Genetic Interactions of Arabidopsis thaliana Damaged DNA Binding Protein 1B (DDB1B) With DDB1A, DET1, and COP1

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ABSTRACT Damaged DNA Binding protein 1 (DDB1–CULLIN4 E3 ubiquitin ligase complexes have been implicated in diverse biological processes in a range of organisms. Arabidopsis thaliana encodes two homologs of DDB1, DDB1A, and DDB1B. In this study we use a viable partial loss of function allele of DDB1B, ddb1b-2, to examine genetic interactions with DDB1A, DET1 and COP1. Although the ddb1b-2 ddb1a double mutant is lethal, ddb1a ddb1b-2/+ and ddb1b-2 ddb1a/+ heterozygotes exhibit few developmental phenotypes but do exhibit decreased tolerance of ultraviolet light. 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 DDB1–CUL4 complexes. Although ddb1a strongly enhances det1 phenotypes in both dark- and light-grown seedlings, ddb1b-2 weakly enhanced the det1 short hypocotyl phenotype in the dark, as well as enhancing anthocyanin levels and suppressing 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 dark and light, enhanced anthocyanin levels in cop1-1 in the light, but had no effect in adults. Thus the requirement for DDB1B varies in the course of development, from COP1-specific effects in hypocotyls to DET1-specific in adults.

KEYWORDS DDB1B, DDB1A, DET1, COP1, light

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. All of the pleiotropic 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 (Chen and Chory 2011; Lau and Deng 2012).

The COP/DET/FUS genes have been cloned and shown to be involved in protein degradation. Six of the COP/DET/FUS family genes encode components of the COP9 signalosome. The COP9 signalosome exhibits high homology to the 19S lid subcomplex 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 repeats 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 photoreceptor Phytochrome A are targeted for degradation via interaction with the COP1 WD40 domain (Yi and
Deng 2005). DET1, a 62-kDa nuclear localized protein, associates with nonacetylated core histones (Benvenuto et al. 2002), has been implicated as a transcriptional repressor (Lau et al. 2011), and exhibits biochemical and genetic interactions with DDB1A. Arabidopsis encodes two homologs of DDB1—DDB1A and DDB1B (Schroeder et al. 2002). DET1 and DDB1A interact with COP10 to form the CDD complex, which in turn interacts with CULLIN4 (Yanagawa et al. 2004; Bernhardt et al. 2006; Chen et al. 2006). Interestingly, COP1 also interacts biochemically with DDB1A and DDB1B as well as CULLIN4 (Chen et al. 2010).

DDB1 and DDB2 are core components of the ultraviolet (UV)-damaged DNA-binding protein complex (DDB) initially identified in human cells. The primary UV-induced DNA lesions include cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts (Kunz et al. 2006). 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 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 human GG-NER, lesion recognition is mediated by the CUL4-DDB1DDB2 complex followed by XPC-HR23B-CEN2 recruitment. In human TC-NER, CUL4-DDB1CSA recognizes the stalled RNA POL II bound to CSB. Thus both subpathways of NER are regulated by a CUL4-DDB1 complex interacting with specific recognition substrates: DDR2 (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). BPB interacts with CUL4 and the clasp-shaped BPA–BPC pocket mediates interactions with a large number of proteins containing WD40 domains, referred to as DCAF proteins (i.e., DDB1-CUL4–associated factor) or DWD proteins (i.e., DDB1 binding WD40 proteins) (Lee and Zhou 2007; Biedermann and Hellmann 2011). The Arabidopsis genome encodes approximately 230 WD40 proteins but only a fraction of them (approximately 86 proteins) have one or more WDxR motifs within the WD40 domain capable of interacting with DDB1 (Lee et al. 2008). Arabidopsis DDB1A and DDB1B are 91% identical and 97% similar at the amino acid level (Schroeder et al. 2002; Bernhardt et al. 2010). The differences between DDB1A and DDB1B are distributed fairly evenly throughout the proteins, with the exception of a region between amino acids 729–766, which decreases to 59% identity and 78% similarity. This region maps to the loop 3b–3c region of DDB1 BPC, which is somewhat variable, and in fact has an extra loop in animal DDB1s. This region is on the bottom of BPC and is not directly involved in interactions yet mapped (Li et al. 2006). Consistent with this, many proteins, including CUL4, DDB2, DET1, COP1, SPA1-SPA4, DWA1-DWA3, FY, PRL1, TRIP-1, VIP3, MS13, and MS14/FVE, have been found to interact with both DDB1A and DDB1B in yeast two-hybrid and/or coimmunoprecipitation experiments (Bernhardt et al. 2006; Lee et al. 2008; Bernhardt et al. 2010; Chen et al. 2010; Lee et al. 2010; Dumbitskashvili et al. 2011; Lee et al. 2011; Pazhouhandez et al. 2011).

Duplication of the DDB1 gene appears to be specific for the Brassicaceae, because clear DDB1A and DDB1B homologs exist in Brassica rapa, Capsella rubella, and Arabidopsis lyrata in addition to Arabidopsis thaliana (www.phytozome.net). Evolution of these genes appears constrained because the Ka/Ks ratio (0.04/0.57) is well below one (Wagner 2002; Singh et al. 2010). DDB1A and DDB1B are expressed throughout Arabidopsis development with DDB1A levels on average twofold greater than those of DDB1B (Al Kateeb and Schroeder 2007; Bernhardt et al. 2010). Null alleles of DDB1A do not exhibit obvious developmental phenotypes while null alleles of DDB1B appear lethal (Schroeder et al. 2002). Up-regulated levels of both DDB1A and DDB1B mRNA are observed after UV irradiation, and mild-to-severe UV sensitivity was observed in ddb1a and ddb2 mutants and over-expression of DDB1A and DDB2 confers increased UV resistance (Koga et al. 2006; Molinier et al. 2008; Al Kateeb and Schroeder 2009). In this study we examine the role of DDB1 by analyzing the genetic interactions of a partial loss of function DDB1B allele with DDB1A, DET1, and COP1.

MATERIALS AND METHODS

Plant materials and growth conditions

All lines in this study were in the Col background of Arabidopsis thaliana. det1-1, ddb1a, and det1 ddb1a were as previously described (Chory et 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 genotyping was as described in Al Kateeb and Schroeder (2007). For ddb1b-2, the T-DNA insertion was detected using LB2 (TTGGGTATGGTTACG TAGTGGGCGCATCC) and UV2.21 (CAGAGAAGGAGCAACAGG GACG) whereas wild-type DDB1B was detected using UV2.21 and DDB1B 3’UTR (AGGGGAGAGGAGAGGTCTTGGGA). Because ddb1a ddb1b-2 is embryonic lethal, these lines were maintained as ddb1b-2 ddb1a/+ and ddb1a ddb1b-2/+ heterozygotes. Seeds were sterilized and plated on Linsmaier and Skoog media (Caisson) supplemented with either 2% sucrose (det1 and cop1 experiments) or 0.6% sucrose (ddb1b-2 ddb1a experiments) and 0.8% Phytoablend (Caisson). After 2 d of stratification at 4°C, plates were transferred to a growth chamber (20°C with 50% relative humidity). Light was provided by fluorescent bulbs (100 μM photons m⁻² sec⁻¹). Short-day conditions correspond to 10-hr light/14-hr dark relative to long-day conditions, which correspond to 16-hr light:8-hr dark. For adult growth, 14-d-old seedlings were transplanted to Sunshine Mix Number 1 (SunGro, Bellevue, WA).

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from 7-d-old seedlings using a RNaseasy plant minikit (QIAGEN) according to the manufacturer’s instructions including a DNase step. Quantity of extracted RNA was measured by spectrophotometric analysis based on UV absorbance. cDNA synthesis and amplification was a one step process using an Access RT-PCR kit (Promega). Semiquantitative RT-PCR was performed at 45°C for 45 min followed by PCR [5 min 94°C, (30 sec 94°C, 50 sec 53°C, 90 sec 72°C) for a gene-specific number of cycles, then 7 min 72°C]. Actin was used as the loading control. PCR products were separated on 1% (w/v) agarose ethidium-bromide-stained gels and band intensities were analyzed using Image Lab 3.0 (Biorad). The following primers were used: DDB1B 2.21 (P3) CAGAGAAGGAGCAACAGG GACG, 2.27 (P4) CABGACCTGCCATTTTATATA, 22 cycles; for DDB1A 10XL (P7) TAAAGAAGTTGATCATGATGTCCTC, 1.4 (P8) GCAGAGTTCATCTTCTGAATATA, 20 cycles; total DDB1A + DDB1B X12 (Cl) GAGGTGTATTCTCCTCAA, 2.20 (C2) TGCCAGACT CTCCACTTGAAC, 22 cycles; CAB2 CAGGAGTGGGCCATCGTG,

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GCCTCTACAACGGAGTGAACCCAA, 21 cycles; and Actin CTGGAA CAAGACTTCTGGGC, GGTGATGAAGCACAATCCAAG, 24 cycles.

Seedling analysis
For hypocotyl length and cotyledon width assays, 7-d-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-d-old seedlings were extracted with 80% acetone overnight, A645 and A663 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.

Adult growth parameters
14-d-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 wk) and plant height, apical dominance, and silique length (at approximately 6 wk) were determined.

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. After irradiation, plants were dark incubated for 3 d 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 d under the same long-day growth conditions as mentioned previously. For light assays, plates were irradiated with 600 J m⁻² UV-C, rotated 90° and incubated vertically under long-day conditions for 2 d. Fresh root growth (starting from the bending point) was measured using NIH Image. For dark assays, plates were UV-C irradiated with 1500 J m⁻², similarly rotated and incubated under dark conditions for 3 d. New growth was detected by bending assay and measured using NIH Image.

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 3 d after transfer to long-day conditions and root growth was measured 7 d after transfer to long-day conditions.

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

Statistical analysis
All experiments were repeated at least three times. Data were compared by Student’s t-test and probabilities of 0.05 or less considered statistically significant.

RESULTS
Interactions between ddb1b and ddb1a
Arabidopsis DDB1A and DDB1B are 91% identical at the amino acid level and are both expressed throughout plant development. ddb1a-null alleles exhibit no obvious developmental defects, suggesting that DDB1B acts redundantly during normal development. However, ddb1b null alleles appear lethal as viable homozygotes cannot be obtained (Schroeder et al. 2002; Al Khateeb and Schroeder 2007). In this study we use a T-DNA allele in the DDB1B gene, ddb1b-2 (SALK_061944), previously described by Bernhardt et al. (2010). This T-DNA insertion results in truncation of the last 112 amino acids of DDB1B, preventing interaction with DDB2, and presumably other WD40 proteins, but not CUL4 (Bernhardt et al. 2010), thus results in a partial loss of function allele (Figure 1A). To examine the role of total DDB1 activity in Arabidopsis growth and development, we combined ddb1b-2 with a previously described ddb1a mutation (Schroeder et al. 2002). This ddb1a allele also retains transcript 5’ of the T-DNA insertion at the beginning of the tenth exon (data not shown), but prevents accumulation of the full length DDB1A transcript (Figure 1B). However, the resulting protein would be only 252 amino acids long (23% of 1088 total amino acids); thus, it would be...
expected to be a stronger allele than that of ddb1b-2. Transcript levels of both DDB1A and DDB1B are unchanged in the ddb1b-2 and ddb1a mutant backgrounds respectively (Figure 1, A and B).

As previously described (Bernhardt et al. 2010; Dumbliauskas et al. 2011), although single ddb1a and ddb1b-2 mutants exhibit no obvious developmental phenotypes, the ddb1a ddb1b-2 double mutant is embryo lethal, preventing analysis of traits later in development. Therefore, we used 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. The respective heterozygotes exhibit decreased levels of DDB1B and DDB1A transcripts [note the segregating ddb1b-2 ddb1a/+ and ddb1a ddb1b-2/+ consist of pooled populations (2/3 +/- and 1/3 +/-/+)] (Figure 1, A and B). Primers in conserved regions of the DDB1 genes reveal that total full-length DDB1 transcript level decreases from wild type to ddb1b-2 to ddb1b-2 ddb1a/+ to ddb1a to ddb1a ddb1b-2/+ (Figure 1C), consistent with data that DDB1A transcript levels are approximately twice those of DDB1B (Al Kateeb and Schroeder 2007).

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 (Supporting Information, Figure S1). Similarly, in light-grown conditions, no phenotypes with respect to hypocotyl length, cotyledon width, anthocyanin or chlorophyll content were observed (Figure S2). In adults, no effects on flowering time (days and leaves), height, silique length, or apical dominance were detected, although a slight increase in rosette diameter was observed in the ddb1b-2 ddb1a/+ heterozygotes (Figure S3). Thus, in our hands, a single wild-type copy of either DDB1A or DDB1B is sufficient for most 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 segregating 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 was scored (Figure 2A). There was no significant difference in percentage dead leaves between the single mutants ddb1b-2 and ddb1a and wild type. Similarly, no differences between ddb1b-2 ddb1a/+ and ddb1b-2 were observed, but ddb1a ddb1b-2/+ exhibited greater levels of tissue death than ddb1a. 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 2 d after UV irradiation when incubated in long-day conditions (Figure 2B). However, increased UV sensitivity in both ddb1b-2 ddb1a/+ and ddb1a ddb1b-2/+ was observed after treatment with a higher dose of UV and 3-d dark incubation (Figure 2C). Note these experiments used segregating ddb1b-2 ddb1a/+ and ddb1a ddb1b-2/+ populations (2/3 +/- and 1/3 +/-/+ ) and thus may underestimate the phenotype of the heterozygotes.

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. Although 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 (Figure 3A). Thus, DDB1A appears to have a critical role in regulation of germination during stress conditions, whereas no significant effect of DDB1B mutation could be detected in either the wild type or ddb1a background. Although ddb1a and ddb1a ddb1b-2/+ exhibited delayed germination, they did not exhibit any root growth phenotypes after 7 d in these conditions (Figure 3B). In fact, root growth in ddb1b-2 was found to be slightly resistant to salt. Finally, we examined the role of DDB1A and DDB1B in heat sensitivity by analyzing the effect of heat on dark-grown hypocotyl length (Figure 3C). ddb1b-2 exhibited mild heat sensitivity while ddb1b-2 ddb1a/+ and ddb1a and ddb1a ddb1b-2/+ all exhibited similar strong sensitivity. Thus both DDB1A and DDB1B contribute to heat tolerance.

**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, \(\text{det1}\) 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 \(\text{ddb1a det1}\) mutants exhibit decreased hypocotyl length and cotyledon width as well as increased anthocyanin levels relative to \(\text{det1}\) single mutants (Figure 4A). However the \(\text{ddb1b-2 det1}\) double mutants did not significantly differ from \(\text{det1}\) with respect to any of these phenotypes. Thus, \(\text{ddb1a}\) has a stronger effect on \(\text{det1}\) phenotypes in the dark than \(\text{ddb1b-2}\).

In light-grown seedlings, \(\text{det1}\) mutants are small with decreased chlorophyll and increased anthocyanin levels. In the light as in the dark, the \(\text{ddb1a det1}\) mutants exhibit decreased cotyledon width and increased anthocyanin levels relative to \(\text{det1}\) (Figure 5). Although the \(\text{ddb1b-2 det1}\) mutants did not differ from \(\text{det1}\) with respect to hypocotyl length or cotyledon width, they did however exhibit enhanced anthocyanin levels, intermediate between those of \(\text{det1}\) and \(\text{ddb1a det1}\) (Figure 5D). Interestingly, \(\text{ddb1b-2 det1}\) mutants exhibited higher chlorophyll levels than \(\text{det1}\), thus \(\text{ddb1b-2}\) partially suppresses the \(\text{det1}\) pale phenotype (Figure 5E). This suppression appears to occur at the transcriptional level, since \(\text{CAB2}\) transcript levels are higher in \(\text{ddb1b-2 det1}\) than in \(\text{det1}\) (Figure 5F).
We also compared the effect of ddb1a and ddb1b-2 on det1 phenotypes in adult plants (Figure 6). det1 mutants exhibit early flowering (Pepper and Chory 1997). Flower bud emergence in det1 occurs at approximately 18 d in long-day conditions in contrast to wild-type plants, where bud emergence occurs at approximately 24 d. Like ddb1a det1 (bud emergence at approximately 22 d), ddb1b-2 det1 double mutants partially suppress early flowering in det1, with bud emergence at approximately 20 d under long-day conditions (Figure 6B). ddb1b-2 also partially suppressed det1 early flowering in short-day conditions (Figure S4A). In terms of leaf number at flowering, ddb1b-2 det1 double mutants flowered at significantly increased leaf number relative to det1 in long day (Figure 6B); however, no effect was observed in short-day conditions (Figure S4B).

det1 adults are dwarf in stature, with reduced rosette diameter, height, and silique length relative to wild type (Figure 6, D–F). All three of these parameters are further decreased in ddb1a det1 double mutants (62%, 75%, and 64% smaller, respectively); thus, ddb1a strongly enhances the det1 dwarf phenotype. ddb1b-2 also enhanced these three det1 phenotypes, but to a lesser extent than ddb1a (27%, 30% and 17% respectively). In addition, ddb1b-2 enhanced these three det1 phenotypes in short-day conditions as well (Figure S4, C–E). det1 also has decreased apical dominance resulting in increased inflorescence number. ddb1b-2 does not affect this phenotype in either long-day or short-day conditions (Figure 6G and Figure S4F).

**Interaction between ddb1b and cop1**

Photomorphogenic protein COP1 has also been shown to form a CUL4-DDB1 complex via interactions between DDB1 and the WD40 motif in the COP1 WD40 domain (Chen et al. 2010). Here we examine ddb1b-2 cop1 genetic interactions during development. ddb1b-2 double mutants were generated with two cop1 alleles: a strong allele (cop1-4, internal deletion potentially altering the conformation of the WD40 domain) and a relatively weak allele (cop1-1, truncated protein lacking the WD40 domain) (McNellis et al. 1994). cop1 mutants, like det1, exhibit a constitutively photomorphogenic phenotype in the dark (Figure 7). In dark-grown seedlings, ddb1b-2 enhanced the short hypocotyl phenotype in cop1-4 but not cop1-1 (Figure 7B). No differences in cotyledon width or anthocyanin content were observed in either ddb1b-2 cop1-4 or ddb1b-2 cop1-1 double mutants relative to their respective single mutants (Figure 7, C and D). In light-grown seedlings (Figure 8), ddb1b-2 decreased both hypocotyl length and cotyledon width in cop1-4 but not cop1-1 (Figure 8, B and C). ddb1b-2 had no significant effect on chlorophyll content in either cop1 allele (Figure 8D). With respect to anthocyanin content, ddb1b-2 had no effect on cop1-4 but enhanced anthocyanin levels in cop1-1 (Figure 8E). In adults, ddb1b-2 did not significantly alter any cop1 phenotype in either long-day or short-day conditions (Figure 9, Figure S4). Thus, genetic interactions between ddb1b-2 and cop1 appear to be developmentally regulated.

Thus ddb1b-2 had no effect on cop1 adult phenotypes but significantly affected det1 adult phenotypes. In contrast, in 7-d-old dark-grown seedlings, ddb1b-2 enhanced the cop1-4 short hypocotyl phenotype but did not significantly affect det1 phenotypes. To examine the effect of seedling age on dark phenotypes, seedlings were grown for 5 or 6 d in the dark and hypocotyl length and cotyledon width measured. After 5 and 6 d, ddb1b-2 now significantly enhanced the det1 short hypocotyl phenotype, as well as that of cop1-4, but not cop1-1 (Figure S5A). Nonetheless the effect of ddb1b-2 on hypocotyl length was consistently stronger in the cop1-4 background, resulting in 30 and 25% reduction in hypocotyl length in 6 and 5 d, respectively, than in the det1 background, where the double mutants were 16 and 13% shorter than the det1 single mutants at 6 and 5 d, respectively. No significant effect of ddb1b-2 on cotyledon width was observed in these conditions (Figure S5B).
DISCUSSION

The purpose of this study was to identify differences between DDB1A and DDB1B in terms of redundant and distinct functions, and to examine genetic interactions with specific DDB1 interactors. Interestingly, whereas our analysis of ddb1a and ddb1b-2 single mutants and segregating heterozygotes detected no phenotypes except for increased rosette diameter in ddb1b-2 ddb1a/+, using the same viable ddb1b-2 allele (SALK_069144), Bernhardt et al. (2010) identified a variety of developmental phenotypes in these lines. These phenotypes included increased dark hypocotyl length in ddb1a and ddb1a ddb1b-2/+, accelerated flowering in long-day conditions in terms of leaf number in all lines, late flowering in long-day conditions 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/+. Pazhouhandeh et al. (2011) observed no flowering phenotypes in long-day conditions with the ddb1b SALK_069144 allele but did detect early flowering in long-day conditions with respect to both days and leaves with ddb1a. The differences between studies could be due to differences in the ddb1a allele used or experimental conditions.

In UV tolerance assays, we did not detect any sensitivity in ddb1b-2 single mutants. Similar results were obtained using the same allele in UV-B and UV-C tolerance assays by Bernhardt et al. (2010) and Castells et al. (2011), respectively. In adult UV tolerance assays, only ddb1a ddb1b-2/+ exhibited UV sensitivity. Similarly, in seedlings treated with UV followed by light incubation, sensitivity was also observed only in ddb1a ddb1b-2/+. Thus in these two assays only the lines with the lowest total DDB1 transcript levels (Figure 1C) exhibited sensitivity.

In light conditions, both photolyase enzymes and NER 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 compared with 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 wild-type copy of DDB1B nor DDB1A is sufficient for wild-type levels of UV tolerance. However, two wild-type alleles of either gene are sufficient.

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. Although DWA1, 2, and 3 all interact with both DDB1A and DDB1B in vitro, we find that germination in ddb1a is...
more sensitive to 100 mM NaCl and 200 mM Mannitol than germination in ddb1b-2 or wild type. Interestingly, the sensitivity of ddb1a in germination had no effect 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.

Figure 7 ddb1b-2 cop1 dark-grown seedlings. (A) From left: Col, ddb1b-2, cop1-4, ddb1b-2 cop1-4, cop1-1, and ddb1b-2 cop1-1. (B) Hypocotyl length (n = 15). (C) Cotyledon width (n = 15). (D) Anthocyanin content (A530/A657 / g fresh weight) (n = 2). Error bars indicate 95% CI; *P ≤ 0.05 of single mutants relative to Col or of ddb1b-2 cop1-4 and ddb1b-2 cop1-1 relative to cop1-4 and cop1-1, respectively.

Figure 8 ddb1b-2 cop1 light-grown seedlings. (A) From left: Col, ddb1b-2, cop1-4, ddb1b-2 cop1-4, cop1-1, and ddb1b-2 cop1-1. (B) Hypocotyl length (n = 15). (C) Cotyledon width (n = 15). (D) Anthocyanin content (A530/A657 / g fresh weight) (n = 2). (E) Chlorophyll content (µg of chlorophyll / mg of fresh weight) (n = 2). Error bars indicate 95% CI; *P ≤ 0.05 of single mutants relative to Col or of ddb1b-2 cop1-4 and ddb1b-2 cop1-1 relative to cop1-4 and cop1-1, respectively.
Schroeder, unpublished data). Consistent with a role for DDB1A in heat tolerance, AtGenExpress data (Kilian et al. 2007) indicates that heat elevates DDB1A transcript levels, but not DDB1B levels. In contrast, neither mannitol nor salt treatment strongly induce DDB1A levels, but DDB1B levels decrease (Figure S6).

DET1 interacts biochemically with CUL4-DDB1 and exhibits genetic interactions with both CUL4 and DDB1A (Schroeder et al. 2002; Chen et al. 2006). Although ddb1a enhances all det1 phenotypes in dark-grown seedlings, ddb1b-2 only weakly enhances the short hypocotyl phenotype. In light-grown seedlings, ddb1b-2 enhanced the det1 high anthocyanin phenotype and suppressed the det1 low chlorophyll phenotype. This suppression appears to occur at the transcriptional level since CAB2 transcript levels are also increased in the ddb1b-2 det1 double mutant. The fact that the ddb1b-2 det1 double mutant suppresses the det1 chlorophyll phenotype suggests the two proteins are not acting together in this instance, and that this suppression may be indirect, for example via another complex. In previous studies, ddb2 was also found to suppress the det1 low chlorophyll phenotype (AI Khateeb and Schroeder 2007). DET1 has recently been shown to act as a transcriptional repressor (Lau et al. 2011), but this does not explain the underexpression of CAB2 in det1 in light, thus DET1 regulation of CAB2 in the light may be indirect. It is not clear whether DDB1 is involved in regulation of transcription by DET1 (Lau et al. 2011). CAB2 promoter analysis has shown that a HY5-binding element is required for DET1 light-regulation of CAB2, and hy5 mutants suppress the det1 pale phenotype (Maxwell et al. 2003).

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 our ddb1a allele is potentially stronger than the ddb1b-2 partial loss of function allele, and that DDB1A is expressed at greater levels than DDB1B throughout development (AI Khateeb and Schroeder 2007; Bernhardt et al. 2010), 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. ddb1b-2 enhanced the short hypocotyl phenotype in both dark and light-grown cop1-4. Because ddb1b-2 had smaller and no effects on det1 dark and light-grown hypocotyl length, respectively, DDB1B appears to be more critical for COP1 function than for DET1 function with respect to regulation of hypocotyl length. In light-grown seedlings, ddb1b-2 enhanced anthocyanin levels in cop1-1 and det1,

**Figure 9** ddb1b-2 cop1 adult phenotypes. (A) From left: Col, ddb1b-2, cop1-4, ddb1b-2 cop1-4, cop1-1, and ddb1b-2 cop1-1. (B) Flowering time (in days). (C) Flowering time (in leaves). (D) Rosette diameter. (E) Plant height. (F) Silique length. (G) Number of stems. For B-G, error bars indicate 95% CI (n = 12); *P ≤ 0.05 of single mutants relative to Col or of ddb1b-2 cop1-4 and ddb1b-2 cop1-1 relative to cop1-4 and cop1-1, respectively.
suggesting that DDB1B has a common role in regulation of light anthocyanin levels. In adults, ddb1b-2 had no effect on any phenotypes in 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 hypocotyls to DET1-specific in adults. Whether this specificity is due to differential levels, cellular localization, or biochemical interactions of DDB1B vs. DDB1A is unknown. In vitro COP1 interacts with both GST-DDB1B and GST-DDB1A, and FLAG-DDB1B communoprecipitates both DET1 and COP1 from light and dark-grown seedlings (Chen et al. 2010). In onion cells, GFP fusions of both DDB1A and DDB1B are localized in both the nucleus and cytoplasm, though a larger proportion of DDB1B is cytoplasmic (Zhang et al. 2008). COP1 is predominantly nuclear in the dark and cytoplasmic in the light, whereas DET1 is exclusively nuclear (Lau and Deng 2012). In addition, the COP1-4 and COP1-1 forms of the COP1 protein exhibit defects in nuclear localization (Stacey et al. 1999, 2000; Nakagawa and Komeda 2004). Thus perhaps cytoplasmic colocalization is the basis of ddb1b cop1i interaction in seedlings, but requires further analysis. 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 compromised in their ability to interact with DDB1 proteins as well as other proteins such as photoreceptors and transcription factors that interact with the COP1 WD40 domain (Yi and Deng 2005). Thus, any ddb1b-2 cop1i 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.

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