to un-irradiated control of the corresponding genotype.

Error bars indicate ±SE, (n=25)

No significant differences were observed between *ddb1b-2 ddb2* relative to *ddb1b-2*, singles relative to *Col-0*

A



B

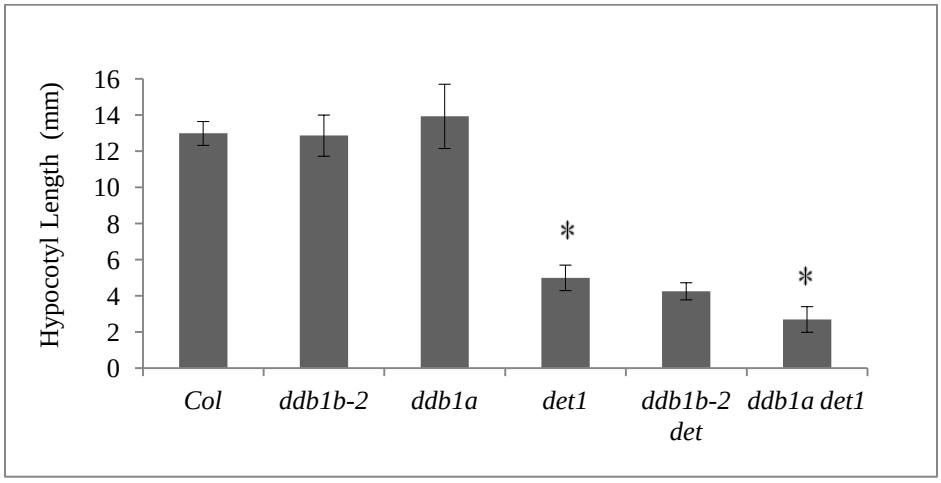


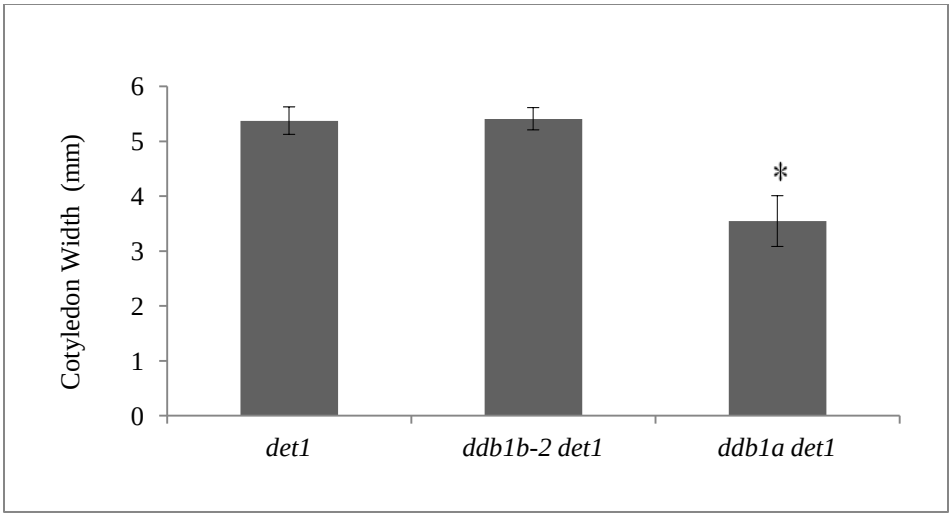
Figure 2.11 Phenotypic characterization of 7 day dark grown seedlings.

A: From left: *Col-0, ddb1b-2, ddb1a, ddb1b-2 det1,* and *ddb1a det1*

B: Hypocotyl Length (in mm)

Error bars indicate 95% C.I., (n=15). * = P≤0.05. *ddb1b-2 det1* and *ddb1a det1* relative to *det1*, single mutants relative to *Col-0*.

C



D

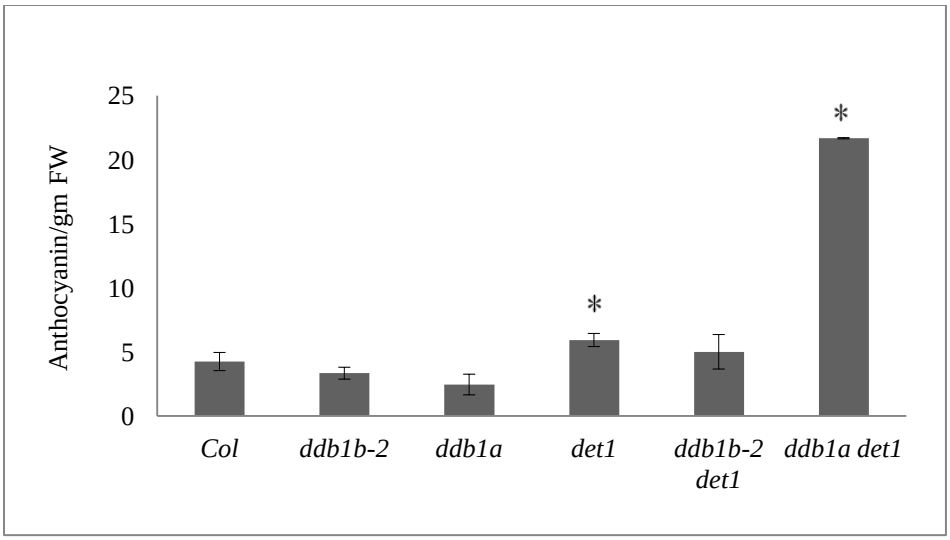


Figure 2.11 Phenotypic characterization of 7 day dark grown seedlings (Cont.)

C: Cotyledon Width (mm)

D: Anthocyanin content /gm FW (n=2)

Error bars indicate 95% C.I., * = P≤0.05

ddb1b-2 det1 and ddb1a det1 relative to *det1*, single mutants relative to *Col-0*.

A

B

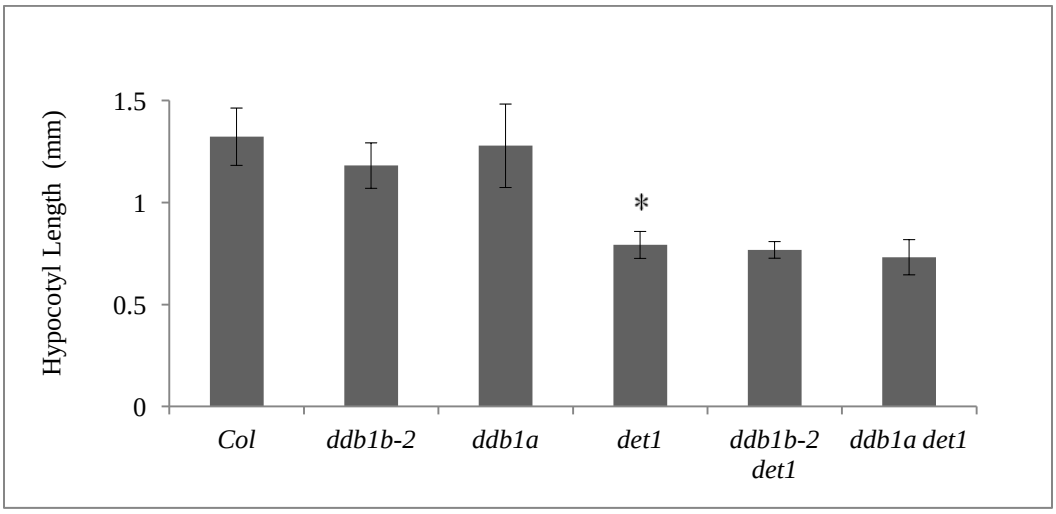


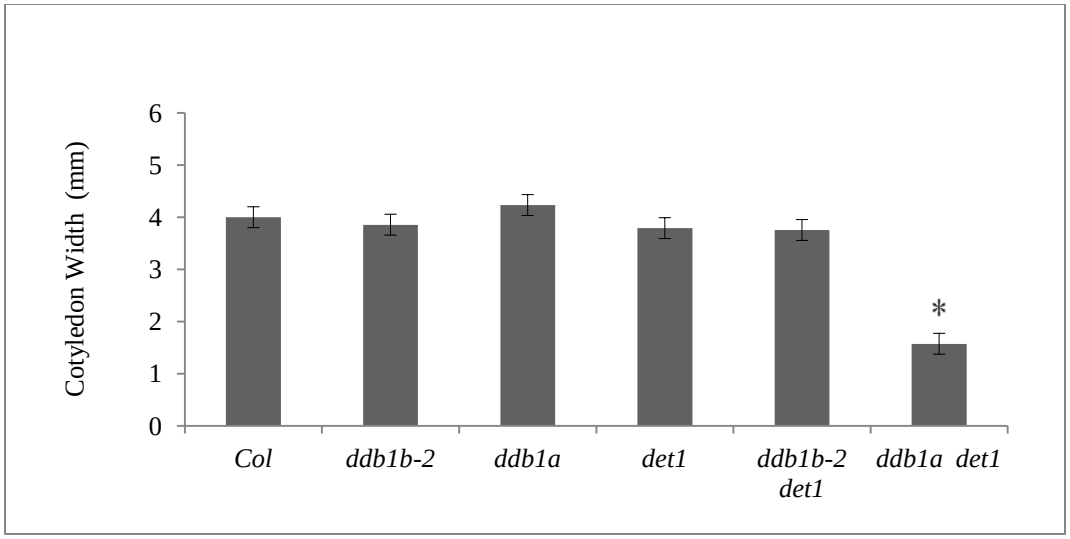
Figure 2.12 Phenotypic characterization of 7 day light grown seedlings.

A: From left: *Col-0, ddb1b-2, ddb1a, ddb1b-2 det1,* and *ddb1a det1*

B: Hypocotyl Length (in mm)

Error bars indicate 95% C.I., (n=15), * = P ≤0.05, *ddb1b-2 det1* and *ddb1a det1* relative to *det1*, single mutants relative to *Col-0*

C



D

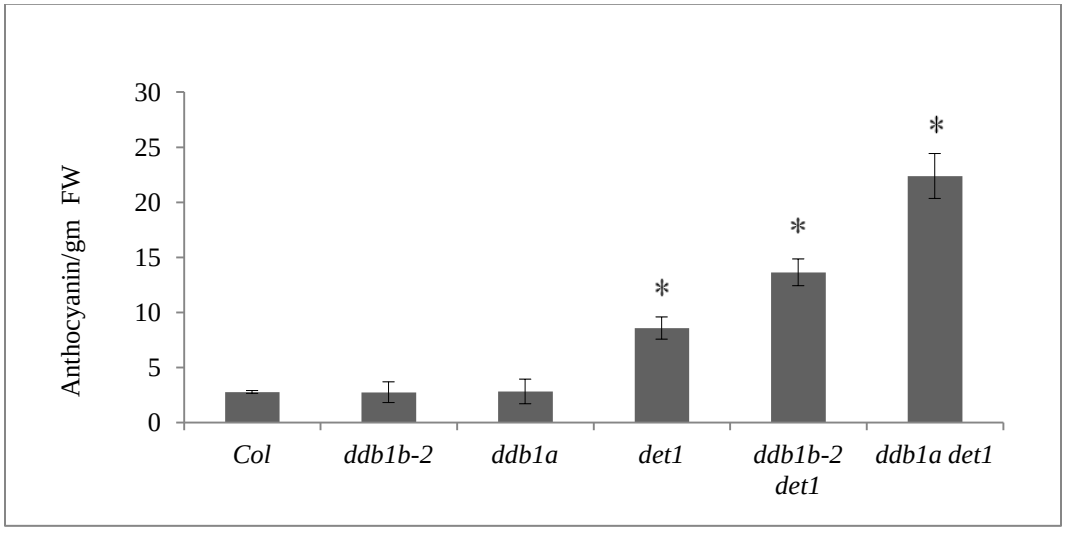


Figure 2.12 Phenotypic characterization of 7 day light grown seedlings (Cont.)

C: Cotyledon Width (in mm); **D:** Anthocyanin Content / gm FW (n=2)

Error bars indicate 95% C.I., * = P ≤ 0.05; *ddb1b-2 det1* and *ddb1a det1* relative to *det1*, single mutants relative to *Col-0*.

E

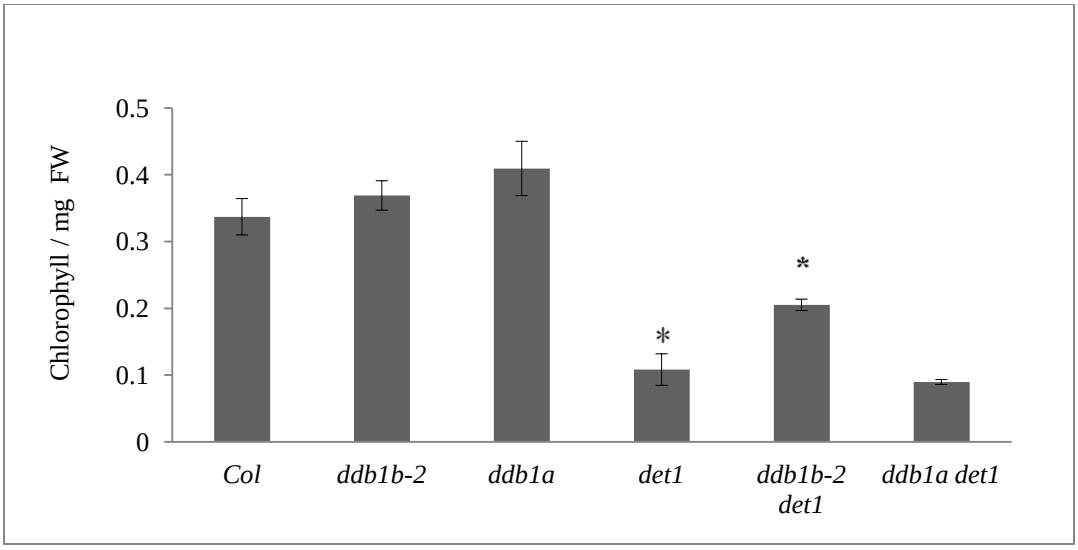


Figure 2.12 Phenotypic characterization of 7 day light grown seedlings (Cont.)

E: Chlorophyll content (µg chlorophyll / mg FW)

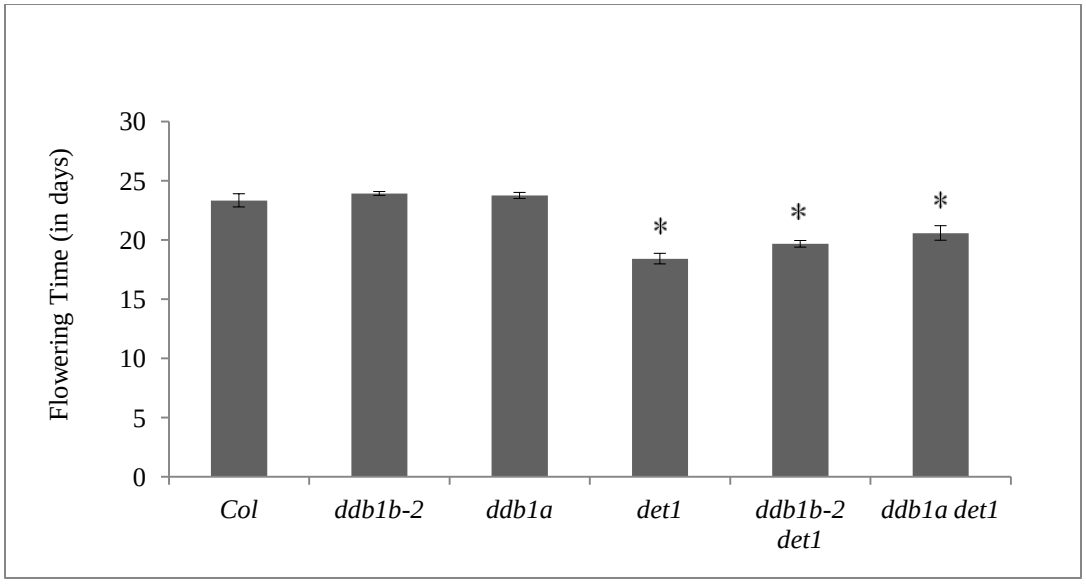
Error bars indicate 95% C.I., (n=2), * = $P \leq 0.05$, *ddb1b-2 det1* and *ddb1a det1* relative to *det1*, single mutants relative to *Col-0*.

A

Figure 2.13 Phenotypic characterization of Adult plants.

A: From left: *Col-0, ddb1b-2, ddb1a, det1, ddb1b-2 det1,* and *ddb1a det1*

B



C

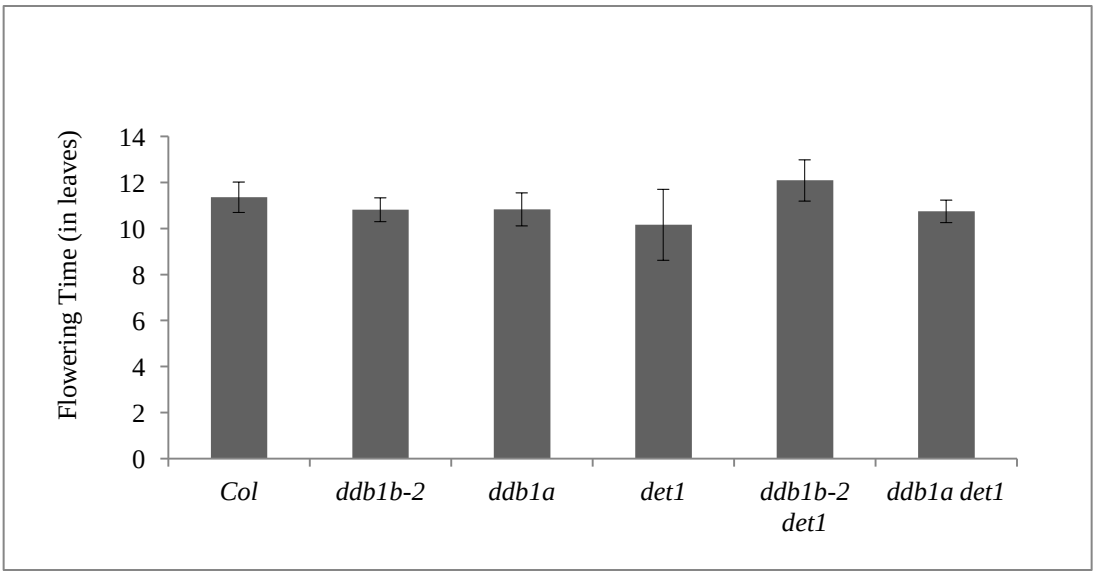
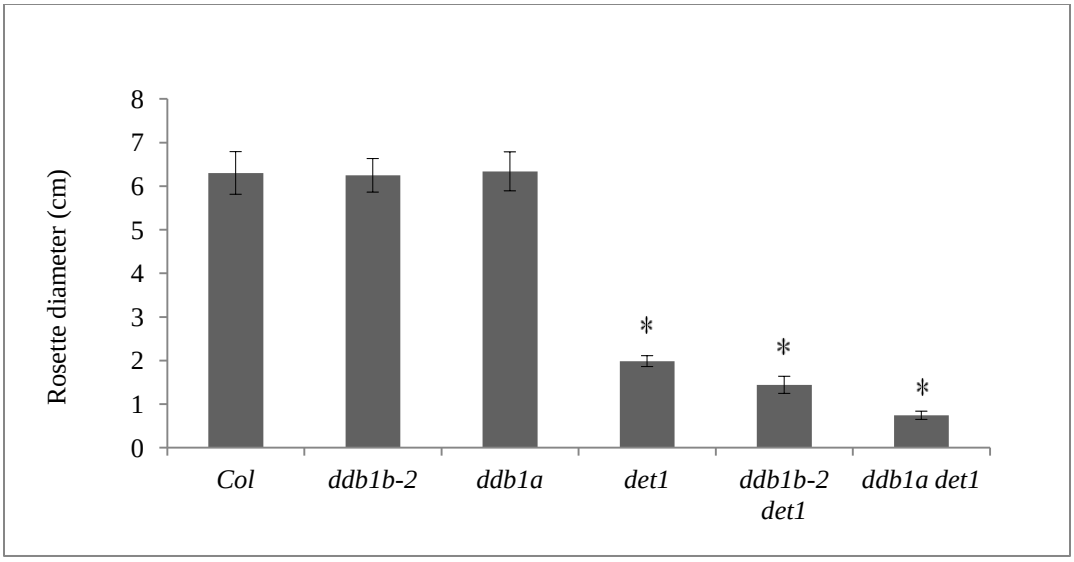


Figure 2.13 Adult Phenotypes (Cont.)

B: Flowering time (in days); **C:** Flowering Time (in leaves)

Error bars indicate 95% C.I., (n=12), * = P ≤ 0.05, *ddb1b-2 det1* and *ddb1a det1* relative to *det1*, single mutants relative to *Col-0*

D



E

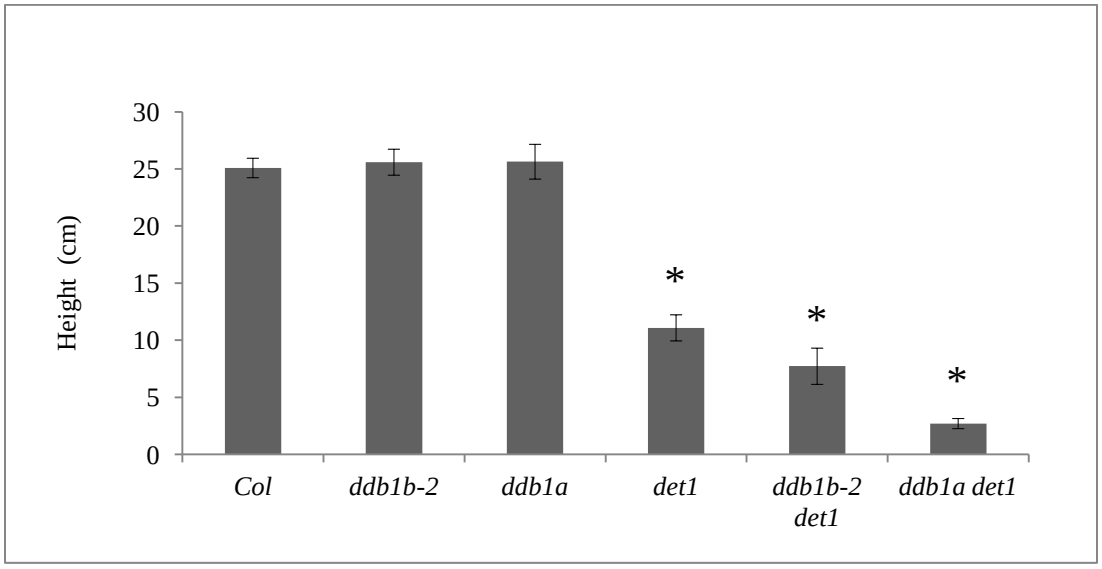
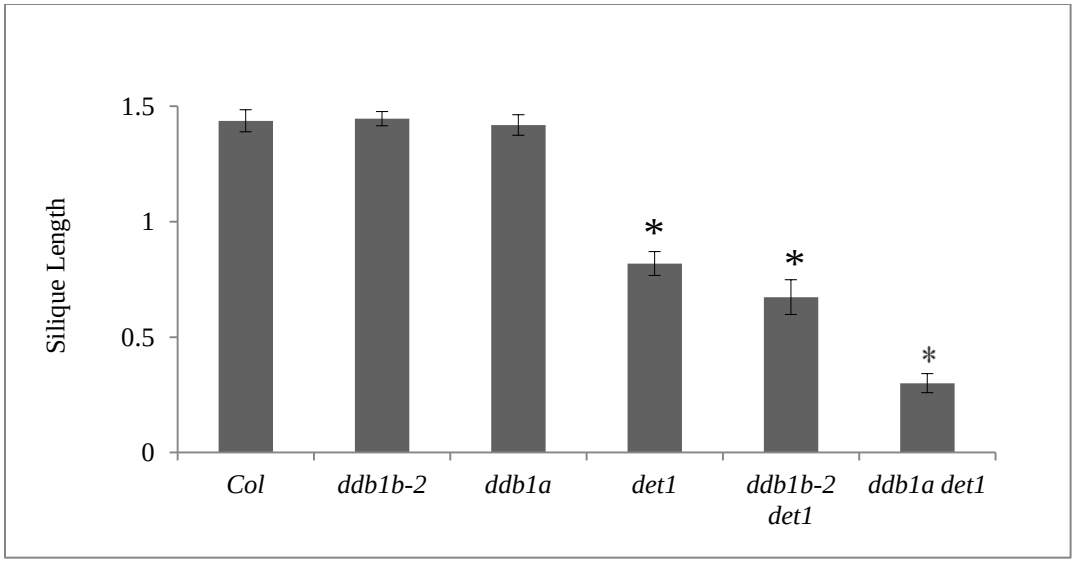


Figure 2.13 Adult Phenotypes (Cont.)

D: Rosette Diameter (in cm); **E:** Height (in cm)

Error bars indicate 95% C.I., (n=12), * = $P \leq 0.05$, *ddb1b-2 det1* and *ddb1a det1* relative to *det1*, single mutants relative to *Col-0*

F



G

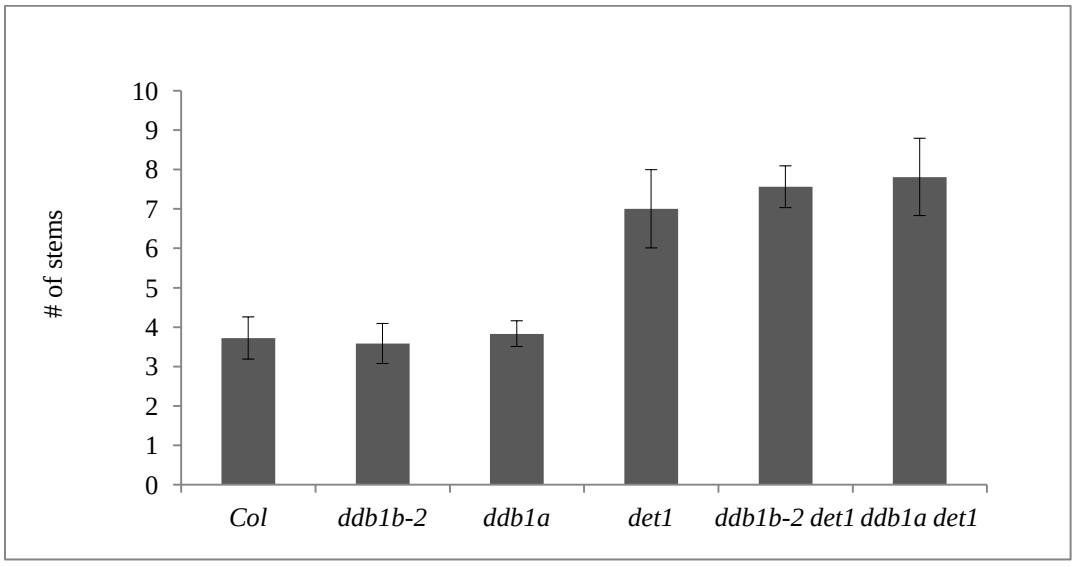
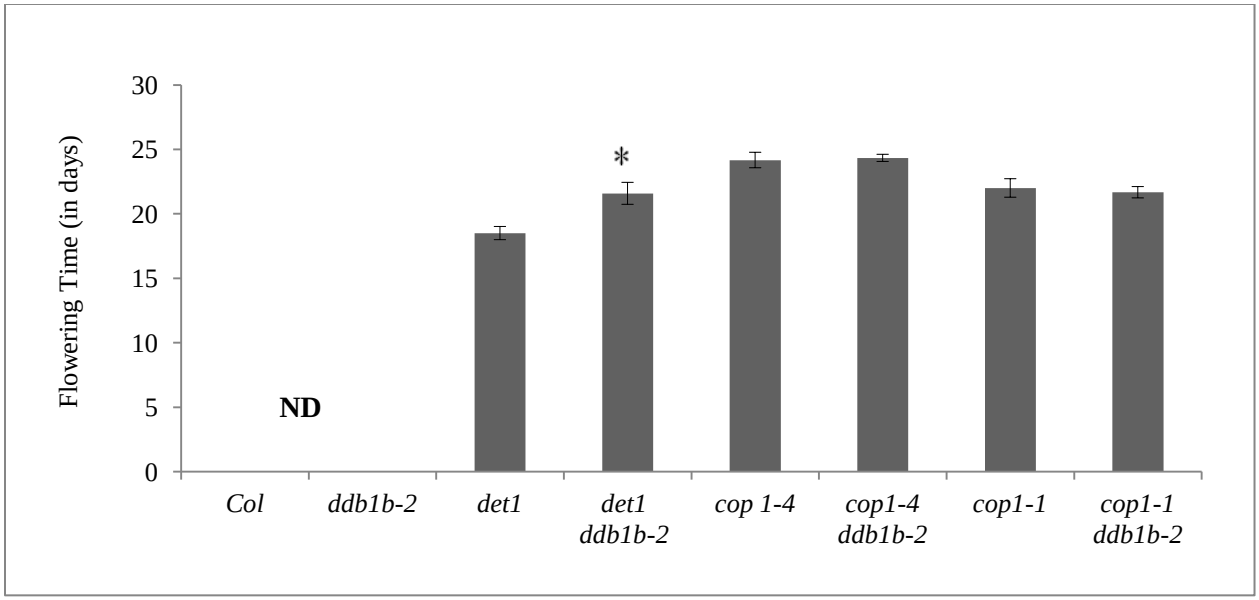


Figure 2.13 Adult Phenotypes (Cont.)

F: Silique length (in cm); **G:** # of stems

Error bars indicate 95% C.I., (n=12), * = $P \leq 0.05$, *ddb1b-2 det1* and *ddb1a det1* relative to *det1*, single mutants relative to *Col-0*

A



B

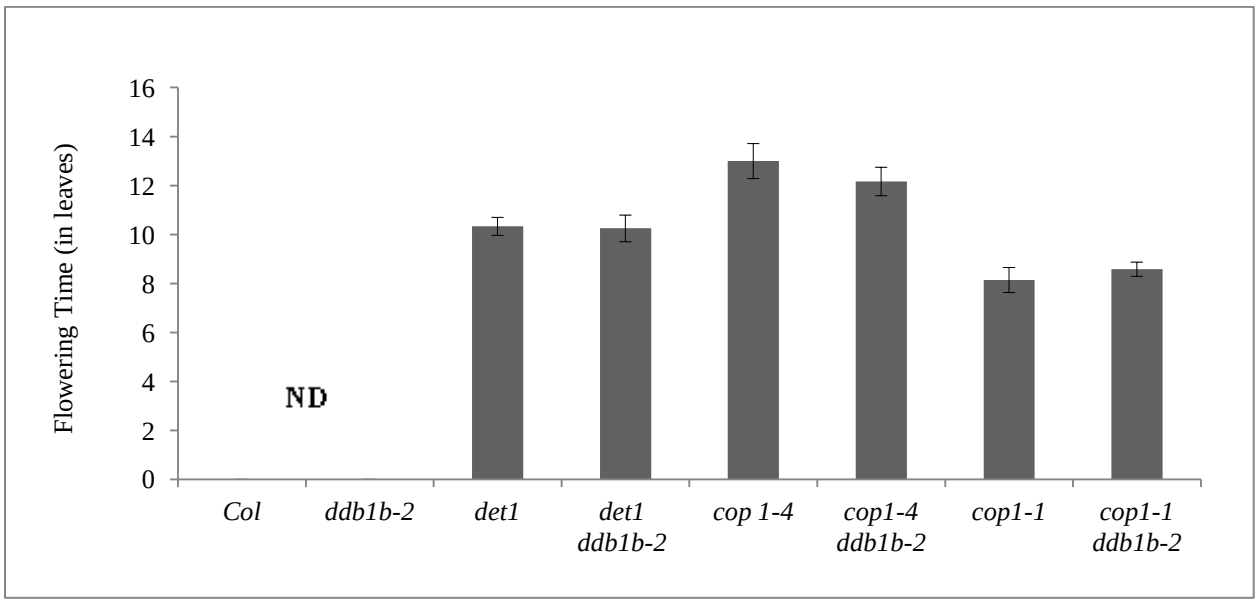
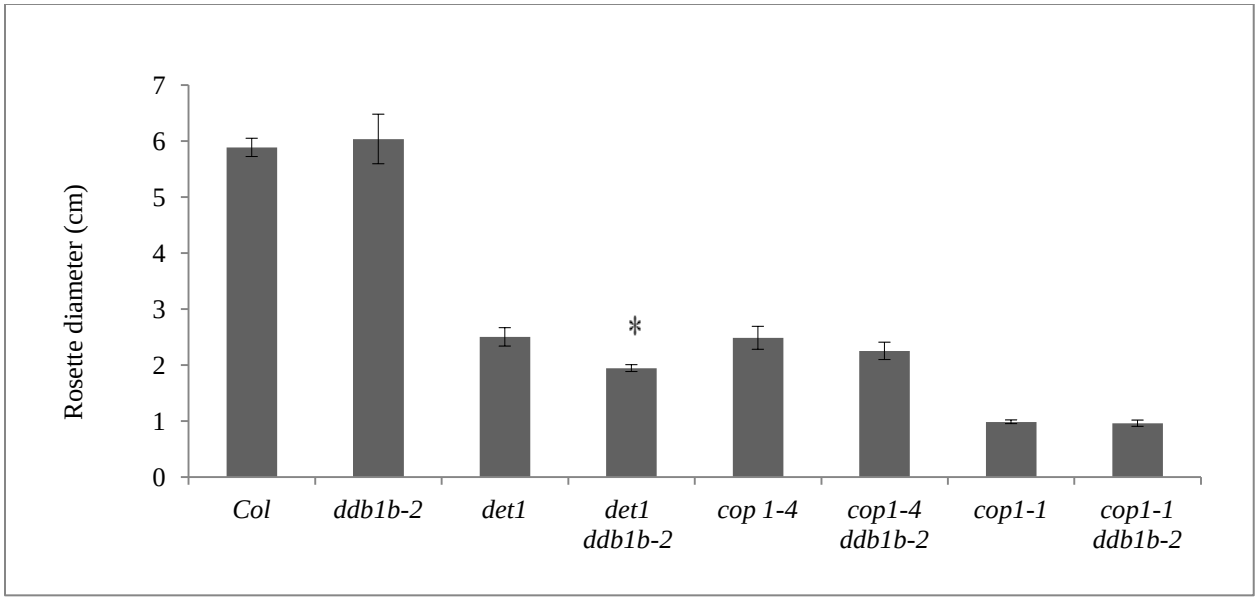


Figure 2.14 Short Day Adult Phenotypes

A: Flowering time (in days); **B:** Flowering Time (in leaves)

Error bars indicate 95% C.I., (n=12), * = $P \leq 0.05$, *ddb1b-2 det1*, *ddb1b-2 cop1-1* and *ddb1b-2 cop1-4* relative to *det1*, *cop1-1* and *cop1-4* respectively. ND = Not Determined

C



D

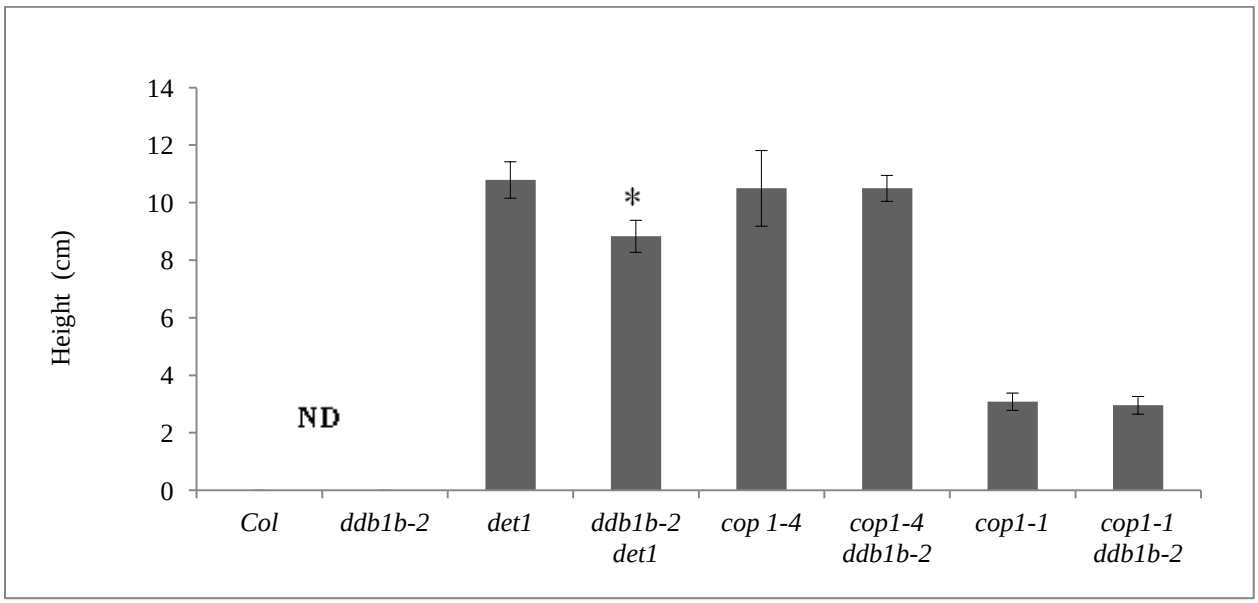
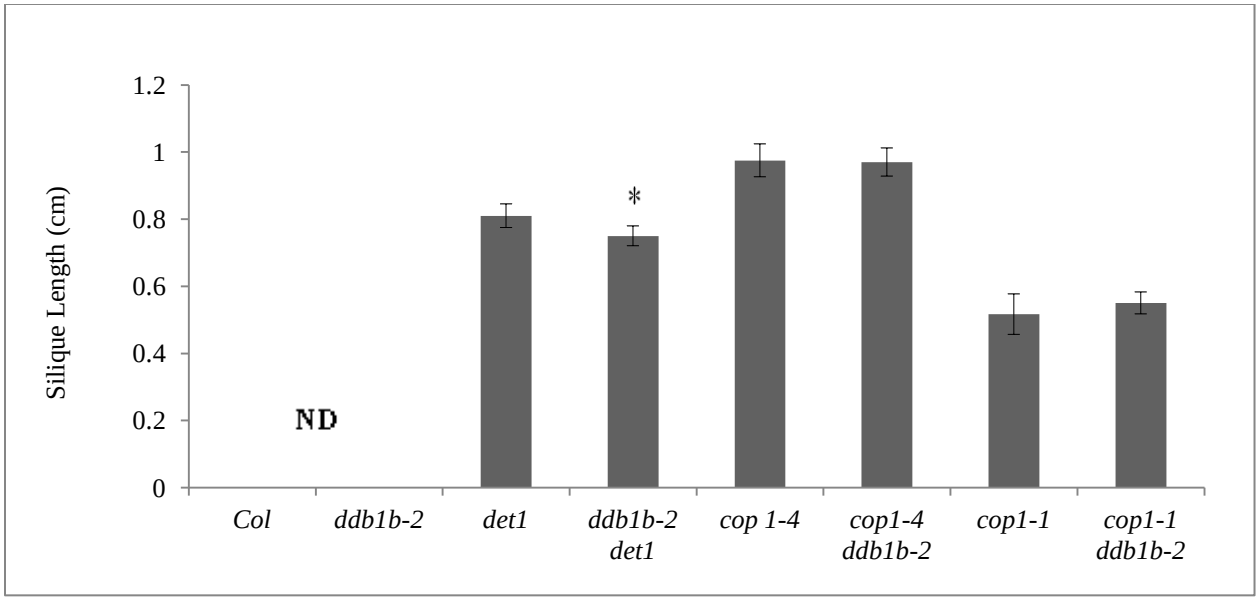


Figure 2.14 Short Day Adult Phenotypes (Cont.)

C: Rosette Diameter (cm); **D:** Height (cm)

Error bars indicate 95% C.I., (n=12), * = $P \leq 0.05$, *ddb1b-2 det1*, *ddb1b-2 cop1-1* and *ddb1b-2 cop1-4* relative to *det1*, *cop1-1* and *cop1-4* respectively. ND = Not Determined

E



F

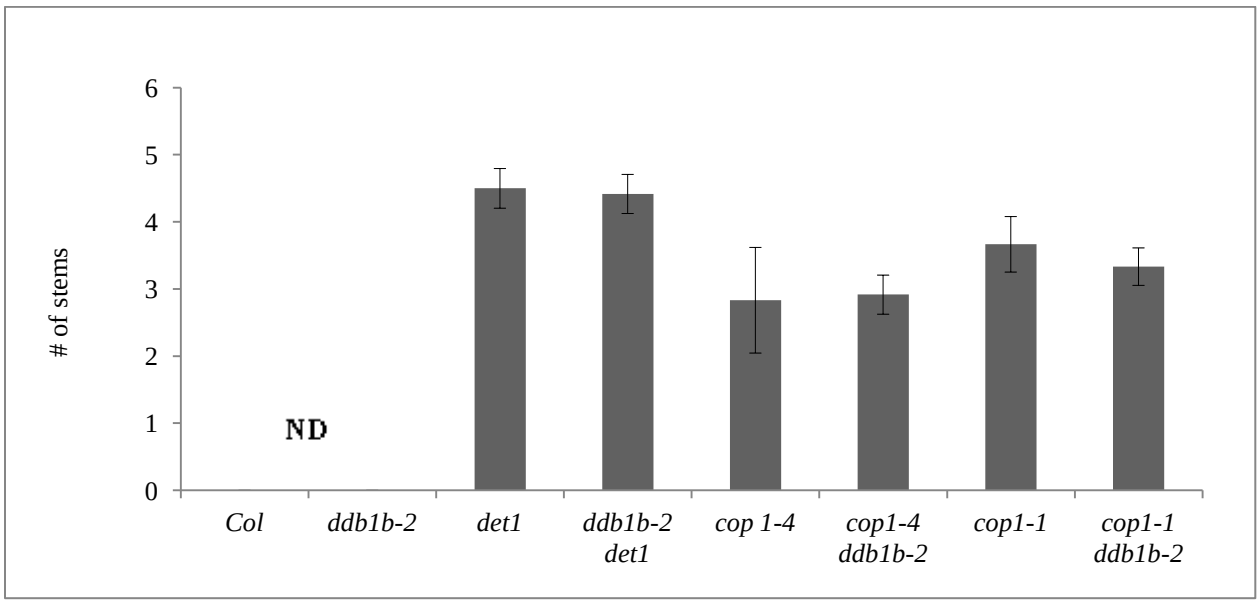
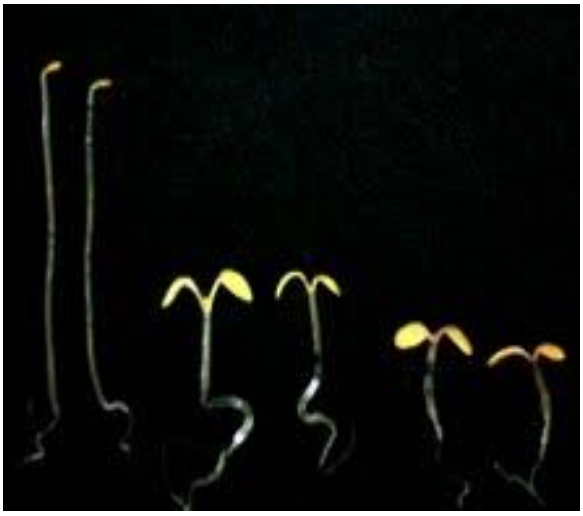


Figure 2.14 Short Day Adult Phenotypes (Cont.)

E: Silique Length (cm); **F:** # of stems

Error bars indicate 95% C.I., (n=12), * = $P \leq 0.05$, *ddb1b-2 det1*, *ddb1b-2 cop1-1* and *ddb1b-2 cop1-4* relative to *det1*, *cop1-1* and *cop1-4* respectively. ND = Not Determined

A



B

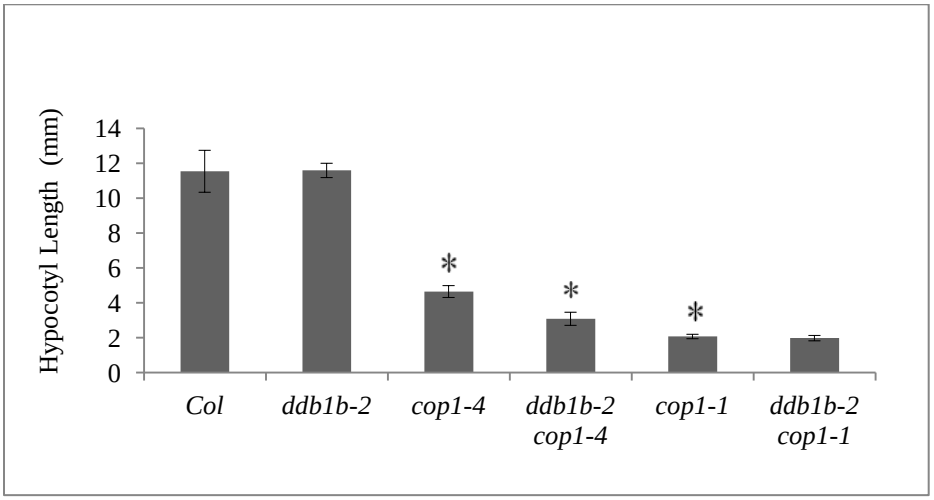


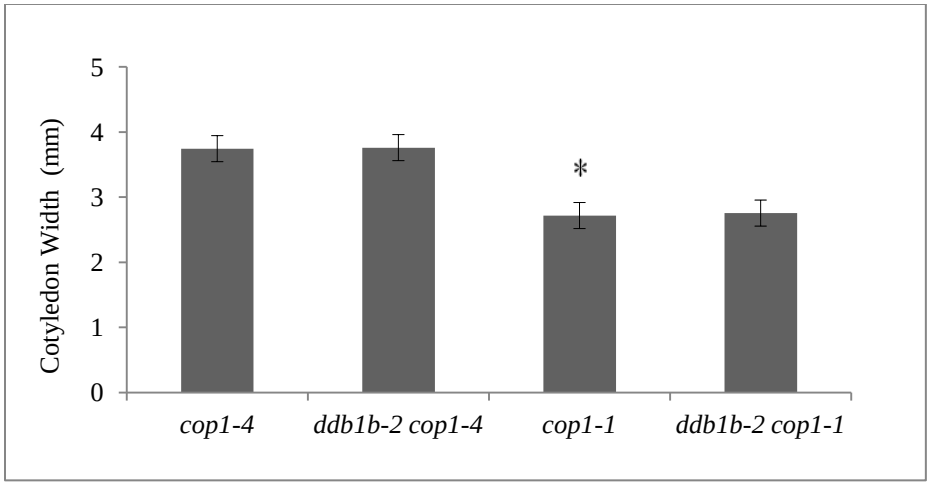
Figure 2.15 Phenotypic characterization of 7 day dark grown seedlings

A: From left: *Col-0*, *ddb1b-2*, *cop1-4*, *ddb1b-2 cop1-4*, *cop1-1*, and *ddb1b-2 cop1-1*

B: Hypocotyl Length (in mm)

Error bars indicate 95% C.I., (n=12), * = P ≤0.05, *ddb1b-2 cop1-4* and *ddb1b-2 cop1-1* relative to *cop1-1* and *cop1-4* respectively, single mutants relative to *Col-0*.

C



D

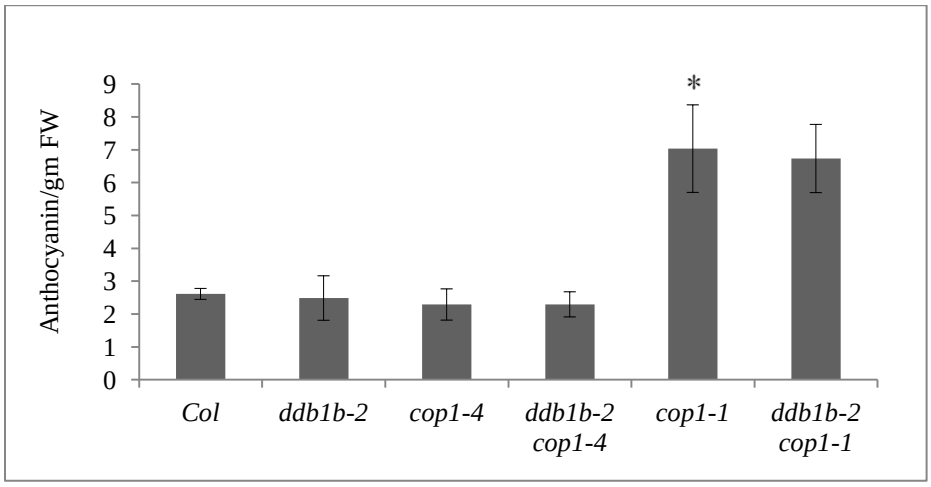


Figure 2.15 Phenotypic characterization of 7 day dark grown seedlings (Cont.)

C: Cotyledon Width (in mm); **D:** Anthocyanin Content / gm FW (n=2)

Error bars indicate 95% C.I., *ddb1b-2 cop1-4* and *ddb1b-2 cop1-1* relative to *cop1-1* and *cop1-4* respectively, single mutants relative to *Col-0*.

A

B

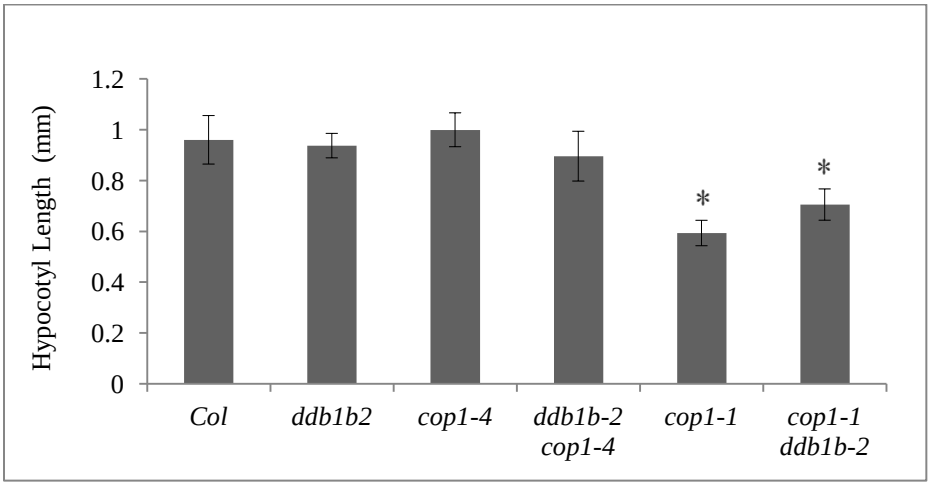


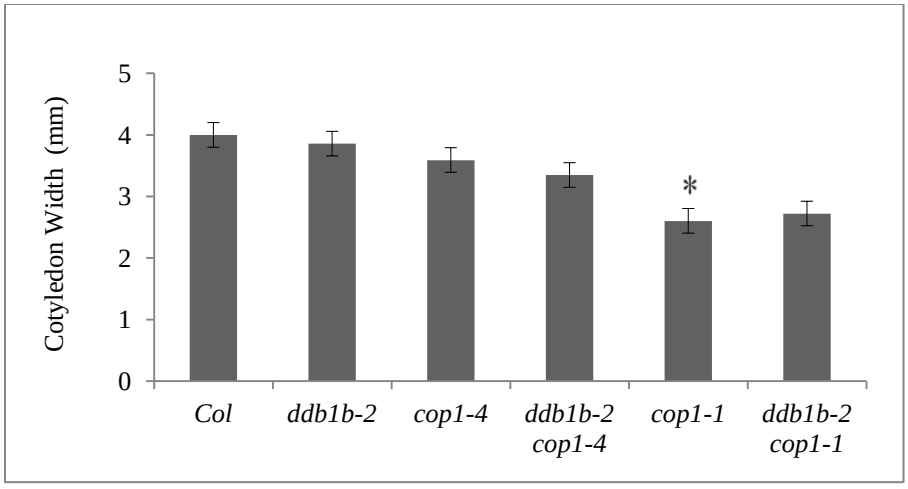
Figure 2.16 Phenotypic characterization of 7 day light grown seedlings.

A: From left: *Col-0, ddb1b-2, cop1-4, ddb1b-2 cop1-4, cop1-1,* and *ddb1b-2 cop1-1*

B: Hypocotyl Length (in mm)

Error bars indicate 95% C.I., (n=15), * = P ≤0.05, *ddb1b-2 cop1-4* and *ddb1b-2 cop1-1* relative to *cop1-1* and *cop1-4* respectively, single mutants relative to *Col-0*.

C



D

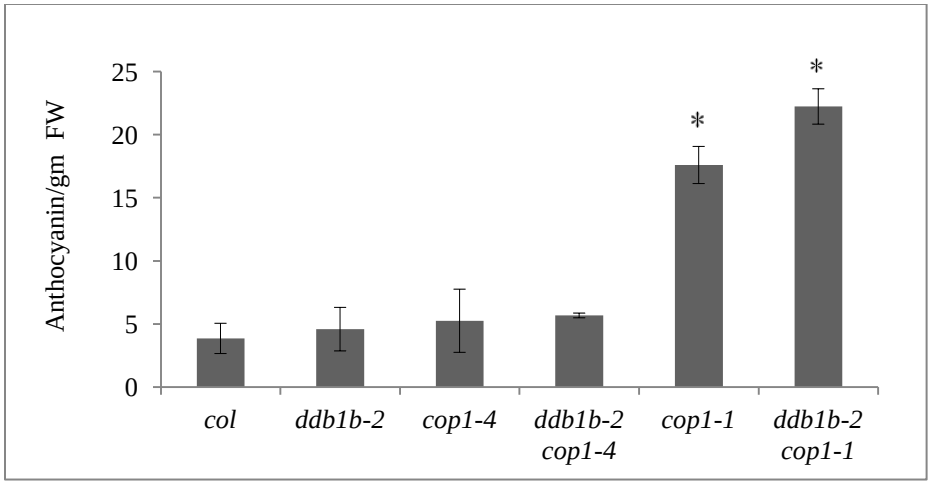


Figure 2.16 Phenotypic characterization of 7 day light grown seedlings (Cont.)

C: Cotyledon Width (in mm); D**:** Anthocyanin Content / gm FW (n=2)

Error bars indicate 95% C.I., * = P ≤0.05; *ddb1b-2 cop1-4* and *ddb1b-2 cop1-1* relative to *cop1-4* and *cop1-1* respectively, single mutants relative to *Col-0*

E

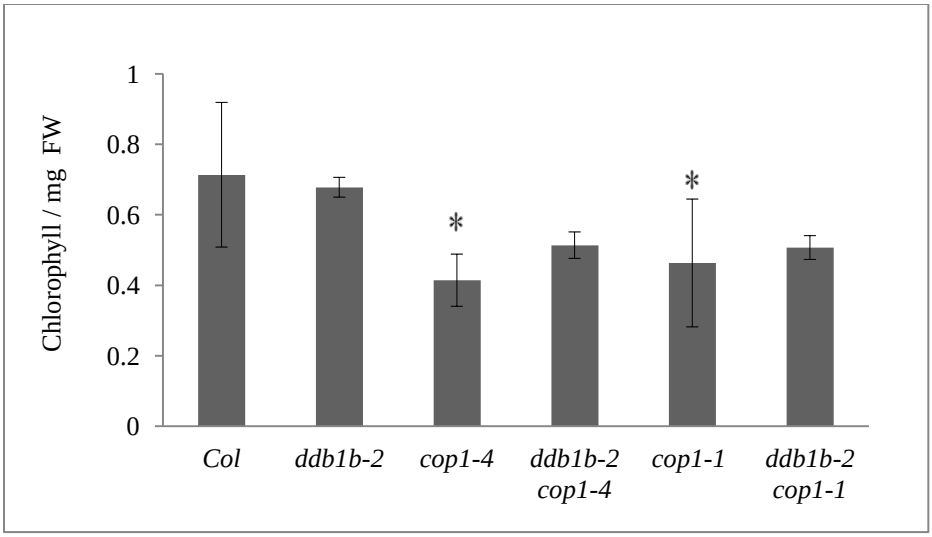


Figure 2.16 Phenotypic characterization of 7 day light grown seedlings (Cont.)

E: Chlorophyll content (µg chlorophyll / mg FW)

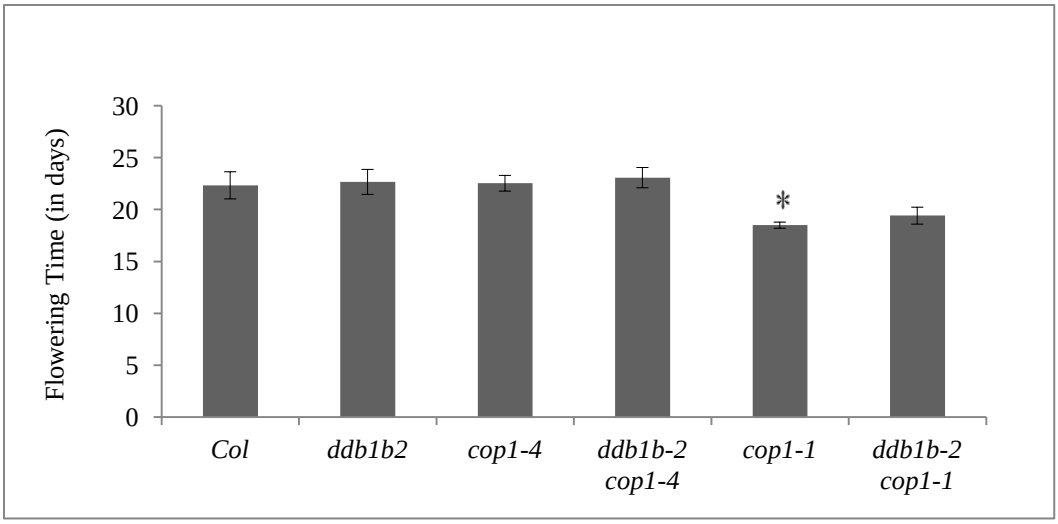
Error bars indicate 95% C.I., (n=2), * = P ≤0.05, *ddb1b-2 cop1-4* and *ddb1b-2 cop1-1* relative to *cop1-4* and *cop1-1* respectively, single mutants relative to *Col-0*

A

Figure 2.17 Phenotypic characterization of Adult plants.

A: From left: *Col-0, ddb1b-2, cop1-4, ddb1b-2 cop1-4, cop1-1, ddb1b-2 cop1-1*

B



C

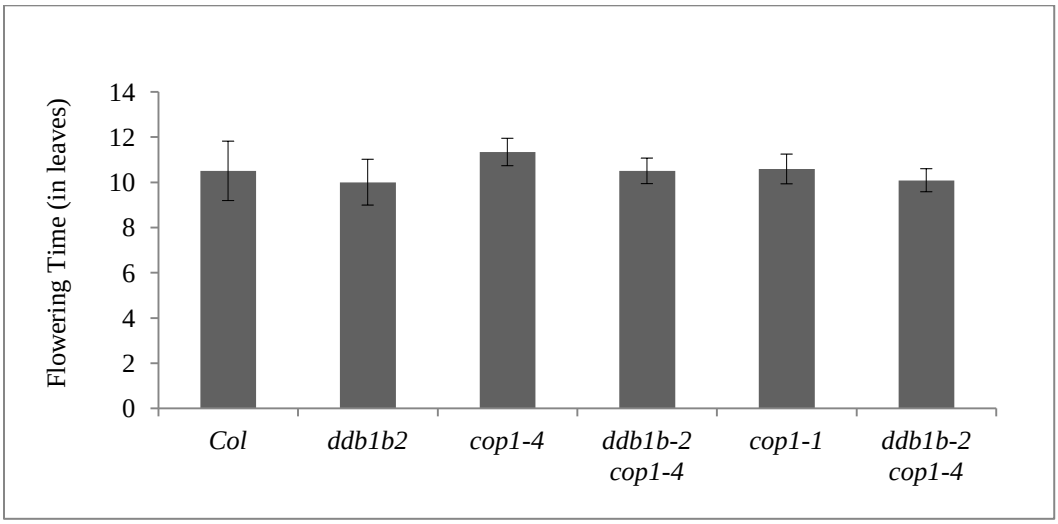
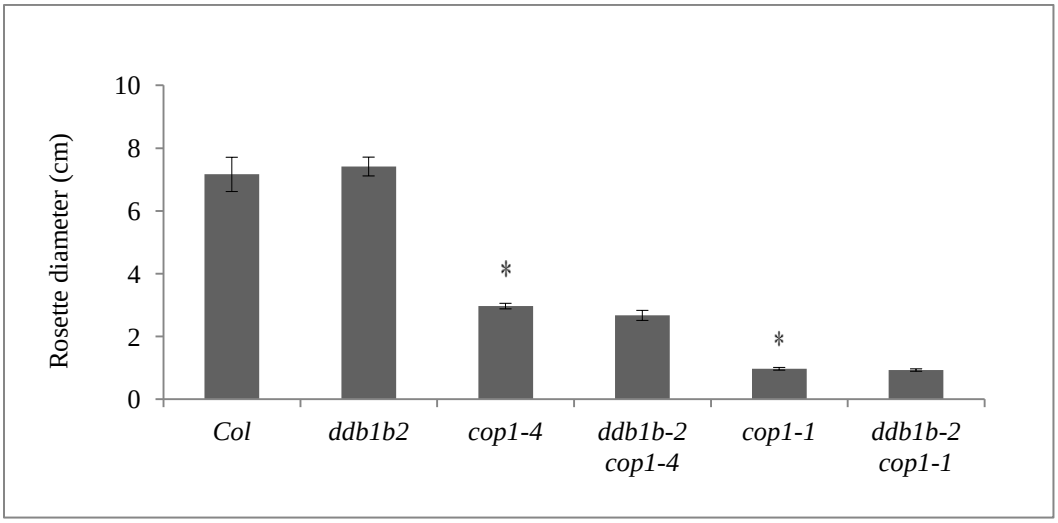


Figure 2.17 Adult Phenotypes (Cont.)

B: Flowering time (in days); **C:** Flowering Time (in leaves)

Error bars indicate 95% C.I., (n=12), * = P ≤0.05, *ddb1b-2 cop1-4* and *ddb1b-2 cop1-1* relative to *cop1-4* and *cop1-1* respectively, single mutants relative to *Col-0*

D



E

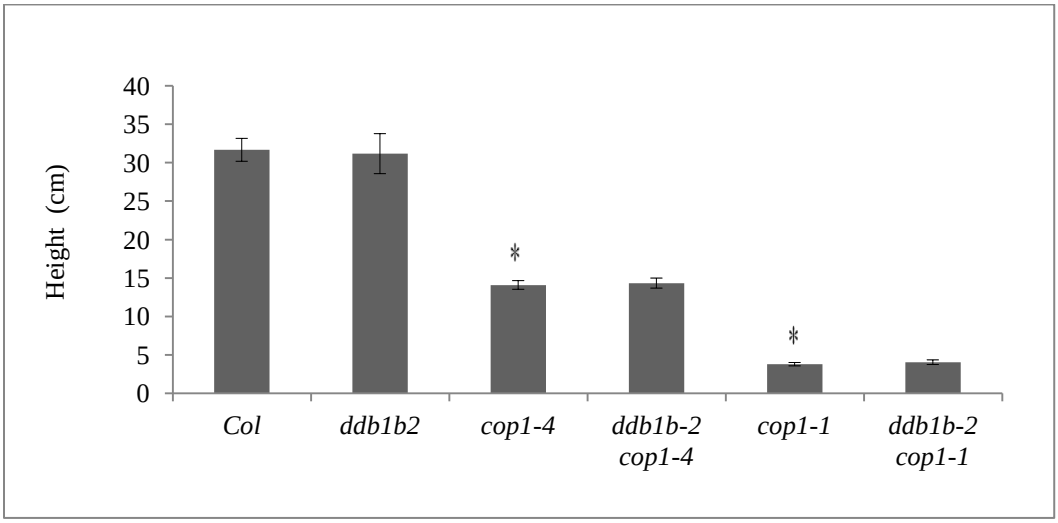
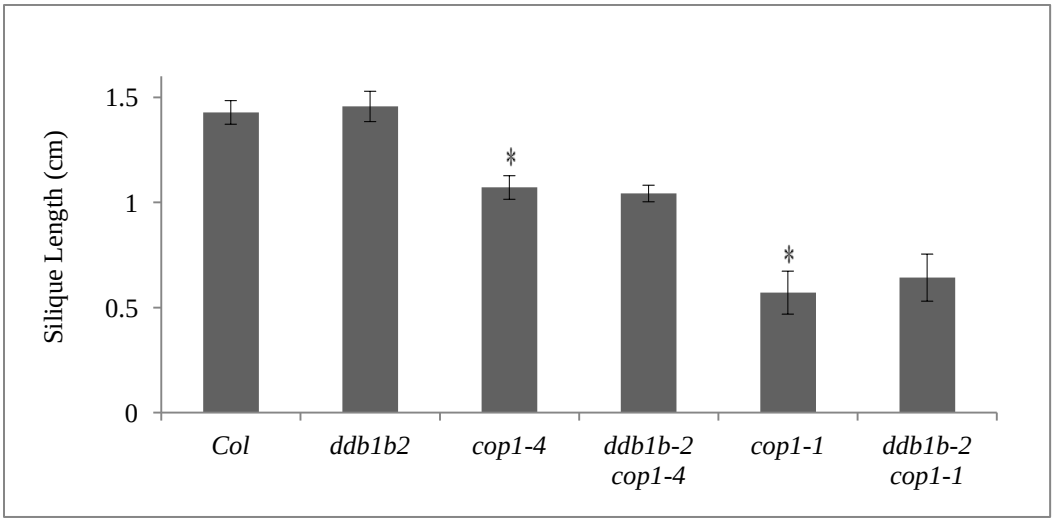


Figure 2.17 Adult Phenotypes (Cont.)

D: Rosette Diameter (in cm); **E:** Height (in cm)

Error bars indicate 95% C.I., (n=12), * = P ≤0.05, *ddb1b-2 cop1-4* and *ddb1b-2 cop1-1* relative to *cop1-4* and *cop1-1* respectively, single mutants relative to *Col-0*

F



G

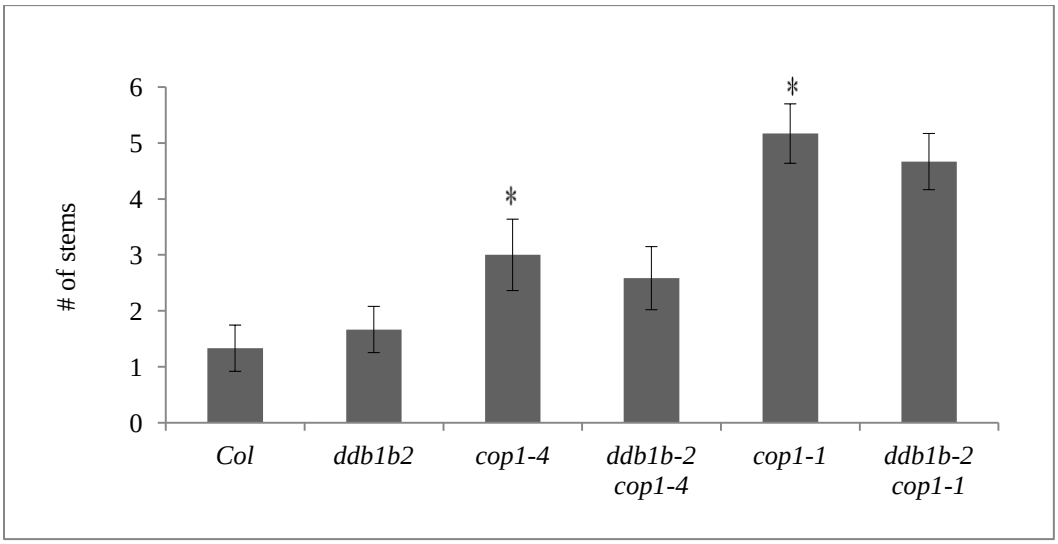


Figure 2.17 Adult Phenotypes (Cont.)

F: Silique length (in cm); **G:** # of stems

Error bars indicate 95% C.I., (n=12), * = P ≤0.05, *ddb1b-2 cop1-4* and *ddb1b-2 cop1-1* relative to *cop1-4* and *cop1-1* respectively, single mutants relative to *Col-0*

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