The Role of DET1 and Damaged DNA Binding Proteins (DDB1 & DDB2) in *Arabidopsis* DNA Repair and Light Signaling

by

Wesam Al Khateeb

**A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
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ABSTRACT

The Role of DET1 and Damaged DNA Binding Proteins (DDB1A and DDB2) in *Arabidopsis* DNA Repair and Light Signaling

During plant development, plants use light to determine the proper timing and duration of each developmental stage. Ultraviolet (UV) radiation is part of the solar radiation that reaches the earth's surface. UV affects plants by interrupting physiological processes or by damaging the genetic material. Plants use various DNA repair mechanisms in order to repair the UV-induced DNA damage. Most of these repair mechanisms are conserved among kingdoms. In humans, proteins called damaged DNA binding protein 1 and 2 (DDB1 and DDB2) are implicated in the disease *Xeroderma pigmentosa* (XP). XP patients are at risk for skin cancer due to an inability to repair UV-damaged DNA. *Arabidopsis* DDB1A and DDB2 show 46% and 30% identity with human DDB1 and DDB2, respectively. However, *Arabidopsis* has two homologs of DDB1: DDB1A and DDB1B. DDB1A has been found in a complex with another protein called DET1, which has a role in visible light signaling. The goal of this thesis was to examine the role of *DET1*, *DDB1A* and *DDB2* in *Arabidopsis* light signaling and DNA repair. To study *DDB2, DDB1A,* and *DET1* genetic interaction during light signalling, a reverse genetic approach was used by generating all possible double and triple mutant combinations. A thorough phenotypic analysis of these mutants under dark, short day and long day conditions led to the identification of two phenotypic categories. The first is *DDB1A*-dependent, where *det1 ddb1a* and *det1 ddb1a ddb2* exhibit similar phenotypes. The second category is *DDB1A*-independent where *det1 ddb1a* show significantly different phenotypes than the triple mutant *det1 ddb1a ddb2*. Our genetic model for the

DET1, *DDB1A* and *DDB2* interaction under visible light states that there is a competition between DET1 and DDB2 for DDB1A/B. *ddb2* mutation liberates DDB1A/B and it become more available for the DET1 complex. Conversely, in the case of *det1* mutation, DDB1A/B is more abundant for DDB2 interaction. Thus the interaction between *DET1* and *DDB2* is through *DDB1A/B*. To examine the role of *DDB1A* in *Arabidopsis* DNA repair, *DDB1A* overexpression lines were used as well as the *ddb1a* null mutant. No significant difference was observed between wildtype and the *ddb1a* mutant in shoot and root assays. *ddb1a* mutants exhibit a slight delay in photoproduct repair. In contrast, *DDB1A* overexpression lines show healthier plants in UV-tolerance assays (shoot and root) and faster DNA repair rates. This indicates that *DDB1A* is important in *Arabidopsis* DNA repair. Then we examined the interaction between *DDB1A/B* and *DDB2* under UV light. *ddb1a*, *ddb2* and the double mutant *ddb1a ddb2* exhibit wildtype phenotypes in UV tolerance assays. Photoproduct analysis indicates that the DDB1A-DDB2 complex is important in pyrimidine 6-4 pyrimidone (6-4PP) and cyclobutane pyrimidine dimer (CPD) damage recognition. In contrast, the DDB1B-DDB2 complex is important in CPD but not in 6-4PP damage recognition after UV exposure. This indicates a subtle interaction between *DDB1A*, *DDB1B* and *DDB2* in DNA repair. Finally we examined if mutation of *det1* in the *ddb2* partial loss of function background affects the *ddb2* phenotype under UV light. The results indicate that *det1* does not appear to modify *ddb2* UV phenotypes. Also, we found mutual mRNA regulation between *DDB1A/B*, *DDB2* and *DET1* in response to UV light. In conclusion, these studies have revealed important roles and complex interactions between *DET1, DDB1A, DDB1B* and *DDB2* during *Arabidopsis* DNA repair and light signaling.

This work is dedicated to someone very special in my life….. She is always there for me.....She has given up her own happiness.....she decided to put her life on hold…..She has taught me the meaning of being a "MOTHER"

This person I am referring to is none other than……..

MY MOTHER

I am forever indebted and very thankful

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LIST OF ABBREVIATIONS

6-4PPs	(6-4) pyrimidinone dimers
95% C. I.	95% Confidence interval
CaMV	Cauliflower mosaic virus
cM	Centimorgan
Col	*Arabidopsis thaliana.* Ecotype Columbia
COP	Constitutive photomorphogenic
CPDs	Cyclobutane pyrimidine dimers
CSA/B	Cockayne syndrome A and B
CSN	The COP9 signalosome
CUL4	Cullin 4
DDB	Damaged DNA-Binding protein complex
DDB1A/B	Damaged DNA-Binding protein 1 A and B
DDB2	Damaged DNA-Binding protein 2
DET1	De-etiolated 1
DWD	DDB1-binding WD40 protein
GFP	Green fluorescent protein
GGR	Global genomic repair
h	Hour
HA	Hemagglutinin
HAT	Histone acetyl transferase
$J.m^{-2}$	Joule per square meter
LD	Long day

mm	millimeter
MS media	Murashige and Skoog medium
NER	Nucleotide excision repair
RBX1	RING-box protein 1
RNAi	RNA Interference
RT-PCR	Reverse transcription polymerase chain reaction
SD	Short day
SE	Standard error
TCR	Transcription-coupled repair
T-DNA	Transferred DNA
UBQ	Ubiquitin
UV	Ultraviolet
UV-B	Ultraviolet class B
UV-C	Ultraviolet class C
XP	*Xeroderma pigmentosum.*

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CHAPTER 1: LITERATURE REVIEW

1. LITERATURE REVIEW

1.1 INTRODUCTION

The solar radiation that reaches the earth's surface includes ultraviolet (UV) radiation and visible radiation (light). Plants adapt differently to these two different types of radiation. The aim of this thesis is to study the role of Deetiolated 1 (DET1), Damaged DNA Binding Protein 1A (DDB1A) and Damaged DNA Binding Protein 2 (DDB2) in *Arabidopsis* development under visible and UV light conditions. In the first part of this chapter I will introduce briefly the effect of visible light on plant growth and development, followed by plant responses to UV light. Afterward, I will discuss the mechanism of DNA damage and some pathways involved in DNA damage repair. Finally, the role of the DDB complex in DNA damage repair will be presented.

1.2 CLIMATE CHANGE

Ultraviolet radiation (UV) is a part of the non-ionizing radiation of the electromagnetic spectrum consisting of around 8–9% of all solar radiation. UV radiation is divided into three wavelength regions: UV-C (200–280 nm) is exceptionally dangerous to all organisms; UV-B (280–320 nm) represents around 1.5% of the total spectrum, but can induce a variety of damaging effects to most organisms; and UV-A (320–400 nm) represents approximately 6.3% of the incoming solar radiation and is the least dangerous fraction of UV radiation (Hollosy, 2002).

UV-C radiation is completely screened out by a thin stratospheric layer that surrounds the earth (ozone layer), 30–40 km above the earth's surface. UV-B radiation (280–315 nm) is also absorbed by the ozone layer, though some UV-B reach the earth's surface. Gases such as SO_2, NO_2 and aerosols also absorb UV-B (McKenzie *et al.*, 2007). In the past few decades, chlorofluorocarbons (CFCs) have been used intensively for refrigerants, aerosol propellants and plastics. This resulted in a quick rise in CFC content in the atmosphere. CFCs break down chemically to generate chlorine atoms that can destroy ozone molecules (Bonzongo and Donkor, 2003). The global ecosystem is affected by ozone depletion due to the increase in UV-B radiation that reaches the earth's surface. Elevated UV-B levels have been shown to increase carbon monoxide, nitrogen oxide and halogenated substance production from several ecosystems (Zepp *et al.*, 2003). A small change in ozone levels could result in a huge increase in biologically effective UV radiation that reaches the earth's surface. It has been suggested that each 1% reduction in ozone level could increase UV-B radiation by 1.3–1.8% (Hollosy, 2002).

Change in the ozone layer is not the only factor that affects the amount of UV-B radiation that reaches the earth's surface. The angle of the sun's rays through the atmosphere has an important impact as well. When the angle is small, the ray's path through the atmosphere is short, which results in minimum absorption. Also, cloud cover and sun–earth separation by aerosols and/or altitude affect UV radiation received at the earth's surface (McKenzie *et al.*, 2003).

1.3 VISIBLE LIGHT RESPONSES

1.3.1 Light perception

In order for a plant to initiate suitable developmental processes they need to monitor the surrounding light conditions. Plants utilize many sophisticated methods of sensing the direction, quantity, quality and duration of light and then utilize this information to generate the appropriate physiological and developmental response (Sullivan and Deng, 2003). Light perception is a complex process; plants have evolved a number of specialized photoreceptors, each characterized by the wavelength of light that it perceives. These include phytochromes, cryptochromes and phototropins.

The phytochrome family was the first family of plant photoreceptors discovered. It is responsible for red/far-red light (600-750 nm) perception. Phytochrome consists of an apoprotein that is attached to a linear tetrapyrrole chromophore by a covalent bond. Phytochromes are present in two forms. Under dark conditions, they are in the red light absorbing form (Pr), upon exposure to red light Pr will convert to the far-red light (Pfr) absorbing form. Pfr is the biologically active form of phytochrome. Exposure of Pfr to far-red light will convert it to Pr (Quail, 1997). Based on stability, there are two classes of phytochromes; phytochrome that degrades rapidly upon exposure to red or white light (type I) and phytochrome that is light stable (type II). It has been shown that *Arabidopsis* has five different phytochrome apoprotein genes, *PHYA–E*, with overlapping functions. All of them except PHYA are type II phytochromes (Sullivan and Deng, 2003).

Blue light and UV-A (320-500 nm) are perceived by cryptochromes and phototropins. Cryptochromes (CRY) are flavoproteins with high similarity to an enzyme called DNA photolyase that is important in DNA damage repair (Eckardt, 2003). Ahmad and Cashmore (1993) showed high homology between CRY1 and photolyase, including more

than 30 % identity observed over the entire 500 amino acid sequence and regions of more than 70-80% identity. Despite the similarities between CRY1 and photolyase, CRY1 protein was found to have no photolyase activity, leading to the conclusion that it is a photosensory receptor and not a DNA repair enzyme (Eckardt, 2003).

Arabidopsis cry1 phyA and *cry1 phyB* double mutants showed reduced response to blue light, which suggests that CRY1 is dependant on phytochrome A and B for action (Ahmad and Cashmore, 1997). Furthermore, it has been shown that both PHYA and PHYB act as blue light photoreceptors and they interact with CRY1 and work together in a signaling pathway for blue and UV-A light (Neff and Chory, 1998). A suggested model for cryptochrome signal transduction is that upon photon reception, a change in protein–protein interactions occurs between cryptochrome and other proteins. This interaction will alter the subcellular localization of light-signaling proteins, as well as gene expression and other cellular processes, resulting in developmental changes in intact plants as a response to blue and UV-A light (Lin, 2002).

Phototropins 1 and 2 (PHOT 1 and 2) are the blue-light receptors in plants. PHOT1 is important in phototropism, blue-light-induced chloroplast relocalization and stomatal opening, as well as blue-light-mediated calcium uptake and membrane depolarization (Briggs and Christie, 2002). A unique feature of phototropins is that they contain two LOV (light, oxygen, and voltage) domains (LOV1 and LOV2). The LOV domain is an approximately 110-amino acid motif that is important in light-sensing and chromophore (flavin)-binding (Liscum *et al.*, 2003).

1.3.2 Effect of light on plant development

1.3.2.1 *Seed germination*

Light perceived by photoreceptors affects growth and development of plants throughout the life cycle. Seed germination in *Arabidopsis*, for example, is enhanced by light. A single exposure of seeds incubated at low temperature to very low fluence rate light will induce seed germination (Botto *et al.*, 1996). It has been shown that PHYA and PHYB play an important role in regulation of *Arabidopsis* seed germination (Sullivan and Deng, 2003).

1.3.2.2 *Seedling development*

Plant development is dependent on the environmental conditions where it is growing. Seedlings implement different developmental programs when grown in light or darkness (Sullivan and Deng, 2003). Light-grown seedlings undergo photomorphogenesis, showing short hypocotyls, open and expanded cotyledons, and photosynthetically active chloroplasts (Terzaghi and Cashmore, 1995). On the other hand, seedlings grown under dark conditions have closed and unexpanded cotyledons, elongated hypocotyls and undeveloped chloroplasts. These seedlings are said to be etiolated. This developmental pattern is known as skotomorphogensis. This process is necessary for newly germinated seedlings to grow through soil to reach the light (Schafer and Bowler, 2002).

1.3.2.3 *Flowering time*

Transition from the vegetative stage to the flowering stage is one of the most significant decisions made by plants during their life cycle. Using forward and reverse genetics, many factors have been found to control floral transition. These include photoperiod, vernalization, gibberellic acid (GA) and/or autonomous pathways (Jaeger *et al.*, 2006).

The effect of day length on flowering time was studied initially more than 150 years ago (Kobayashi and Weigel, 2007). Flowering plants are grouped into three categories according to flowering transition. The first category, known as short-day plants, includes plants where short days induce flowering or long days stop it; the second category, named long-day plants, where long days induce flowering or short days stop it; and the third category, called day-neutral plants, are day length independent (Kobayashi and Weigel, 2007).

Using the natural genetic variation in *Arabidopsis* flowering time and the huge number of early and late flowering time mutants that have been identified using molecular genetics, several flowering time pathways have been discovered. Genes that regulate these pathways have been cloned and characterized, including *FT*, *SOC1* and *LFY* (Putterill *et al.*, 2004).

1.3.3 Molecular genetics of seedling light response

The seedling stage is crucial in any plant life cycle because it is the transition from the embryo stage to the adult stage. For plant molecular geneticists, plant seedlings are also important, due to the ease of phenotypic analysis, reduced space and time required,

and the availability of mutant screens. Many genes have been cloned in *Arabidopsis* that show an important role in seedling development.

The etiolation/de-etiolation developmental switch appears to be under the control of at least 10 genes. Mutation of these genes results in seedlings with a de-etiolated (*det*) or constitutive photomorphogenic (*cop*) phenotype even if they are grown under dark conditions (McNellis and Deng, 1995) (Figure 1.1). This *COP/DET/FUS* gene family has been widely studied.

det mutants (de-etiolated) were identified in a mutagenized population of *Arabidopsis thaliana* seeds. After 7 days of dark growth some seedlings were found that had short stems with opened cotyledons and are light purple in color. After a series of screens, eight *det* mutants were identified and shown to fall into two complementation groups: *det1* and *det2*. In addition, *det1* homozygous seedlings are smaller and paler than the wildtype plants in the light. They also show reduced apical dominance and daylength insensitive flowering as adults. This recessive mutation (*det1*) also regulates a number of light regulated traits such as germination, leaf development and gene expression (Chory *et al.*, 1989). Around one thousand genes are either positively or negatively expressed in *det1* compared to the wildtype (Schroeder *et al.*, 2002). Mutation of *det1* also results in an increase in expression level of pathogen defense genes (*SHS*, *LOX* and *PAL*). An explanation of this change in expression level of these genes could be due to the specific effect of *det1* mutations on light signal transduction pathways (Mayer *et al.*, 1996).

DET1 is a nuclear protein with a molecular weight of 62 kDa (Pepper *et al.*, 1994). Schroeder *et al.* (2002) investigated the structure and function of DET1 in *Arabidopsis*.

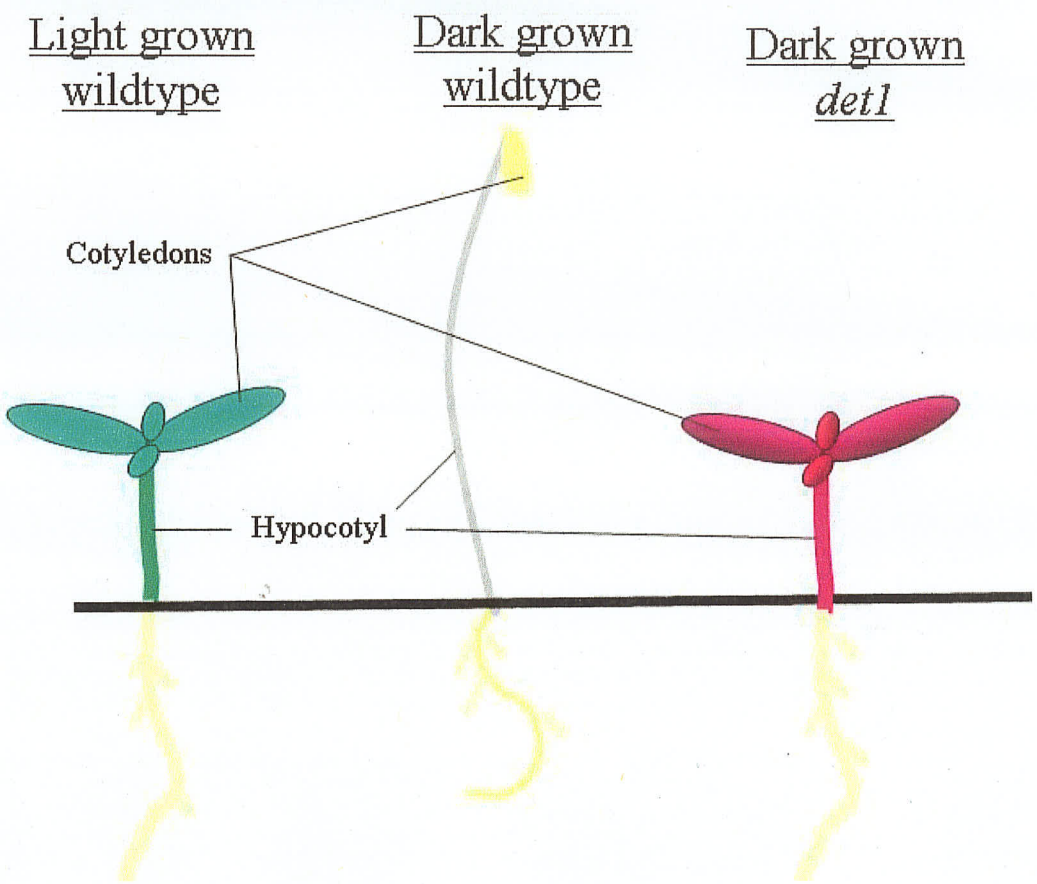


Figure 1.1: Wildtype and *det1* seedling growth under dark and light conditions

They found using an epitope-tagging approach that DET1 is present with other proteins in a complex with an approximate mass of 350 kDa. Purification of this 350 kDa protein complex by immunoaffinity followed by mass spectrometric analysis identified an approximately 120 kDa copurifying protein which was found to be the plant homolog of UV-Damaged DNA Binding protein 1 (DDB1).

cop1 mutants also display photomorphogenic traits when grown under dark conditions. Similar to *det1* mutants, *cop1* seedlings exhibit short hypocotyls and open cotyledons in the dark (Deng and Quail, 1992). It has been shown in *Arabidopsis* that COP1 interacts with HY5, a transcription factor and a positive regulator of photomorphogenesis and promotes its turnover (Figure 1.2) (Holm *et al.*, 2002). Cloning of COP1, DET1 and COP9 revealed that these proteins are involved in protein ubiquitination processes (Yanagawa *et al.*, 2005).

The ubiquitin-proteasome pathway is the principal regulatory mechanism of protein abundance inside the cell. The 26S proteasome is responsible for proteolysis. It recognizes substrates that are tagged with polyubiquitin. Ubiquitation requires the E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase (Schwechheimer and Schwager, 2004). E3 ligases determine the specificity of the whole degradation pathway by binding to specific substrates. It has been suggested that plants have more than 1000 E3 ubiquitin ligases. COP1 can act as an E3 ubiquitin ligase (Yanagawa *et al.*, 2005). The COP9 signalosome (CSN) is a multiprotein complex composed of eight subunits which associates with and supports the activity of multiple

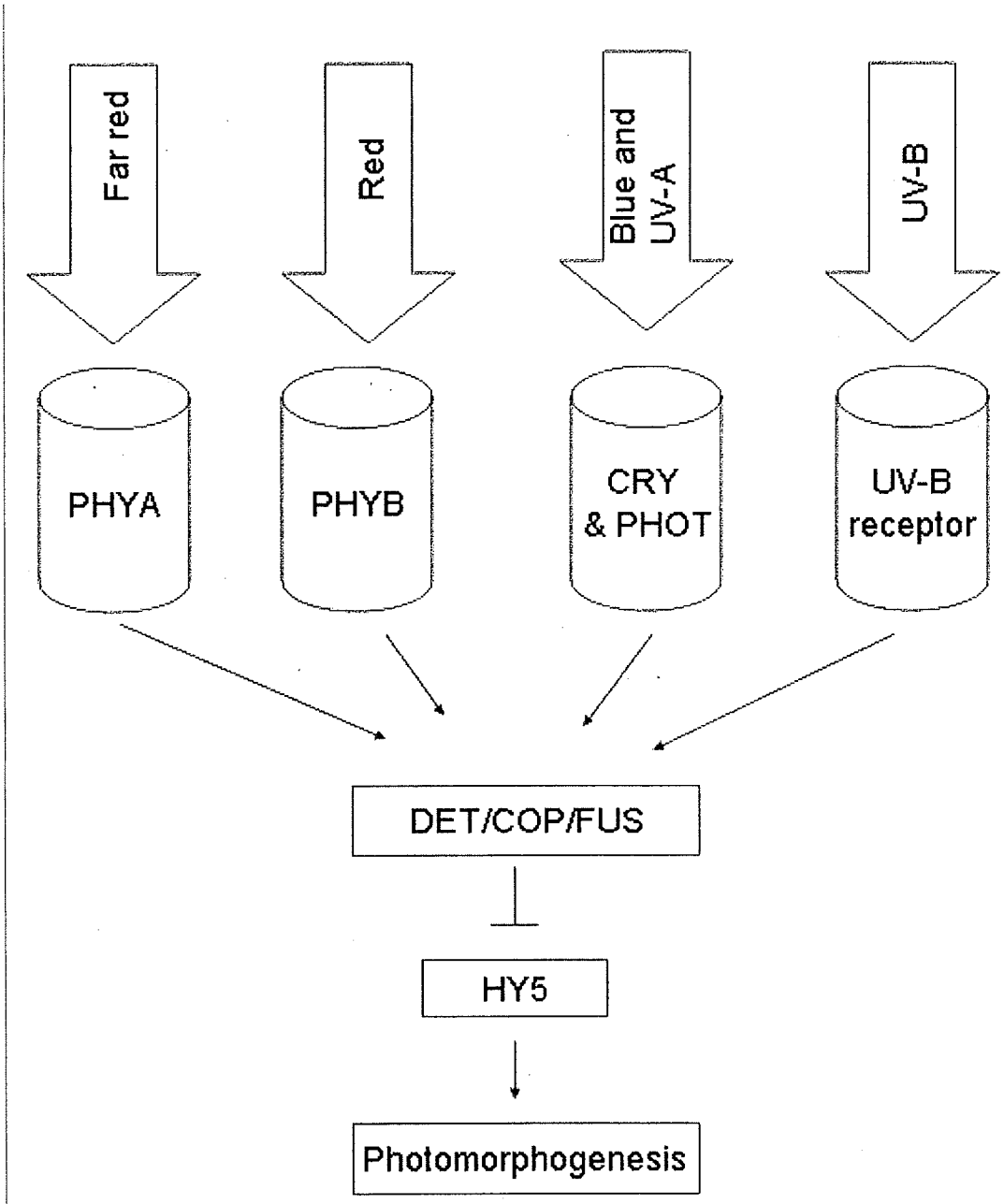


Figure 1.2: Light signalling pathways that regulate photomorphogenesis in *Arabidopsis*.

cullin-containing E3 ubiquitin ligase complexes (Schwechheimer and Villalobos, 2004).

In humans, it has been shown that DDB1, Cullin4 (CUL4) and ROC1 form E3 ligases that regulate cell-cycle progression, replication and DNA damage response. Many WD40-repeat proteins were found to interact with this complex and work as adaptors to recognize substrates for degradation (Higa *et al.*, 2006). DET1 and DDB2 are examples of these adaptor proteins in humans (Wertz *et al.*, 2004). In *Arabidopsis* DDB1A associates with DET1 and COP10 (Yanagawa *et al.*, 2004). It has been shown that *DET1* inhibits degradation of LHY protein that is involved in the circadian clock (Song and Carre, 2005). *Arabidopsis* DET1 and DDB1A interact genetically (Schroeder *et al.*, 2002) as well as in a yeast two-hybrid assay (Bernhardt *et al.*, 2006).

1.4 UV LIGHT RESPONSES

1.4.1 UV light perception

Although many studies have been conducted on the phytochrome, cryptochrome and phototropin light-sensing systems, the UV-B photoreceptor (absorbing light at 280–320 nm) is not yet known (Figure 1.2). This may be simply due to the complex effects of UV-B light, which include damage to diverse cellular constituents, and a lack of obvious screenable phenotypes. Studies have shown that identification and characterization of the precise photosensory processes which are involved in UV-B responses are very difficult (Ulm and Nagy, 2005).

Many studies have suggested the presence of UV-B photoreceptor molecules, even though the identification of this receptor is still mysterious. Many hypotheses have been

suggested. One study proposed the total absence of such a specific UV-B-absorbing macromolecule, so plant UV-B responses are due to DNA damage, reactive oxygen species (ROS) production or membrane damage. Others suggested that the red and/or blue light receptors could also absorb UV-B light and respond to absorption of photons in this wavelength (Brosche and Strid, 2003).

Mackerness *et al.* (2001) suggested a model for UV-B signal transduction. They proposed that UV-B radiation activates many signaling pathways specific for UV-B radiation and other pathways that are required for general stress signaling. After perception of UV-B radiation by a UV-B receptor the signal transduction pathway starts, which is followed by several steps involving calcium, calmodulin, and protein phosphorylation, and in other cases possibly a specific catalytic formation of ROS and salicylic acid in the cell are involved.

One major difficulty that has made the progress in the study of UV light perception slow is the identification of specific UV-B morphogenic responses. Many experiments have been done on UV responses with different time intervals. Most studies emphasized fast responses (within hours), examples of these are cotyledon bending, hypocotyl elongation inhibition and cotyledon opening (Frohnmeyer and Staiger, 2003). On the other hand, slow responses (weeks) such as axillary branching, leaf thickening and changes in root-shoot ratio were also studied. Different questions arose from these studies such as whether these different responses interacted with each other, and whether a single molecular mechanism or multiple independent mechanisms control the different responses (Jansen, 2002). Boccalandro *et al.* (2001) studied the effects of low UV-B levels on *Arabidopsis* seedlings. Results showed that the effect of red light on cotyledon

opening and hypocotyl elongation in de-etiolating *phyB* mutants was enhanced by exposure to UV-B light. This suggests that the effects of UV-B on cotyledon opening and hypocotyl growth inhibition are mediated through different photosensory mechanisms than phytochrome.

Chalcone synthase (*CHS*) expression is regulated by UV and blue light exposure. CHS is the primary step in flavonoid synthesis (Jenkins, 1997). UV signal transduction was studied by analyzing *CHS* gene expression under different wavelengths. Results showed that UV-A, UV-B and blue light induce *CHS* gene expression, but not red or far red light (Christie and Jenkins, 1996). Moreover, expression of *CHS* in *cry1* mutants retains wildtype levels after exposure to UV-B. This suggests that UV-B has a separate photoreception system than UV-A (Fuglevand *et al.*, 1996). Studies have shown that calcium could also be involved in UV-B signal transduction. In parsley (*Petroselinum crispum*) cell cultures, short exposure to UV-B significantly increased calcium levels. This increase was correlated with enhancement of *CHS* expression (Frohnmeyer and Staiger, 2003).

Reactive oxygen species (ROS), salicylic acid (SA), jasmonic acid (JA) and ethylene could be key regulators of gene expression in response to UV-B exposure. ROS have crucial roles as second messengers in UV-B signal transduction. Increased levels of ROS resulted in synthesis of SA, JA and ethylene in plant cells (Mackerness, 2000). Other proteins with flavin or pterin chromophores (or both) could be the UV-B receptor. Pterins are proposed as strong candidates (Krizek, 2004).

The role of COP1 in UV-B signaling was studied in *Arabidopsis*. Exposure of wildtype to low levels of UV-B irradiation levels showed modification in the expression

profile of many genes and increase in flavonoid accumulation. In contrast, *cop1* mutants exhibit no change in gene expression and flavonoid level. These results indicate that COP1 could be a positive regulator in response to UV-B during plant photomorphogenesis (Oravecz *et al.*, 2006). However, it has been shown recently that the expression of other genes in response to UV-B exposure is COP1 independent including *ANAC13* (Safrany *et al.*, 2008).

In summary, studies have indicated a strong interaction between UV-A, UV-B and blue light signal transduction pathways, but unfortunately, little information is available about UV-B signal transduction.

1.4.2 Effect of UV light on plant morphology

UV-B radiation induces a series of morphogenic changes in plants. These include leaf thickening, cotyledon curling, inhibition of hypocotyl and stem elongation, leaf expansion, axillary branching and changes in the root/shoot ratio (Marcel and Jansen, 2002; Frohnmeyer and Staiger, 2003). In addition, UV radiation affects plant reproduction through increased flowering (Day *et al.*, 1999; Petropoulou *et al.*, 2001). The response of seven ecotypes of *Arabidopsis thaliana* collected from different geographic locations in Europe to UV-B was studied. When seedlings were grown under supplementary UV-B radiation, they exhibited decreased plant height, rosette diameter and vegetative and reproductive tissue dry mass (Torabinejd and Caldwell, 2000).

In rice, *Oryza sativa* L., UV-B radiation resulted in visible damage symptoms on the leaves. These included formation of necrotic spots, accumulation of pigments, and

eventually desiccation of the damaged leaves (Teranishi *et al.*, 2004). In a different study, de la Rosa *et al.* (2003) investigated the effects of increased UV-B radiation (levels equivalent to a 25% depletion of stratospheric ozone) on the growth of silver birch (*Betula pendula* Roth) seedlings grown in the greenhouse. No visible symptoms of UV-B damage were observed throughout the experiment, height and dry weight of seedlings were also not affected by UV-B radiation. On the other hand, a significant shift in dry weight allocation toward the roots was observed which resulted in a lower shoot/root ratio and leaf area ratio in plants treated with UV-B.

Plant species respond in different ways to UV irradiation. Supplemental UV-B irradiation decreases shoot height of *Triticum aestivum* and *Avena sativa,* but increases it in *Amaranthus retroflexus* and *Kochia scoparia*. However, UV-B increased tillering in all of the four species (Barnes *et al.*, 1990). Different responses were observed on plant root/shoot ratio. Root/shoot increased in sweetgum (*Liquidambar*) after UV-B exposure, but decreased in cassava (*Manihot esculenta*) (Marcel and Jansen, 2002).

Besides the harmful effect of UV-B radiation on plants, it enhances some of the photomorphogenic responses such as inhibition of hypocotyl elongation, twisting of the cotyledons and induction of gene expression (Ballare *et al.*, 1995). Kim *et al.* (1998) found that the hypocotyl length of wildtype *Arabidopsis thaliana* seedlings grown under UV-B radiation was 50% shorter than seedlings grown under dark conditions. In contrast, the hypocotyl length of dark grown *det1* and *cop1* seedlings didn't show significant differences in hypocotyl length under dark or UV-B radiation.

1.4.3 Effect of UV light on plant pigments

Pigments have the ability to absorb low levels of UV-B radiation. These levels will not induce plant cell damage but, simultaneously, can produce signals that will follow the signal transduction pathway and induce DNA transcription resulting in changes in the expression profile (Brosche and Strid, 2003). UV-B exposure resulted in induction of many genes involved in protection against UV damage. These included genes encoding enzymes involved in flavonoid production which will decrease the penetration level of UV-B into the tissues (Mackerness, 2000).

1.4.4 Effect of UV light on plant gene expression

Perception of environmental signals by plants and the transduction of the perceived information, from the location of interaction into the cell, are key factors in the regulation of many gene expression processes. Studying gene expression after UV-B exposure is considered an important method to investigate plant response to UV-B. Studies of differential gene regulation after UV-B exposure have mainly focused on photosynthetic genes and genes encoding proteins required in the biosynthesis of flavonoid compounds (Brosche and Strid, 2003).

Plant exposure to UV-B light results in a reduction in photosynthesis rate. This could be a result of reduction in RUBISCO synthesis or due to the increased degradation rate of this enzyme under UV-B light. The decreased level of transcripts encoding the small (*rbcS*) and large subunits (*rbcL)* of this enzyme or the reduction of transcription of

light harvesting complex proteins results in reduction in the photosynthetic system effectiveness (Mackerness, 2000).

Studies of the effect of UV light on gene expression in maize showed that around 347 genes exhibit change (at least 2-fold) in the pattern of gene expression under UV light compared to control conditions. Out of these, 265 were up-regulated and only 80 were down-regulated. Differences in gene expression were found among plant organs. Leaves for example, which are directly exposed to UV-B, showed up-regulation of certain genes that are down regulated in roots (Casati and Walbot, 2004).

1.4.5 Mechanisms of UV protection

As a protective mechanism against potentially damaging irradiation, plants produce UV-absorbing compounds (Hahlbrock and Scheel, 1989). These compounds are secondary metabolites (mainly phenolic compounds, flavonoids, and hydroxycinnamate esters) which accumulate in the vacuoles of epidermal cells and reduce the penetration of UV radiation into deeper cell layers. Humans use sunscreen with UV-absorbing agents to mimic the powerful plant protection responses (Frohnmeyer and Staiger, 2003). Synthesis of isoflavonoids in legumes is thought to be induced by DNA damage because the wavelength dependency of the response is similar to that for DNA absorption, and acceleration of DNA repair by photoreactivating light can lower the magnitude of the response to UV (Beggs and Wellmann, 1994). Exposure of rice plants to UV radiation resulted in a significant difference in the relative levels of phenolic compounds produced in plant extracts. Tolerant cultivars accumulated higher levels of phenolics than the

sensitive ones (Tanner *et al.*, 1997). Mackerness and Jordan (1999) found that UV-B exposure resulted in a decline in protein levels (e.g. RUBISCO) and disruption of the chloroplast membranes.

In an attempt to enhance the understanding of plant protection mechanisms against UV-B radiation, researchers investigated the role of plant pigments (mainly flavonoids) in UV protection. Flavonoids are not only important in fruit and flower coloration, but they are also involved in a wide range of other biological tasks in plants. Flavonoids are UV-absorbing compounds and accumulate primarily in the epidermal cells of plant tissues after UV exposure. The ability of flavonoids to absorb ultraviolet radiation suggests they may function as shields against potentially harmful radiation (Winkel-Shirley, 2002).

In *Arabidopsis,* the significance of flavonoids in UV protection has also been studied thoroughly. Mutants which are unable to synthesize flavonoids were exposed to UV radiation. These plants were found to be highly sensitive to UV (Harvaux and Kloppstech, 2001). Furthermore, a flavonoid overexpression line of *Arabidopsis* was generated and exposed to high UV-B levels. A high degree of tolerance and a positive correlation between flavonoid content and UV tolerance were observed (Bieza and Lois, 2001). Where seedlings of *Solanum melongena* were grown under UV-B irradiation, no significant effect of UV-B was observed on chlorophyll content in the cotyledons. On the other hand, flavonoid content increased significantly. Furthermore, they found a negative correlation between the level of DNA damage and flavonoid content (Takeda *et al.*, 1994). This suggests a major role of flavonoids as UV-B protectants.

1.5 DNA DAMAGE AND REPAIR

1.5.1 DNA damage

During its life cycle, the living organism's genome is subjected to a wide range of stresses, of either endogenous or environmental origin. The genetic material (DNA), which is composed of sugar residues, phosphodiester linkages and the purine and pyrimidine bases that give informational content to the genome, can suffer from damage. This damage can result in toxic or mutagenic effects (Bray and West, 2005). The modification of the genetic material which is caused by DNA damage is considered to be harmful to all living organisms. There are two main kinds of damage: spontaneous and environmental damage. Spontaneous damage occurs during DNA metabolism and pairing. Environmental damage on the other hand is caused by physical and/or chemical agents such as alkylating agents, UV radiation and fungal or bacterial toxins (Tuteja *et al.*, 2001).

Various types of DNA damage have been found including: photoproduct formation (UV irradiation); base modifications (e.g. methylation); mispairs (mistakes in DNA synthesis); cross-linked nucleotides (DNA-protein) and single and double-stranded DNA breaks (Cagney *et al.*, 2006).

Many of the effects of UV radiation on plants are the direct result of cellular damage caused by UV photons. UV photons can cause unusual photoproducts in macromolecules such as proteins and DNA, and also induce the production of potentially harmful active oxygen species (Boccalandro *et al.*, 2001).

Exposure of living tissues and cells to UV radiation is probably the best studied model system for investigating the biological effects of DNA damage and repair. Although UV-C is completely eliminated by the ozone layer, it is the most widely used source of producing DNA damage due to the maximum absorption of DNA molecules of this radiation, shorter time of damage induction than UV-B and UV-A, its availability from regular germicidal lamps and cheap intensity measurement instrumentation (Chatterjee, 1997) .

DNA molecules are particularly sensitive to UV radiation because UV absorption results in phototransformations, which cause bulky lesions in the DNA strand (Frohnmeyer and Staiger, 2003). DNA lesions have been identified as photoproducts resulting from the formation of covalent bonds between adjacent pyrimidine bases. These photoproducts are cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) which correspond to 75 and 25% of the total UV-induced damage products, respectively (Thoma, 1999).

CPDs and 6–4PPs affect proper base pairing, which results in blocking key cellular processes such as transcription and replication. In addition, these lesions may reduce RNA synthesis, stop or slow down cell cycle progression and induction of apoptosis (Friedberg *et al.*, 1995).

The biological effects of these lesions have been widely investigated in many organisms. It has been shown that pyrimidine dimers have the ability to inhibit the progress of DNA polymerases. At the point where the polymerase finds a lesion during the replication process, the enzyme will try to install the nucleotide which is opposite to the lesion. The aim of that step is to recognize the damaged lesion as mismatched bases

(Britt, 1995).

1.5.2 DNA repair pathways

To avoid the toxic effects of damaged DNA produced by endogenous and environmental toxic agents, most organisms have developed a complex network of repair mechanisms including photoreactivation, excision, and recombination repair (Friedberg *et al.*, 1995). Activation of DNA repair pathways is not the only response to DNA damage. Damaged DNA can activate transcription, cell cycle checkpoints, and chromatin remodelling (Rouse and Jackson, 2002).

1.5.2.1 *Mismatch repair pathway*

The DNA mismatch repair pathway (MMR) plays a significant role in maintaining genetic stability of prokaryotes and eukaryotes. It is a post-transcriptional DNA repair mechanism that is responsible for repairing mismatched bases that are mistakenly incorporated during DNA replication (Jiricny, 2006). Because MMR is necessary for cell cycle arrest and/or programmed cell death in response to some DNA damage types, defects in MMR in human cells will result in hereditary human cancers (Li, 2008). The biochemical and genetic factors in *E. coli* MMR have been broadly studied. Thus *E. coli* MMR is a useful system for understanding the MMR mechanism. The major proteins involved in *E. coli* MMR are MutS, MutL and MutH. MutS can recognize DNA mismatches and small nucleotide insertion/deletion mispairs. The

physical interaction between MutS and MutL enhances mismatch recognition, and also recruits and activates MutH (Li, 2008).

1.5.2.2 *Base excision repair*

The most frequent damage to cellular DNA is base damage. Base excision repair (BER) has the ability to repair and replace a single nucleotide or up to 13 nucleotides. The repair process in general starts with removal of the damaged base using glycosylases, then strand incision of the apurinic or apurimidinic (AP) site using endonuclease (APE1). The gap filling is achieved by DNA polymerases and finally, strand ligation by many proteins including the XRCC1/ligase III complex (Hakem, 2008).

1.5.2.3 *Double strand break (DSB) repair pathways*

DSBs are harmful lesions in both DNA strands that can result from a variety of endogenous and exogenous agents. Ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS) are human syndromes that are a result of defective responses to DNA double-strand breaks. Unrepaired DSBs affect the nervous and immune systems and will exhibit cancer predisposition (McKinnon and Caldecott, 2007). Two mechanisims are involved in DNA DSBs repair: homologous recombination (HR) or non-homologous end joining (NHEJ).

a. Homologous recombination (HR): HR is a multistep pathway that involves many proteins. Defects in HR can result in dramatic consequences, such as the human syndromes AT-like disorder (ATLD) and the NBS (Thompson and Schild, 2002). HR is an error-free pathway that uses a sister chromatid as template DNA to accomplish accurate repair. At the beginning of the HR pathway, Mre11–Rad50–Nbs1 (MRN) makes the 5'-3' cut, Rad51 starts the homology search and strand invasion, and Rad52 and Rad54 proteins promote strand invasion and recombination (Phillips and McKinnon, 2007). It has been shown that the cause of ATLD and NBS is linked to defects in the MRN complex. The MRN complex is important in DSBs NHEJ, telomere maintenance and cell cycle checkpoint activation (Hakem, 2008).

b. Non-homologous end joining (NHEJ): In contrast to HR, NHEJ is an error-prone DSB repair pathway that requires DNA-dependent protein kinase (DNA-PK) and the DNA ligase IV (Lig4) complex, which together assist in re-joining of broken non-compatible DNA ends. In the beginning, the heterodimer Ku70/80 recognizes and binds to the DSB. Then the DNA-PK complex will form by Ku70/80 and DNA-PKcs interaction. Two molecules of DNA-PKs will hold the DNA ends before ligation. The XRCC4/DNA ligase IV complex makes the DNA ligation later on (Phillips and McKinnon, 2007).

1.5.2.4 *Photorepair*

Photoreactivation is a repair system used to remove UV lesions in many organisms. Repair of these UV lesions is catalyzed by an enzyme called photoreactivating enzyme or DNA photolyase. Photolyases rapidly convert UV lesions back to the original undamaged bases in a simple reaction using visible light as a source of energy. This enzyme is found in bacteria, lower eukaryotes and plants. Two types of photolyase have been found, one specific for cyclobutane pyrimidine dimers (CPD photolyase) and another specific for pyrimidine (6-4) pyrimidone photoproducts [(6-4) photolyase] (Yasui and Eker, 1997).

Photoreactivation in *Arabidopsis* involves the existence of two active photolyases, specific for CPDs or 6–4 photoproducts, respectively (Jiang *et al.*, 1997). The repair of (6-4) photoproducts appears more complex than the repair of CPDs because (6-4) photoproduct repair requires the breaking of a covalent bond, then the transfer of a hydroxyl or amino group from the 5′ base end to the 3′ base end (Deisenhofer, 2000). All photolyases are monomeric proteins having molecular masses between 53 to 66 kDa (454–614 amino acid residues) depending on the organism. A plant photolyase cDNA was cloned from wild mustard. It showed a high degree of identity with previously cloned microbial photolyases (Sancar, 1990).

In beans (*Vicia faba)*, photolyase activity was enhanced after plants were exposed to different light regimens. Two fold enhancement was observed after a brief exposure to red light. However, the opposite effect was observed after exposure to far-red light. This result suggests that photolyase induced activity is mediated through phytochrome (Britt *et al.*, 1993).

1.5.2.5 *Nucleotide excision repair*

A flexible and light-independent repair mechanism is called nucleotide excision repair (NER). NER operates to remove many types of lesions, including UV-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4 photoproducts) (Ford *et al.*, 1997).

NER is a complex multi-step process (Figure 1.3) that involves the concerted action of more than 20 proteins. NER involves several distinct steps: damage recognition (see below), DNA unwinding by two DNA helicases, called XPB and XPD, then excision of the damaged DNA, which requires two nucleases. The first nuclease XPF: ERCC1 makes a cut at the 5' end of the DNA lesion. At the 3' end of the DNA lesion another nuclease called XPG makes the cut. Finally gap filling and strand ligation is achieved by a variety of enzymes including DNA polymerase (Shuck *et al.*, 2008).

The NER system consists of two subpathways. The first one is global genomic repair (GGR), which is involved in the repair of DNA lesions across the genome (this repair pathway is not actively targeted to specific regions of the genome). The second repair pathway is transcription-coupled repair (TCR), which is targeted to areas of active transcription and involves repair activity that is directed to DNA lesions in the transcribed strand of active genes (Hoffen *et al.*, 2003).

Various genes involved in NER have been isolated from humans, yeast, and plants (Figure 1.3). These genes show significant similarity in sequence, suggesting that the NER pathway is essential in many if not all organisms (Britt, 1996). In the past decade,

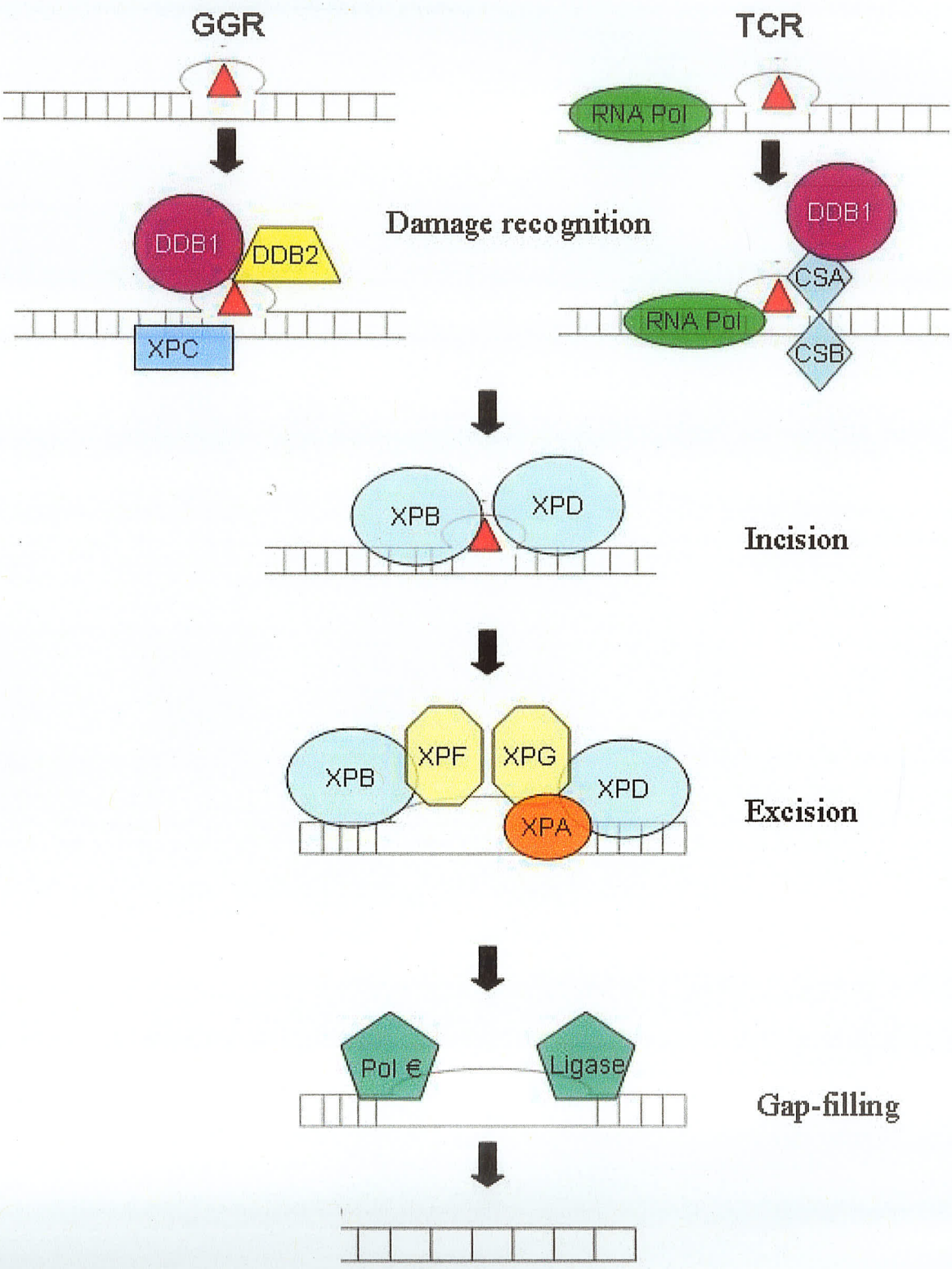


Figure 1.3: **Nucleotide excision repair pathway (NER)**. GGR: global genome repair. TCR: transcription-coupled repair

NER in plants have been widely investigated. Biochemical and genetic evidence suggests that the NER mechanism is involved in plant DNA repair (Kunz *et al.*, 2006).

Xeroderma pigmentosum is a photosensitive disorder in humans resulting from inherited defects in NER genes. It is subdivided into seven complementation groups (XP-A to -G). XP patients display an increased sensitivity of the skin to UV light. In addition, XP patients have a highly elevated risk of developing UV-induced skin cancer. In global genomic repair, many proteins are involved in damage recognition, such as XPC, XPA, replication protein A (RPA) and the damaged DNA binding protein complex (DDB: DDB1 and DDB2). XPA is a 31-kDa protein that can bind double-stranded DNA and interact with RPA, ERCC1, and TFIIH. RPA is a single-stranded DNA binding protein that is important in DNA replication, recombination, damage recognition and dual incision. It has been shown that RPA changes the enzymatic activity of many proteins, such as RNA and DNA polymerases, helicases, and nucleases. XPC is a large DNA-binding protein (106-kDa) with a preference for single-stranded, damaged DNA. It has been shown that XPC is found in a complex with HR23B, which is important for XPC stabilization during excision repair (Reardon and Sancar, 2005). The role of the damaged DNA binding protein complex will be discussed later in this chapter.

The transcription coupled repair pathway (TCR) is a process that targets DNA lesions that are located on transcribed strands of active genes and are responsible for stalling of the elongating RNA polymerase. In general, it has been found that TCR is more rapid and/or more efficient in removal of certain types of DNA damage from the actively transcribed strands of expressed genes compared with the nontranscribed strands

(Mellon *et al.*, 1987). TCR is a highly conserved repair pathway identified in a variety of organisms including bacteria, yeast, mammals and plants.

Cockayne syndrome (CS) is a rare autosomal recessive disease with a wide variety of symptoms including physical and mental retardation, developmental abnormalities, UV-sensitivity, brain degeneration and pigmentary degeneration of the retina followed by pronounced cachexia leading to early death. At the cellular level, CS patients are unable to continue damage-inhibited DNA and RNA synthesis after exposure to UV-light. Two CS complementation groups, CSA and CSB, have been identified. Patients cells in these groups lack RNA synthesis recovery after UV-light irradiation and exhibit mild UV sensitivity (Fousteri and Mullenders, 2008).

The CSA gene encodes a protein containing WD-40 repeats, a motif that is involved in protein-protein interactions. It has been shown that CSA is part of an E3-ubiquitin ligase (E3-Ub ligase) complex containing DDB1, Cullin 4A and ROC1/Rbx1 (O'Connell and Harper, 2007). On the other hand, the CSB gene encodes a 168 kDa protein and is a member of the SWI/SNF family of ATP-dependent chromatin remodellers (Selby and Sancar, 1997).

The mechanism of the TCR pathway is complex and involves many proteins. In the absence of UV, RNA polymerase II interacts with CSB and possibly XPG to promote transcription elongation. This interaction becomes more stable upon UV irradiation. Subsequently, CSB is recruited to the lesion where RNA polymerase II is stalled. Other proteins are also recruited such as HAT p300, TFIIH, XPG, RPA, and inactive CSA/DDB1- CSN E3-Ub ligase complex. Other NER components are also recruited to the site of stalled RNA polymerase II. The assembly of these factors will generate an

efficient repair complex that has the ability to generate a new 3′ end mRNA which will resume transcription after elimination of the damaged DNA. An increased interaction of CSA, DDB1, and CSN1 with CSB and RNA polymerase II was observed in chromatin of UV-exposed TCR-proficient cells. This interaction was found to be mediated through direct interaction of CSA with stalled RNA polymerase II but it is CSB dependent (Fousteri *et al.*, 2006).

1.6 DDB COMPLEX

The Damaged DNA-Binding protein complex (DDB) is important in DNA repair. DDB may function to alter chromatin structure and recruit nucleotide excision repair (NER) factors to DNA damage sites (Takata *et al.*, 2004). This complex can distinguish many DNA lesions which are induced by treatment with DNA-damaging factors (Fujiwara *et al.*, 1999). In humans, DDB is a heterodimeric protein complex consisting of a 127 kDa subunit (DDB1) and a 48 kDa subunit (DDB2). The two DDB subunits have a higher affinity for various types of DNA lesions compared with the other damage recognition factors (XPA, RPA, XPC-HR23B, and TFIIH) (Wakasugi *et al.*, 2001).

While DDB has been implicated in DNA repair, the mechanism by which DDB participates in DNA repair has remained unclear. A possible role of DDB in NER presumably is in damage recognition (Wakasugi *et al.*, 2001). El-Mahdy *et al.* (2006) reported that DDB2 protein localizes to the site of UV-induced lesions within minutes after UV-irradiation. Furthermore, the binding activity of XPC to the damaged lesion is accelerated by the presence of DDB2.

Mutations in the p48 subunit of DDB (DDB2) have been characterized from XP-E patients (Datta *et al.*, 2001). The repair-deficiencies in XP-E cells are milder compared to other groups of *Xeroderma pigmentosum*. XP-E cells showed a 50–60% reduction in the repair activity compared to normal cells (Keeney *et al.*, 1994).

Cloning of DDB homologues in rice (*Oryza sativa* cv. Nipponbare) showed a protein with a molecular mass of 122 kDa and 1090 amino acid residues that encodes DDB1 and another protein with molecular mass of 65 kDa and 584 amino acid residues that encodes DDB2 (Ishibashi *et al.*, 2003). Comparison of rice UV-DDB1 amino acid sequence with other previously reported eukaryotic DDB1 resulted in 78.0%, 53.0%, 53.2%, and 32.3% amino acid sequence identity with *Arabidopsis thaliana*, *Homo sapiens*, *Chlorocebus aethiops* and *Caenorhabditis elegans* DDB1, respectively. On the other hand, rice DDB2 showed only 62.6% and 23.0% identity with *A. thaliana* and *H. sapiens* DDB2, respectively. The *DDB1* transcript was detected in young leaves, roots and more strongly in root tips of rice (Ishibashi *et al.*, 2003).

In *Arabidopsis*, two homologues of DDB1 have been found: DDB1A and DDB1B, these two homologues are almost identical at the amino acid level (91%). *DDB1A* ESTs are detected in roots, siliques, adult shoots and seeds of *Arabidopsis*. Schroeder *et al.* (2002) studied *ddb1a* and *ddb1b* mutants in *Arabidopsis* using T-DNA insertions. They found that *ddb1a* mutants showed no obvious phenotypic differences from the wildtype plants. In contrast, *ddb1b* mutants were appear to be lethal.

In *Drosophila*, Takata *et al.* (2004) tried to generate a *DDB1* mutant using the RNAi method. Unfortunately all the *DDB1* gene knocked down flies were dead, suggesting a crucial role of DDB1 in development. Yoon *et al.* (2005) generated a mouse strain

deficient in DDB2. Those mutants showed high susceptibility to skin cancer after exposure to UV irradiation. In addition, the CPD repair rate was found to be less than that of the wildtype mice.

UV irradiation in humans induces *DDB2* transcription in a process that is p53 dependent (Liu *et al.*, 2000). p53 is a tumor suppressor protein that controls cell cycle arrest, apoptosis and DNA repair after DNA damage. The role of p53 in the NER pathway is not completely understood. Nevertheless, p53 has been reported to regulate expression of NER genes such as *XPC* and *DDB2* (*XPE*) in human cells (Adimoolam *et al.*, 2003). In plants, no homolog of p53 has been found so far, therefore other plant factors could be performing the p53 functions (Whittle *et al.*, 2001).

In humans DDB2 is located in the nucleus, while DDB1 is primarily cytoplasmic. DDB1 translocates to the nucleus after exposure to UV irradiation by a mechanism that is believed to be DDB2-dependent (Liu *et al.*, 2000). DDB2 is degraded at early stages after UV exposure in human cells by CUL-4A (Rapic-Otrin *et al.*, 2002). CUL-4A is a member of the cullin family, subunits of ubiquitin-protein ligases. CUL-4A is localized in the cytoplasm and expressed at high levels in many tumor cells. Chen *et al.* (2001) found that CUL-4A can physically interact with the damaged DNA-binding protein complex (DDB). Groisman *et al.* (2003) found CUL-4A in a complex with DDB1, DDB2 and the COP9 signalosome. They reported that the degradation by ubiquitin ligase of DDB2 is regulated by the COP9 signalosome.

Many proteins have been found to interact with DDB1 and form a variety of complexes. DDB1 is a component of the newly identified multisubunit complexes that contain cullin-4 and other proteins. These complexes display an E3 ligase activity that

recognizes specific substrates and mediates their degradation (Lee and Zhou, 2007). DDB1 has been shown to act as a linker to recruit receptor proteins (WD40) via a conserved protein motif (the DWD box) to the E3 ligase machinery (He *et al.*, 2006). The DDB1–Cul4A ligase has been shown to target a variety of substrates such as the DNA replication licensing factor Cdt1 (Hu *et al.*, 2004) and several histones (Kapetanaki *et al.*, 2006). In humans, in addition to DDB1, the CUL4A complex can contain DET1, a highly conserved protein. This complex is involved in ubiquitination and degradation of the proto-oncogenic transcription factor c-Jun (Wertz *et al.*, 2004). Also, DDB1 is integrated into nearly identical complexes with DDB2 and CSA with important roles in GGR and TCR, respectively (Groisman *et al.*, 2003). Furthermore, DDB1 (and its partner DDB2) interact in vivo and in vitro with p300 which has histone acetyltransferase (HAT) activity (Datta *et al.*, 2001). Another study (Rapic-Otrin *et al.*, 2002) showed that DDB1 – p300 interaction is DDB1 dependent but not DDB2 dependent. DDB1 may form another complex with SPT3-TAF_{II}31-GCN5L acetylase (STAGA). This complex may be involved in the NER pathway by facilitating the assembly of the repair machinery on the nucleosome by chromatin unfolding (Martinez *et al.*, 2001).

The main goal of this study was to investigate the role of DET1 and Damaged DNA Binding Proteins (DDB1A & DDB2) in *Arabidopsis* DNA repair and light signaling. In order to achieve this broad goal, we subdivided it into different experiments, each of which answers a question and as a whole they meet the primary goal. Different experiments were designed to:

1- Study the effect of light on growth and development of single, double and triple *Arabidopsis* mutants of *det1, ddb1a* and *ddb2*

2- Study the effect of *DDB1A* overexpression on *Arabidopsis* growth and development

3- Study the effect of *DDB1A* overexpression on *Arabidopsis* DNA repair

4- Investigate the role of *DDB2* on *Arabidopsis* DNA repair

5- Study the genetic interaction between *DDB2* and *DET1* under UV irradiation

CHAPTER 2: *DDB2*, *DDB1A* AND *DET1* EXHIBIT COMPLEX INTERACTIONS DURING *ARABIDOPSIS* DEVELOPMENT

2. *DDB2*, *DDB1A* AND *DET1* EXHIBIT COMPLEX INTERACTIONS DURING *ARABIDOPSIS* DEVELOPMENT

2.1 ABSTRACT

Damaged DNA Binding proteins 1 and 2 (DDB1 and DDB2) are subunits of the Damaged DNA Binding protein complex (DDB). DDB1 is also found in the same complex as DE-ETIOLATED 1 (DET1), a negative regulator of light-mediated responses in plants. *Arabidopsis* has two *DDB1* homologues, *DDB1A* and *DDB1B*. *ddb1a* single mutants have no visible phenotype while *ddb1b* mutants are lethal. We have identified a partial loss of function allele of *DDB2*. To understand the genetic interaction between *DDB2, DDB1A,* and *DET1* during *Arabidopsis* light signaling, we generated single, double and triple mutants. *det1 ddb2* partially enhances the short hypocotyl and suppresses the high anthocyanin content of dark grown *det1*, and suppresses the low chlorophyll content, early flowering time (days), and small rosette diameter of light grown *det1*. No significant differences were observed between *det1 ddb1a* and *det1 ddb1a ddb2* in rosette diameter, dark hypocotyl length and anthocyanin content suggesting these are *DDB1A*-dependent phenotypes. In contrast, *det1 ddb1a ddb2* showed higher chlorophyll content and later flowering time than *det1 ddb1a*, indicating these are *DDB1A*-independent phenotypes. We propose that the DDB1A-dependent phenotypes indicate a competition between DDB2- and DET1- containing complexes for available DDB1A, while for DDB1A-independent phenotypes DDB1B is able to fulfill this role.

2.2 INTRODUCTION

Plant development is dependent on environmental conditions. Because light is the energy source for plant growth, plants have evolved highly sensitive mechanisms for perceiving light. This information is used to regulate development and to maximize light utilization for photosynthesis. The transition from the vegetative to the reproductive stage is also regulated by light. Seedlings implement different developmental programs when grown in light or darkness. Light-grown seedlings undergo photomorphogenesis, exhibiting short hypocotyls, open and expanded cotyledons, and photosynthetically active chloroplasts. In contrast, seedlings grown under dark conditions are etiolated, having closed and unexpanded cotyledons, elongated hypocotyls and undeveloped chloroplasts. This developmental pattern is known as skotomorphogenesis (Chen *et al.,* 2004).

This developmental switch (etiolation/de-etiolation) is under the control of at least 10 genes *(COP/DET/FUS).* Molecular genetic studies in *Arabidopsis* indicate that these proteins function downstream of the photoreceptors to repress photomorphogenesis in the absence of light. Mutation of these genes results in seedlings with a de-etiolated (*det*) or constitutive photomorphogenic (*cop*) phenotype when grown under dark conditions. The null mutations of these genes are seedling lethal with high anthocyanin levels (*fus*) (Wang and Deng, 2002). COP1 is a WD-40 and RING finger protein with E3 ubiquitin ligase activity, which targets photoreceptors and downstream transcription factors for ubiquitination and subsequent degradation. The COP9 signalosome (CSN) is a multiprotein complex composed of eight subunits which associates with and supports the activity of multiple cullin-containing E3 ubiquitin ligase complexes. In *Arabidopsis*,

mutants in CSN components also exhibit the constitutive photomorphogenic/de-etiolated/fusca (*cop/det/fus*) phenotype (Schwechheimer and Calderon, 2004).

De-etiolated 1 (*det1-1*) partial loss of function mutants exhibit short hypocotyls, open cotyledons and high anthocyanin levels in the dark (Chory *et al.*, 1989). Under light conditions, *det1-1* seedlings are smaller and paler than wildtype. In addition, they show reduced apical dominance, day length insensitive flowering (Pepper and Chory, 1997) and defects in germination, expression of light regulated genes and chloroplast development (Chory and Peto, 1990). Approximately one thousand genes are either up or down regulated in *det1-1* compared to wildtype (Schroeder *et al.*, 2002).

DET1 is a nuclear protein (Pepper *et al.*, 1994) present in a complex with an approximate mass of 350 kDa. In tobacco BY2 cells, this complex includes the plant homolog of UV-Damaged DNA Binding protein 1 (DDB1). In *Arabidopsis*, two homologues of DDB1 have been found: DDB1A and DDB1B, which are 91% identical at the amino acid level. *Arabidopsis* DDB1A matches tobacco DDB1 more closely than *Arabidopsis* DDB1B (Schroeder *et al.*, 2002). *DDB1A* is expressed at higher levels (almost 2 fold) than *DDB1B* throughout the *Arabidopsis* life cycle in all organs studied (Figure 2.1 A). *ddb1a* and *ddb1b* mutants in *Arabidopsis* were studied using T-DNA insertions. *ddb1a* mutants show no obvious phenotype in a wildtype background, but mutation of *DDB1A* in the *det1* background enhanced *det1* phenotypes. In contrast to *ddb1a*, *ddb1b* mutants are lethal, suggesting a crucial role for DDB1B during *Arabidopsis* development. DDB1 is evolutionarily conserved as *Arabidopsis* DDB1A is 83% and 46% identical at the amino acid level with rice and human DDB1, respectively (Schroeder *et al.*, 2002).

The *Arabidopsis* DDB1A protein is homologous to human DDB1 (127 kDa), a component, along with DDB2 (48 kDa), of the damaged DNA-binding protein complex (DDB). DDB1 is present at higher levels than DDB2 in human cells (Liu *et al.,* 2000). DDB1 is present in the cytoplasm, but upon UV irradiation, translocates to the nucleus. Loss of DDB2 function prevents accumulation of DDB1 in the nucleus (Shiyanov *et al.,* 1999), whereas loss of DDB1 function had no effect on binding activity of DDB2 to the damaged DNA (Li *et al.,* 2006a). The suggested role of the DDB complex is to recognize DNA lesions, initiating nucleotide excision repair (NER). In humans, the rare inherited disease *Xeroderma pigmentosa* group E (XPE) results from mutation of *DDB2.* XP patients display an increased skin sensitivity to UV light and are at highly elevated risk of developing UV-induced skin cancer (Cleaver, 2005).

Recently, human DDB1 and DDB2 were found to be components of an E3 Ubiquitin ligase. DDB1, along with CUL4 and ROC1, is a component of several types of E3 ligases, including one with DDB2 and another with the transcriptional coupled repair factor CSA. Both these E3 ligase complexes are regulated by the COP9 signalosome (Groisman *et al.,* 2003). Subsequently, DDB1-CUL4 complexes were found to interact with many WD40-repeat proteins and use them as adaptors to recognize substrates for proteolysis (Higa *et al.,* 2006; He *et al.,* 2006; Jin *et al.,* 2006; Angers *et al.,* 2006). Human DDB1 is also found in a complex with CUL4, ROC1, DET1 and COP1 (Wertz *et al.,* 2004). *Arabidopsis* DDB1A and DET1 copurify with the E2 Ub conjugase variant COP10 (Yanagawa *et al.,* 2004) and these proteins have recently been found to form a complex with AtCUL4 and RBX1 (ROC1) (Bernhardt *et al.,* 2006; Chen *et al.,* 2006). Thus DDB1 appears to be a central component of CUL4 E3 ligases.

The DDB complex is present not only in humans, but also in other organisms such as Rice (*Oryza sativa* cv. Nipponbare) (Ishibashi *et al.,* 2003). *Arabidopsis* DDB2 shows 59% and 30% identity with rice and human DDB2, respectively. In this study we examined the role of *Arabidopsis DDB2* and its interaction with *DDB1A* and *DET1* in light signaling. All combinations of double and triple mutants were generated in order to understand the genetic interaction between these genes. Plants were grown and analyzed at different developmental stages. Comparison between the mutants revealed complex interactions between these genes. In some cases the modulation of *det1* phenotypes by *ddb2* was *DDB1A* dependent, in other cases it was *DDB1A* independent. We interpret these results as consistent with a model whereby separate DET1 and DDB2 containing complexes compete for DDB1A, in the case of the dependent phenotypes, and where DDB1B is able to fill this role, in the case of DDB1A-independent phenotypes.

2.3 MATERIALS AND METHODS

2.3.1 Plant materials and growth conditions

All mutations used in this experiment were in the *Col* background of *Arabidopsis thaliana.* The *DET1* partial loss of function allele *det1-1* and *ddb1a* T-DNA mutant were described previously (Pepper *et al.,* 1994; Schroeder *et al.,* 2002) and the *ddb2* allele (SALK_040408) was obtained from the *Arabidopsis* Stock Center (http://www.arabidopsis.org/). *cop1-4* was kindly provided by X. W. Deng, Yale University. Seedlings were grown in a growth chamber at 20° and 50% relative humidity. Light was provided by fluorescent bulbs (100 μmol photons. m^{-2}. s^{-1}). Plants were grown

in Sunshine mix number 1 (SunGro, Bellevue, WA). Short day (SD) conditions corresponded to 10 h light and 14 h dark, long day conditions (LD) conditions consisted of 16 h light and 8 h dark.

2.3.2 Construction of double and triple mutants

Double-mutants: The *det1 ddb1a* mutant was generated as described in Schroeder *et al.* (2002), *cop1-4 ddb2*, *det1 ddb2* and *ddb1a ddb2* double mutants were derived from genetic crosses of their corresponding single-parental mutants. Because all mutations analyzed were recessive, double homozygous plants were identified in the F_2 generation, where they occur in a ratio close to 1:15. Putative double mutants in the F_2 generation were selected based on mutant phenotypes and PCR genotyping. For example, for the *ddb2 x det1* cross, ~100 F_2 seeds were plated, segregating *det1* homozygotes identified by their dwarf stature, transplanted and genotyped for *ddb2*. In the F_3 generation, several independent *det1 ddb2* double mutant lines consistently exhibited shorter hypocotyls and decreased anthocyanin in dark grown seedlings and increased chlorophyll in light-grown seedlings relative to their *det1 DDB2/-* siblings.

Triple-mutant: Pollen from plants homozygous for *ddb2* was used to fertilize flowers of *det1 ddb1a* plants. As expected, all F_1 plants showed wildtype phenotype. PCR was used to confirm the presence of the *ddb2* insertion. Plants heterozygous for *ddb2* and *det1 ddb1a* were selfed to produce F_2 (*DET1* and *DDB1A* are linked, approximately 10 cM apart (Schroeder *et al.,* 2002)). *det1 ddb1a* homozygotes were identified as extreme dwarfs and were then genotyped to identify *ddb2* homozygotes. Due to the infertility of

this triple mutant, stocks are maintained as a segregating population homozygous for *ddb2* and heterozygous for *det1 ddb1a.*

PCR reactions were conducted to confirm the *ddb1a* and *ddb2* insertions. DNA was extracted according to Weigel and Glazebrook (2002). *ddb1a* insertions were detected using LB2 (5'–TTGGGTGATGGTTCACGTAGTGGGCCATCG-3') and UV1.17 (5'–ACTGGGCTCAACTAGAAAATATGGAACAA-3') while UV1.17 and UV1.1 (5' –GTCTTGACTGTGCATTTCAGAGTGCTTAT-3') were used to detect the wildtype *DDB1A*. For *ddb2*, LB2 and DDB2.1 (5'–TTGGGTGATGGTTCACGTAGT GGGCCATCG-3') were used, while DDB2.1 and DDB2.3 (5'–ACGACGTGTTTT GTCGGTGTGGAAGAA-3') were used for wildtype *DDB2*.

2.3.3 RNA extraction and RT-PCR

Total RNA was extracted from 7-day-old seedlings using the RNeasy plant minikit (Qiagen) according to the manufacturer's instructions. Quality and quantity of isolated RNA was checked by denaturing gel electrophoresis and by spectrophotometric analysis. cDNA synthesis and PCR-amplification were performed in the same reaction using Access RT-PCR kit (Promega) according to the manufacturer's instructions. *DDB2* specific primers in exon 2 (5'- ACAGCCTGGCCATGAAGCTGGA-3') and in exon 6 (5'-CCTGCCATCCATCAGGGTTGAG -3') were used. cDNA synthesis was performed at 45° for 45 min, followed by PCR (5 min 94°, 30x (30 sec 94°, 50 sec 67°, 2 min 72°), 2 min 72°). To detect relative differences in transcript levels, amplification was performed when the PCR product was accumulating exponentially with respect to cycle number (30

cycles). *UBQ10* was used as an internal control (5'-GATCTTTGCCGGAAAACAATTG GAGGATGGT-3' and 5'-CGACTTGTCATTAGA AAGAAAGAGATAACAGG-3') (5 min 94°, 22x (1 min 94°, 1 min 64°, 1 min 72°), 2 min 72°). PCR products were separated on 1% (w/v) agarose gels, the intensities of ethidium bromide-stained bands were determined using ImageJ software (1.36b National Institutes of Health, USA).

2.3.4 Hypocotyl elongation assay

Seeds were plated on Murashige and Skoog (MS) media (1X MS salts, 0.8% phytoagar, 2% sucrose) after sterilization and stratified at 4° for two days. Plates were transferred to a growth chamber and grown either under light (16 h light 80 μmol. m^{-2}. sec^{-1}) or dark conditions (after 6 hr light as germination enhancer). Seven days later, hypocotyl length was measured for at least 10 seedlings using NIH image software.

2.3.5 Pigment analysis

For chlorophyll measurement, 7-day-old seedlings were extracted with 80% acetone overnight, the A_{645} and A_{663} determined in a spectrophotometer (Model 2100 pro, Ultrospec) and chlorophyll content calculated according to the method of Mackinney (1941). Anthocyanin was determined using standard technique (Fankhauser and Casal, 2004). Experiments were repeated at least three times with four replicates per line in each experiment. Each replicate contained at least 50 mg of plant tissue.

2.3.6 Growth parameter measurements

Seedlings were transplanted from plates to soil at 14 days old. For each line, 18 plants were used for growth parameter analysis. Flowering time was scored for each plant as number of days until the first bud became visible. Also, the total number of rosette and cauline leaves on the main inflorescence was counted. Plants were moved regularly to random positions in the growth chamber to prevent any positional effects on plant growth. The following traits were also recorded: shoot fresh weight (4 weeks old), rosette diameter (4 weeks old), total number of inflorescences and height (6 weeks old).

2.3.7 Statistical Analysis

All experiments were repeated at least three times. Results presented are means with 95% confidence intervals of ten to eighteen replicates. Means were compared by student *t* test. Probabilities of 0.05 or less were considered to be statistically significant.

2.4 RESULTS

To examine the role of *DDB2* in light signaling and interaction with *DDB1A* and *DET1*, we studied the physiological role of these proteins at different stages of *Arabidopsis* development. Gene expression data available at the Genevestigator website (https://www.genevestigator.ethz.ch) shows that *DDB2* is expressed in all *Arabidopsis* organs, as are *DDB1A, DDB1B* and *DET1* (Figure 2.1 A). A T-DNA insertion in *DDB2*

(SALK_040408) was obtained from the *Arabidopsis* Stock Center (http://www.arabidopsis.org). This insertion is located 180 bp upstream of the *DDB2* ATG (Figure 2.1 B). PCR genotyping was performed to confirm this insertion (Figure S2.1). RT-PCR analysis showed that *DDB2* expression was reduced by approximately two fold in *ddb2* mutants compared to the wildtype (Figure 2.1 C,D). Loss of function of *DDB2* in other mutant backgrounds results in similar relative levels of expression (Figure 2.1 C,D). This data suggests that this T-DNA insertion results in partial loss of function of *DDB2*. This result is in contrast to Koga *et al.* (2006) who observed complete absence of *DDB2* expression in the same T-DNA insertion line (SALK_040408). This variation in transcript level may be due to co-suppression.

For all the growth parameters measured in the following sections, no significant differences were observed between wildtype, the single mutants *ddb2*, *ddb1a* and the double mutant *ddb1a ddb2*. Since we had previously shown that *ddb1a* exhibited no phenotype on its own yet enhanced the phenotypes of the *DET1* partial loss of function allele *det1-1*, we generated the *det1-1 ddb2* double mutant. The triple mutant was generated to determine if *det1-1 ddb2* phenotypes were *DDB1A* dependent or independent.

2.4.1 Dark grown seedlings

Hypocotyl elongation: In the absence of light, wildtype plants show increased hypocotyl elongation, but *det1* mutants lack this response and have short hypocotyls. Lines carrying mutations in *DET1*, *DDB1A* and *DDB2* singly or in combination were

grown under dark conditions for 7 days to study the role of these proteins in de-etiolation and light signaling (Figure 2.2). Plants with partial loss of function of *DDB2* in the wildtype or *ddb1a* background did not differ significantly from wildtype. In contrast, mutation of *DDB2* in the *det1* background resulted in enhancement of the *det1* short hypocotyl phenotype ($P \leq 0.001$), resulting in 31% shorter hypocotyls. However, the triple mutant *det1 ddb1a ddb2* did not differ significantly from the double mutant *det1 ddb1a.* Thus enhancement of the *det1* short hypocotyl phenotype by *ddb2* is a *DDB1A*-dependent phenotype.

Anthocyanin content: We studied anthocyanin accumulation in seedlings, which ordinarily does not accumulate when plants are grown under dark conditions. Very low levels of anthocyanin were detected in Col-0, *ddb2*, *ddb1a* and *ddb1a ddb2* seedlings (Figure 2.2 C). *det1* showed higher levels of anthocyanin than the wildtype (18 fold). The *ddb2* mutation in the *det1* background suppressed this increase in anthocyanin content, showing only 28% of the levels observed in the *det1* single mutant. In contrast to *ddb2* partial loss of function in the *det1* background, *ddb1a* loss of function in the same background showed an increase in anthocyanin content, with 6.5 fold enhancement. The anthocyanin content of the triple mutant *det1 ddb1a ddb2* exhibited no significant difference from the double mutant *det1 ddb1a.* Thus, *ddb2* exhibits *DDB1A*-dependent suppression of *det1* dark anthocyanin accumulation.

2.4.2 Light grown seedlings

Hypocotyl elongation: In light, *det1* seedlings are also shorter than wildtype. Growth of wildtype and *ddb2*, *ddb1a* and *ddb1a ddb2* mutants under long day conditions for 7 days showed no significant differences (Figure 2.3 A,B). Loss of function of *DDB2* in the *det1* background did not affect the *det1* short hypocotyl. In contrast, mutation of *DDB1A* in the same background reduced hypocotyl elongation ($P \leq 0.05$). No significant differences were observed between *det1 ddb1a* and *det1 ddb1a ddb2*. Thus *ddb2* has no effect on this phenotype.

Anthocyanin content: When grown under light conditions, seedlings accumulate anthocyanin. Col-0, *ddb2*, *ddb1a* and *ddb1a ddb2* had similar levels while *det1* showed more than a 60% increase compared to wildtype (Figure 2.3 A,C). While no significant difference was observed between *det1* and *det1 ddb2*, the increased anthocyanin content of *det1* was dramatically enhanced in *det1 ddb1a*. However *ddb2* significantly suppressed ($P \leq 0.03$) anthocyanin accumulation in the *det1 ddb1a* double mutant as levels in the triple mutant were only 76% that of the double mutant. Thus, *ddb2* partially suppresses anthocyanin accumulation in light grown *det1 ddb1a* seedlings but not the *det1* single mutant.

Chlorophyll content: In light, *det1* mutants accumulate less chlorophyll than wildtype. Wildtype, *ddb2*, *ddb1a* and the double mutant *ddb1a ddb2* all showed similar levels of chlorophyll accumulation (Figure 2.3 D). *det1* showed a decrease compared to the previous genotypes with only 34% of wildtype chlorophyll content. Loss of function of *DDB2* in the *det1* background partially suppressed the pale color of *det1* and resulted in 60% more chlorophyll in the double mutant. In contrast, loss of function of *DDB1A* in the *det1* background showed similar chlorophyll content as *det1* alone. Like the effect of

the *ddb2* mutation on the *det1* background, loss of function of *DDB2* in the *det1 ddb1a* background resulted in more chlorophyll accumulation in the triple mutant *det1 ddb1a ddb2* (2 fold). This suggests that suppression of the *det1* pale phenotype by *ddb2* is *DDB1A* independent.

2.4.3 Adult plants

Rosette diameter: Adult *det1* are also smaller than wildtype, so after one month of growth under either long day or short day conditions, rosette diameter was measured for all genotypes. Analysis of rosette diameter for different genotypes showed similar trends when either grown under long or short day conditions. In general, plants grown under long day conditions showed larger rosette diameter than those grown in short day. *ddb2, ddb1a,* and *dd1a ddb2* loss of function mutants did not differ significantly from wildtype (Figure 2.4 A,B). This result is in contrast to Koga *et al.* (2006), who found that *DDB2* mutation resulted in reduction in leaf length and width. Although our growth conditions vary slightly from theirs, we find this result to be independent of growth conditions (Figure S2.2). Our weaker phenotype is consistent with the fact that we still observe approximately 50% of wildtype *DDB2* expression in our *ddb2* mutants while Koga *et al.* (2006) used an RNA null. *det1* showed smaller rosette diameter than wildtype (Figure 2.4 A,B). The *ddb2* mutation partially suppressed the *det1* small rosette diameter in both long and short day conditions ($P \leq 0.01$). In contrast, the *ddb1a* mutation significantly enhanced this phenotype in the *det1* background, resulting in smaller rosette diameter. Mutation of *ddb2* in the *det1 ddb1a* background has no significant effect under both conditions.

Therefore, the partial suppression of the *det1* small rosette diameter by *ddb2* is *DDB1A* dependent.

Flowering time: To examine the role and interaction of these proteins in controlling *Arabidopsis* flowering time, we compared wildtype, single mutants, double mutants and the triple mutant grown in short and long days. *Arabidopsis* is a facultative long day plant, so flowering in wildtype is accelerated in long days. We found that wildtype, *ddb2*, *ddb1a* and *ddb1a ddb2* start flowering after 26 and 62 days on average in long and short day conditions, respectively (Figure 2.4 C). Loss of function of *DET1* resulted in early flowering time compared to the wildtype (18 or 22 days, respectively). The double mutant *det1 ddb2* showed significant suppression ($P \leq 0.01$) of *det1* early flowering time (20 and 27 days respectively). No significant difference was observed between *det1 ddb2* and *det1 ddb1a* in both photoperiods. The triple mutant *det1 ddb1a ddb2* showed significant ($P \leq 0.01$) suppression of early *det1 ddb1a* flowering time, flowering at 24 and 32 days respectively. Thus suppression of *det1* early flowering time (days) by *ddb2* is a *DDB1A*-independent phenotype.

In addition, flowering time was measured by counting the number of rosette leaves plus cauline leaves on the main inflorescence. *ddb2*, *ddb1a* and *ddb1a ddb2* had the same number of leaves as wildtype at flowering time. *ddb2* suppressed significantly ($P \leq 0.05$) the early flowering time of *det1* in terms of number of leaves at flowering under long day conditions (Figure S2.3) but not under short days (data not shown). No significant differences were observed between the double mutant *det1 ddb1a* and the triple mutant *det1 ddb1a ddb2* in either condition. Therefore, suppression of *det1* early

flowering time (number of leaves) by *ddb2* is a *DDB1A*-dependent phenotype in long day conditions but has no effect in short day conditions.

Height: After full adult height had been achieved under long or short day conditions, plant height was measured from the soil surface to the last flower on the inflorescence. *ddb2*, *ddb1a* and *ddb1a ddb2* did not differ from wildtype (Figure 2.4 A,D). *det1* mutants are 35% shorter than wildtype. No significant difference was observed between *det1* and *det1 ddb2*. In contrast, *det1 ddb1a* significantly enhanced the short *det1* phenotype under both photoperiods. Loss of function of *DDB2* in the *det1 ddb1a* background resulted in further enhancement of the short phenotype ($P \leq 0.01$). *det1 ddb1a ddb2* plants showing only 50% of *det1 ddb1a* height under long day and 21% under short day conditions. Thus *ddb2* enhances the dwarf phenotype of the double mutant *det1 ddb1a*.

2.3.4 Fertility assessment

The observation that the double mutant *det1 ddb1a* produced few and very small seed-containing siliques encouraged us to look thoroughly at floral development in the different genotypes. Long day grown wildtype, *ddb2*, *ddb1a* and *ddb1a ddb2* did not have significantly different silique lengths (Figure 2.5 A). *det1* exhibit shorter silique length, but mutation of *ddb2* in this background partially suppresses the short silique phenotype ($P \leq 0.01$). The double mutant *det1 ddb1a* showed very short siliques (4.75 mm). Loss of function of *DDB2* in the *det1 ddb1a* background enhanced this short silique phenotype and resulted in 1.63 mm siliques on average. After dissecting at least 6 siliques from each

genotype, we counted the number of seeds in each half. No significant differences were observed between wildtype, *ddb2*, *ddb1a* and *ddb1a ddb2,* with 29 -33 seeds in each half silique (Figure 2.5 B). The *det1* mutant showed lower seed numbers compared to the previous genotypes. Again, the *ddb2* mutation in the *det1* background suppressed this phenotype and resulted in more seeds. The *det1 ddb1a* siliques showed a lower seed number (9.17 seeds on average per half silique). On the other hand, *det1 ddb1a ddb2* siliques were found to be pale in color and dry; dissecting these siliques showed that none of them developed seeds.

These observations led us to examine flowers of these genotypes. Early flowers of wildtype, *ddb2*, *ddb1a* and *ddb1a ddb2* showed long petals and long stamens that are very close to the stigma in terms of height (Figure 2.5 C). *det1* flowers showed slightly shorter stamens than the wildtype or the other single mutants. *det1 ddb2* flowers were found to have longer stamens than *det1*, relatively similar to wildtype. So *ddb2* mutation in the *det1* background suppressed the short stamens, and subsequently facilitates the fertilization process. In contrast to *det1 ddb2*, mutation of *DDB1A* in the *det1* background reduced stamen elongation. Dissecting *det1 ddb1a* flowers showed that the stamens exhibit approximately 1/3 the stigma length, as well as short petals. In later flowers, *det1 ddb1a* stamens were found to be longer and closer to the stigma, resulting in partial fertility. *det1 ddb1a ddb2* flowers showed reduced size and petal length and short stamens through out their life cycle. Thus, *ddb2* suppresses the related *det1* phenotypes of short silique length, reduced number of seeds and short stamens. This suppression is not observed in the absence of *DDB1A*, in fact these phenotypes are enhanced in the triple mutant.

Other growth traits were also assessed, such as number of inflorescences produced by each plant and fresh weight at one month of age under long or short day conditions. For these traits, loss of function of *DDB2* had no effect in any background (Figure S2.4, S2.5).

2.4.5 Interactions with other de-etiolated mutants

In order to determine if *ddb2* interacts with *det1* specifically or non-specifically modifies the de-etiolated phenotype, we generated the *cop1-4 ddb2* line. *cop1-4* is a partial loss of function allele that is phenotypically similar to *det1-1*, exhibiting short hypocotyls when grown in the dark. However, loss of function of *DDB2* in the *cop1-4* background resulted in no significant effect on hypocotyl length under dark conditions (Figure 2.6 A,B) or anthocyanin content (Figure S2.6) at the seedling stage under light conditions. Analysis of adult plants showed no significant differences between *cop1-4* and *cop1-4 ddb2* in flowering time, rosette diameter, number of inflorescences, height and silique length (Figure S2.6). Thus *ddb2* appears to specifically modify *det1*, rather than the de-etiolated phenotype in general.

2.5 DISCUSSION

Arabidopsis Damaged DNA Binding protein 1A (DDB1A) has been found in a complex with DET1 (Schroeder *et al.,* 2002) and COP10 (Yanagawa *et al.,* 2004). Also, DDB1 has been found in another complex with Damaged DNA Binding protein 2

(DDB2) involved in DNA repair (Cleaver, 2005). Due to the fact that DDB1 is involved in the formation of both complexes, we were interested in studying the interaction between DET1 and DDB2 through DDB1. To test this hypothesis, we generated the double and triple mutants of these genes. Our analysis indicates several modes of interaction between *DDB2*, *DDB1A* and *DET1*.

2.5.1 Suggested models of interaction

DDB1A dependent: For some phenotypes, loss of function of *DDB2* in the *det1* background resulted in significant changes, while loss of function of *DDB2* in the *det1 ddb1a* background showed no effect. This suggests that modulation of these phenotypes by *ddb2* is *DDB1A* dependent. These phenotypes include dark hypocotyl elongation, dark anthocyanin content and rosette diameter. Our hypothesis for the basis of this behavior is as follows. Due to the fact that the *det1-1* mutation is not a null mutation, there are small amounts of this protein still active in the cell. This small portion of DET1 may compete with DDB2 for binding with DDB1A. Mutation of DDB2 may increase the availability of DDB1A inside the cell to interact with DET1. Since loss of *DDB1A* resulted in reduced DET1 activity, more DDB1A liberated from the DDB2 complex may increase DET1 activity. This model explains the basis of the suppression that we observed of *det1* by *ddb2*. However, for dark grown hypocotyls, loss of function of *DDB2* was found to enhance the *det1* phenotype. In this case, perhaps the increase in DDB1A results in an increase in inactive complexes or increased degradation of ligase components. There is evidence that overexpression of E3 ligase components can result in loss of function

phenotypes (e.g. Gray *et al.,* 2002). Alternatively, both DET1-dependent and/or DDB2-dependent pathways may be required for optimal dark hypocotyl development.

DDB1A independent: For some phenotypes loss of function of *DDB2* resulted in significant changes in both the *det1* and *det1 ddb1a* backgrounds. This pattern of interaction suggests that regulation of these phenotypes by *ddb2* is *DDB1A* independent. These phenotypes include light chlorophyll and anthocyanin content, flowering time (days), and height. Our hypothesis for the basis of this behavior is as follows. *Arabidopsis* is the only organism with two versions of the DDB1 protein (DDB1A and DDB1B). *DDB1B* is expressed at lower levels than *DDB1A* (Figure 2.1 A). While *DDB1A* loss of function results in no obvious phenotype in wildtype background, *DDB1B* loss of function is lethal (Schroeder *et al.,* 2002), thus little is known about the role of *DDB1B* in light signaling. Perhaps DDB2 interacts with DDB1B in the absence of DDB1A. Thus the DDB1A-independent phenotypes may be acting via DDB1B. If the redundant action of DDB1B results in DDB1A-independent phenotypes, this implies that DDB1B is unable to compensate for DDB1A-dependent phenotypes. For example, light-grown seedlings exhibit independent phenotypes (chlorophyll, anthocyanin) while dark grown seedlings exhibit dependent phenotypes (hypocotyl, anthocyanin). Genevestigator data shows that expression of *DDB1A* and *DDB1B* does not vary significantly between light and dark, *DDB1A* is expressed at twice the level of *DDB1B* in both cases. Perhaps DDB1B is inactivated post-transcriptionally in dark-grown seedlings resulting in DDB1A-dependent phenotypes.

These models suggest that both DET1 and DDB2 are able to interact with DDB1A and DDB1B. Support for this hypothesis is as follows. *Arabidopsis* DET1 and

DDB1A interact genetically (Schroeder *et al.,* 2002) as well as in a yeast two-hybrid assay (Bernhardt *et al.,* 2006). In addition DET1, DDB1A, COP10, CUL4 and RBX1 are able to form an active E3 Ub ligase complex (Chen *et al.,* 2006). While no direct interaction between DET1 and DDB1B has been demonstrated, Bernhardt *et al.* (2006) showed that both DDB1A and DDB1B interact with At-CUL4 in an in vitro pull down assay, and myc-tagged DET1 immunoprecipitates with CUL4 from plant extracts. Residues required for interaction between human DET1 and DDB1 have recently been identified - DDB1 910-913 MALY in β-propeller C (Jin *et al.,* 2006), and *Arabidopsis* DDB1A, DDB1B and rice DDB1 all contain the same variation of this sequence – LALY (Schroeder *et al.,* 2002). With regard to DDB2/DDB1 interaction, Bernhardt *et al.* (2006) also showed that At DDB2 can interact with DDB1A in yeast two hybrid assay. No direct interaction between DDB2 and DDB1B has been demonstrated, nor have the human DDB1 residues required for interaction with DDB2 been identified, but competition experiments have shown that DDB2 competes with the viral protein SV5 for DDB1 interaction (Leupin *et al.,* 2003) and SV5 has been shown to interact with the β–propeller C of DDB1 (Li *et al.,* 2006b). Several recent studies however have identified a WDXR motif at approximately residue 273 in human DDB2 and other DDB1-interacting WD40 proteins (DCAFs) that is required for interaction with DDB1 (Jin *et al.,* 2006; Angers *et al.,* 2006; Higa *et al.,* 2006; He *et al.,* 2006). This motif is also conserved in rice and *Arabidopsis* DDB2 (Ishibashi *et al.,* 2003). Thus DDB1 appears to act as a scaffold to recruit specific factors, including DET1 and DDB2, to CUL4 E3 ligase complexes. Interaction with these specific factors appears to primarily occur via the β–propeller C domain of DDB1. In *Arabidopsis*, *DDB1A* appears to be expressed at

nearly twice the level of *DDB1B* throughout development (Figure 2.1 A), and both DET1 and DDB2 can interact with DDB1A in vitro (Bernhardt *et al.,* 2006). Our genetic analysis suggests this interaction is functionally important.

2.5.2 Modulation of dark hypocotyl elongation

det1 seedlings show short hypocotyls when grown under dark conditions, this phenotype is similar to light grown wildtype seedlings (Chory *et al.,* 1989). Photoreceptors mutations, which alone show long hypocotyls, in the *det1* background exhibit the *det1* phenotype. This suggests that *DET1* is functioning downstream of the photoreceptors (Wang and Deng, 2002).

Enhancers of the *det1* dark hypocotyl phenotype include *ddb1a* (Schroeder *et al.,* 2002), and a weak allele (*cop1-6*) of the WD-40 protein COP1. The double mutant *cop1-6 det1-1* exhibits dark purple cotyledons, very short hypocotyls and adult lethality (Ang and Deng, 1994). Similarly, we have shown that loss of function of *DDB2*, another member of the WD-40 protein family, enhances the short hypocotyl phenotype of *det1*. All these enhancers appear to be acting near *DET1* in the light signaling pathway.

Suppressors of the *det1* dark hypocotyl phenotype include *CONSTANS-LIKE3* (*col3*). Dark grown *det1-1 col3* exhibit 55% longer hypocotyls than *det1-1* alone (Datta *et al.,* 2006). Loss of function alleles of the transcription factor HY5 also suppress the *det1* dark phenotype (Chory, 1992; Pepper and Chory, 1997). Mutation of *TED3* in the *det1* background also completely suppress the short *det1* hypocotyl (Pepper and Chory, 1997).

TED3 encodes a peroxisomal protein (Pex2p) involved in *Arabidopsis* development (Hu *et al.,* 2002). These suppressors act downstream of *DET1* in light signaling.

2.5.3 Regulation of chlorophyll content

Light grown *det1* seedlings exhibit a pale phenotype compared to wildtype (Chory *et al.,* 1989). This correlates with a decrease in *CAB* mRNA expression (Chory and Peto, 1990). The region of the *CAB2* promoter involved in DET1 up-regulation of light expression has been mapped to the CAB Upstream Factor-1 element (CUF-1). The CUF-1 element is bound by the transcription factor HY5, and *hy5* mutants also under-express *CAB2* in the light (Maxwell *et al.,* 2003). Other studies indicate that circadian regulation of *CAB* transcription by DET1 might be the post-transcriptional. Degradation of the LHY (Late elongated hypocotyl) factor is accelerated in *det1-1* mutants (Song and Carre, 2005).

Our results show that mutation of *DDB2* in *det1* or *det1 ddb1a* backgrounds significantly enhanced chlorophyll content of these seedlings (i.e. suppressed the *det1* pale phenotype). It will be interesting to determine the basis of this suppression.

2.5.4 Regulation of anthocyanin content

The anthocyanin biosynthetic pathway is well studied. Several transcription factors that regulate anthocyanin biosynthesis have been identified. In addition, many environmental conditions regulate this pathway. Light is an important factor that

regulates anthocyanin accumulation in plant cells (Koes *et al.,* 2005). As shown previously, *det1* seedlings have more anthocyanin than wildtype. Chory and Peto (1990) reported that the anthocyanin biosynthetic gene *Chalcone synthase (CHS)* is ectopically expressed in leaf mesophyll cells in *det1* seedlings. Chory *et al.* (1989) studied *CHS* gene expression of dark grown seedlings and found that *det1* seedlings have more (20-50 fold) *CHS* mRNA than the wildtype.

In light-grown seedlings, we observed no significant effect of *ddb2* on *det1* anthocyanin levels, but *ddb2* suppressed anthocyanin accumulation in the *det1 ddb1a* double mutant. In contrast, in dark grown seedlings *ddb2* suppressed anthocyanin accumulation in a *DDB1A*-dependent fashion. Interestingly, while *ddb2* regulation of both anthocyanin levels and hypocotyl elongation was found to be *DDB1A*-dependent in dark-grown seedlings, opposing effects were observed. Loss of function of *DDB2* suppressed the high anthocyanin content of *det1* but enhanced the *det1* short hypocotyl phenotype. This opposing trend was also observed for another mutation. Loss of function of *COL3* in the *det1* background enhanced the high anthocyanin content of *det1* but suppressed the short hypocotyl length of dark-grown seedlings (Datta *et al.,* 2006).

2.5.5 Regulation of fertility parameters

Mutation of *DDB1A* in the *det1* background results in reduced floral development and seed production (Schroeder *et al.,* 2002). Mutation of *DDB2* suppressed the short stamens and reduced dehiscence, number of seeds and silique length in *det1* single mutants, but enhanced those phenotypes in the *det1 ddb1a* double mutant. These

phenotypes were similar to those observed for loss of function alleles of Jasmonic acid (JA) biosynthesis or signaling genes. JA, a lipid-derived signaling compound present in most plant species, has been found to play a major role in anther development. One model suggests that JA regulates water flow in the stamens and petals which will further regulate flower opening and stamen maturation. On the other hand, regulation of programmed cell death in the anther by JA as dehiscence proceeds has also been proposed (Scott *et al.,* 2004). Similar phenotypes were also observed in an overexpression line of RBX1 (Gray *et al.,* 2002). RBX1 (also known as ROC1) is RING-domain protein and a component of cullin-containing E3 ubiquitin ligases including the SCF-type complex COI1 involved in JA signalling (Schwechheimer and Calderon, 2004) and the human DET1, COP1, DDB1, CUL4A complex (Wertz *et al.,* 2004).

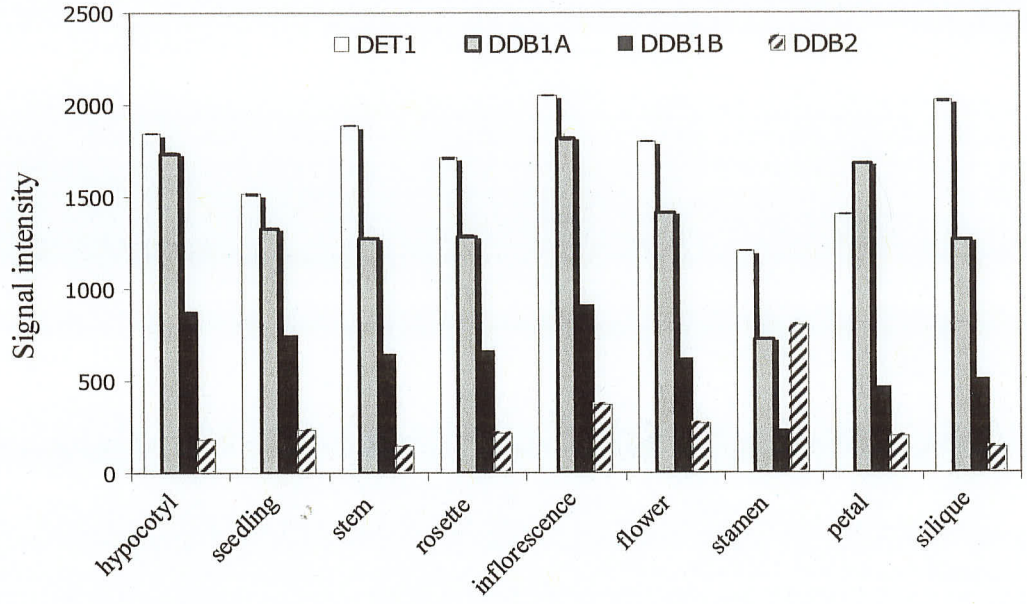
2.5.6 DDB1 and DDB2 loss of function mutants in other organisms

DDB1 and DDB2 were originally identified due to their role in nucleotide excision repair. Human XPE patients have loss of function alleles of *DDB2* (Cleaver, 2005). *DDB2* knockout mice, although viable, also have increased tumor formation (Yoon *et al.,* 2005; Itoh *et al.*, 2004). Decrease in leaf width and length, and increase in UV sensitivity were observed in null *ddb2* mutants in *Arabidopsis* (Koga *et al.,* 2006). While good DDB2 homologues have been identified only in vertebrates and higher plants, DDB1 homologues also exist in *Drosophila, C. elegans,* and *S. pombe*. Recently, loss of function of *DDB1* in human cells has been found result in defects in UV-damage repair (Li *et al.,* 2006a) and increased DNA double-strand break accumulation throughout

the genome (Lovejoy *et al.,* 2006). *DDB1* knock out flies are lethal, suggesting a crucial role for DDB1 in *Drosophila* development (Takata *et al.,* 2004). RNAi screens in *C. elegans* have shown that loss of *DDB1* results in embryonic and larval lethality (Kim and Kipreos, 2006). Yeast (*Schizosaccharomyces pombe*) *DDB1* was found to have a significant role in preventing mutation and genome stability (Holmberg *et al.,* 2005), as well as a role in cell division and replication control (Bondar *et al.,* 2003; Zolezzi *et al.,* 2002). In tomato (*Lycopersicon esculentum*), *High Pigment 1* (*HP-1*) is homologous to *Arabidopsis* and human *DDB1*. *hp-1* mutants exhibit highly pigmented fruit and short hypocotyls like mutants in the tomato *DET1* gene, *HP-2* (Lieberman *et al.,* 2004; Liu *et al.,* 2004). Thus, the role of DDB2 appears to be specific to DNA repair, while DDB1 appears to have addition roles during development. This is consistent with biochemical evidence that DDB2 specifically recruits the DDB1/CUL4 E3 ligase to DNA damage (Kapetanaki *et al.,* 2006), while DDB1/CUL4 can form complexes with multiple targeting factors, such as CSA (Groisman *et al.,* 2003) and DET1 (Wertz *et al.,* 2004; Chen *et al.,* 2006; Bernhardt *et al.,* 2006).

Here we show that the *ddb2* and *ddb1a* single and double mutants exhibit no developmental phenotypes on their own, but in the *det1-1* background, strong (*ddb1a*) or more subtle (*ddb2*) developmental effects can be observed. We propose that these effects are due to changes in the activity of the DET1 complex, either directly (*ddb1a*) or indirectly (*ddb2*) via modulations of the DDB1A/DDB1B pool.

A



B

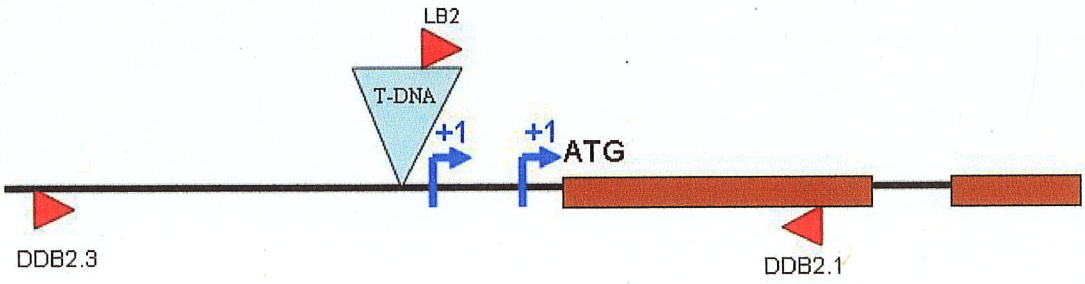
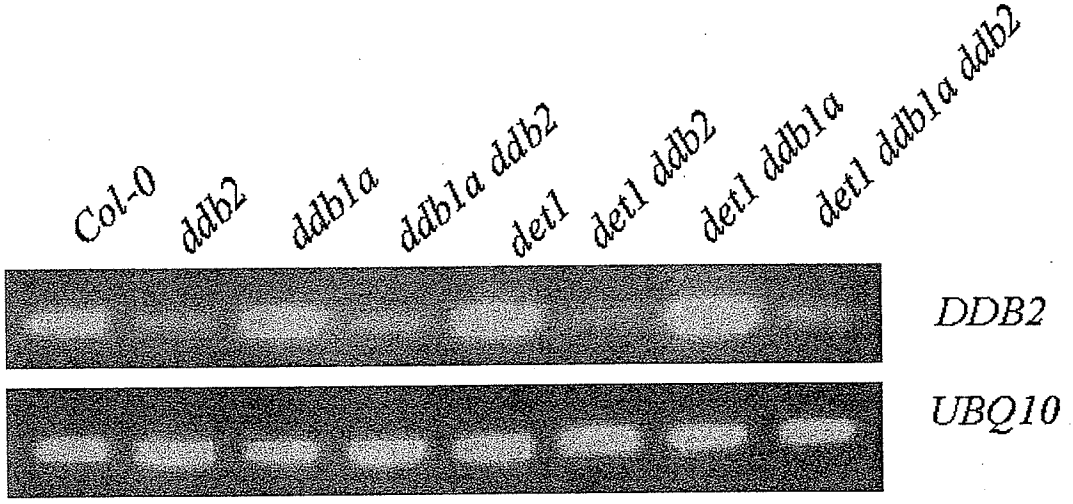


Figure 2.1 Characterization of *Arabidopsis DDB2*. **A**: Gene expression data as obtained from Genevestigator database (https://www.genevestigator.ethz.ch). **B**: Schematic representation of 5' end of the *DDB2* gene (At5g58760). Transcription start sites (+1) based on Genbank accession numbers BX832566 and AK175124. Exons are shown as boxes and introns and upstream sequences as lines. Position of the T-DNA insertion (SALK_040408) is indicated, along with primers used for genotyping.

C



D

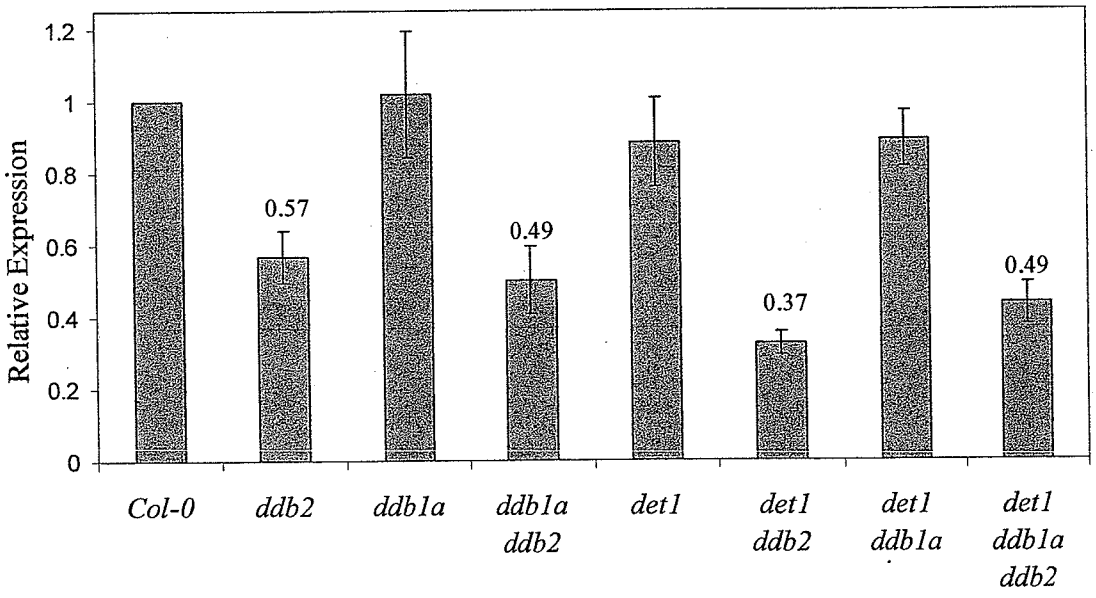
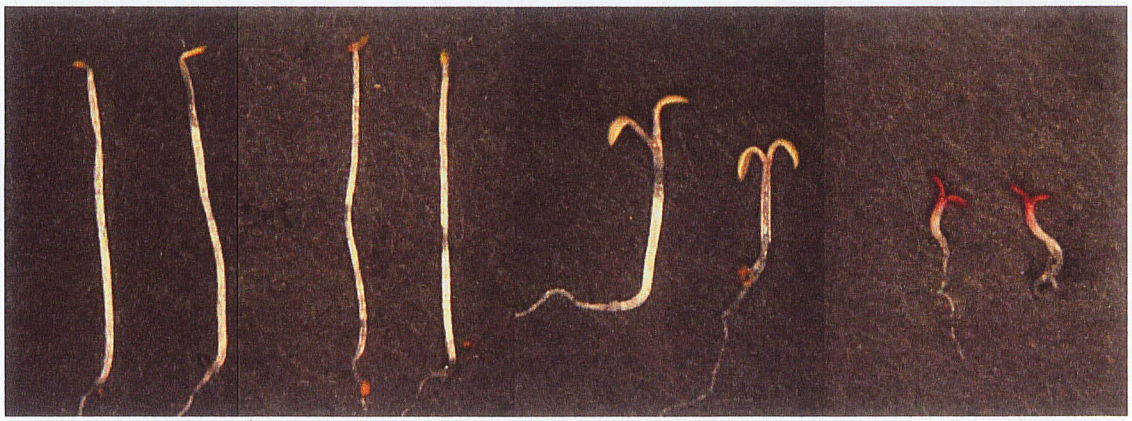


Figure 2.1 Characterization of *Arabidopsis DDB2*. (cont.) **C**: RT-PCR products for *DDB2* and *UBQ10* of 7-day-old seedlings grown under long day conditions. **D**: Quantification of *DDB2* transcript level (*DDB2* values normalized to *UBQ10* levels, relative to Col-0). Data are shown as the means ± SE of three different technical repeats. Numbers above error bars indicate expression relative to *DDB2* wildtype control.

A



B

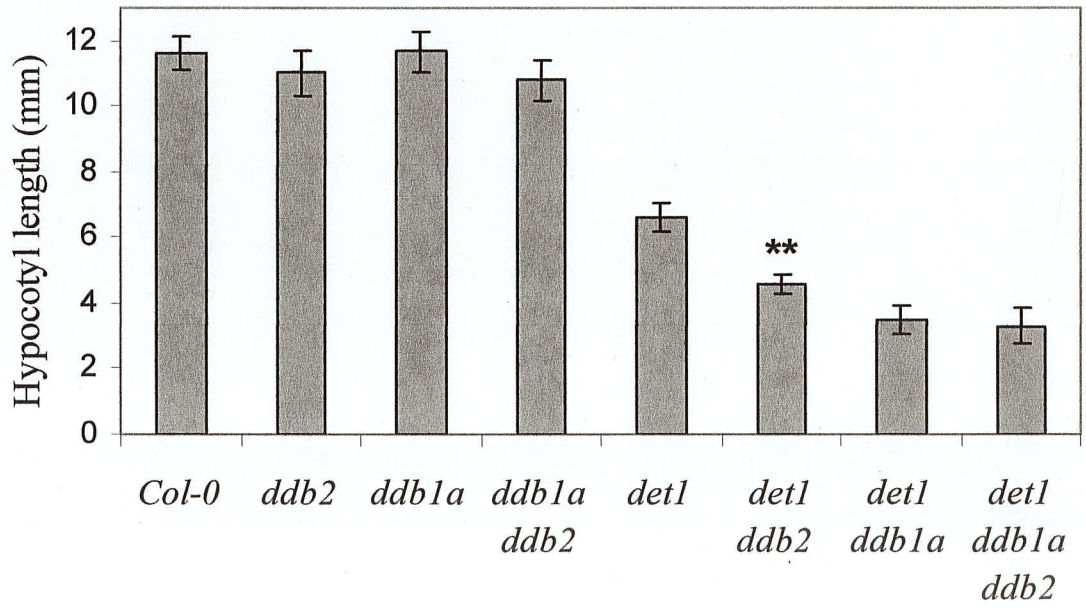


Figure 2.2 Phenotypic analysis of 7-day-old dark grown seedlings.
A: From left to right Col-0, *ddb2*, *ddb1a*, *ddb1a ddb2*, *det1, det1 ddb2, det1 ddb1a* and *det1 ddb1a ddb2.* **B**: Hypocotyl length. Error bars indicate 95% C. I. **=P≤0.01 relative to *DDB2* wildtype control.

C

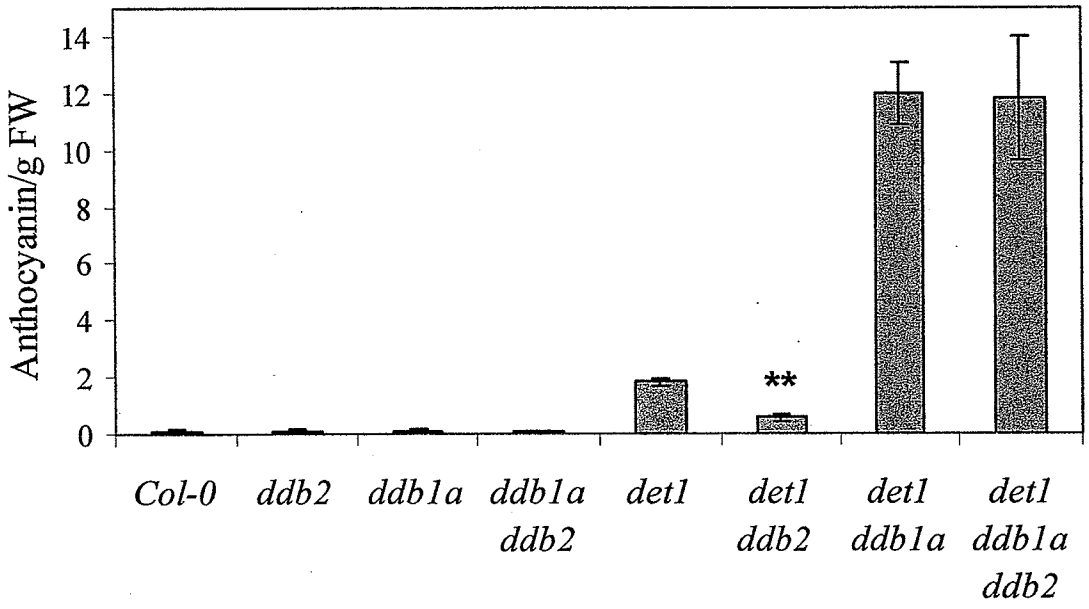
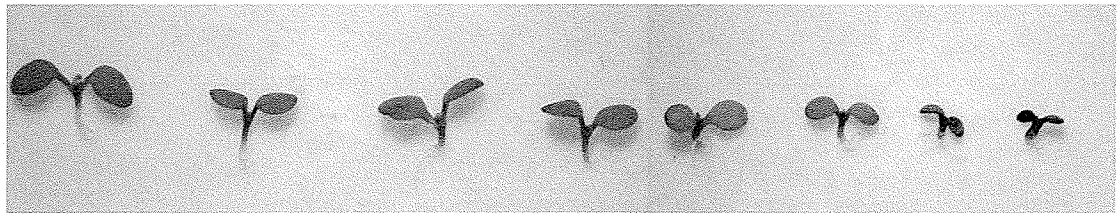


Figure 2.2 Phenotypic analysis of 7-day-old dark grown seedlings (cont.). C: Anthocyanin content ($A_{530} - A_{657}$/g fresh weight). Error bars indicate 95% C. I. **=P≤0.01 relative to *DDB2* wildtype control.

A



B

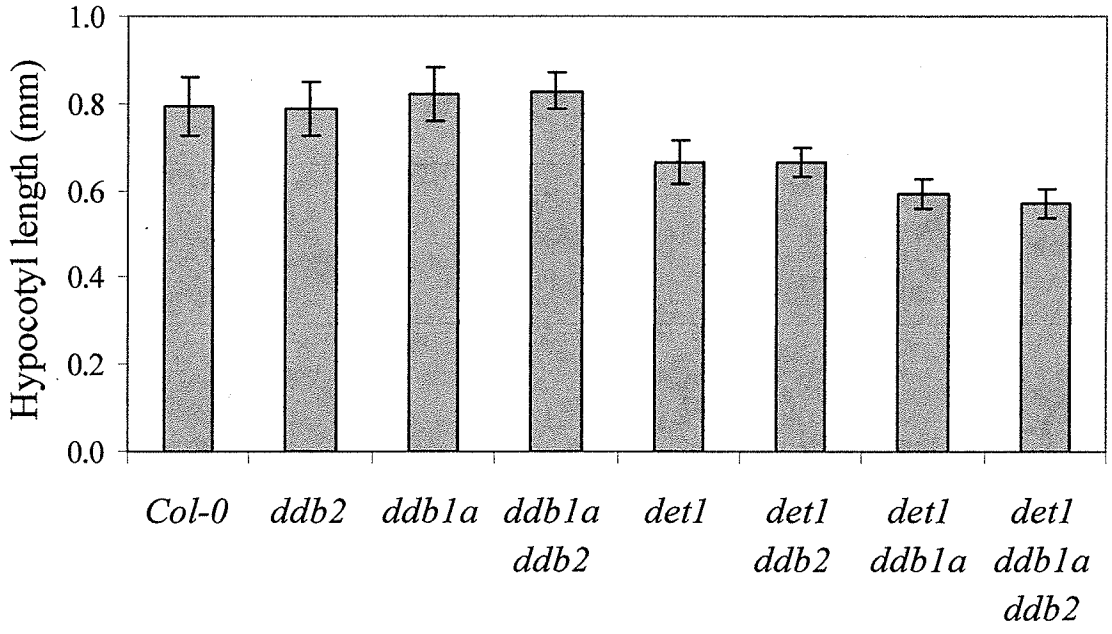
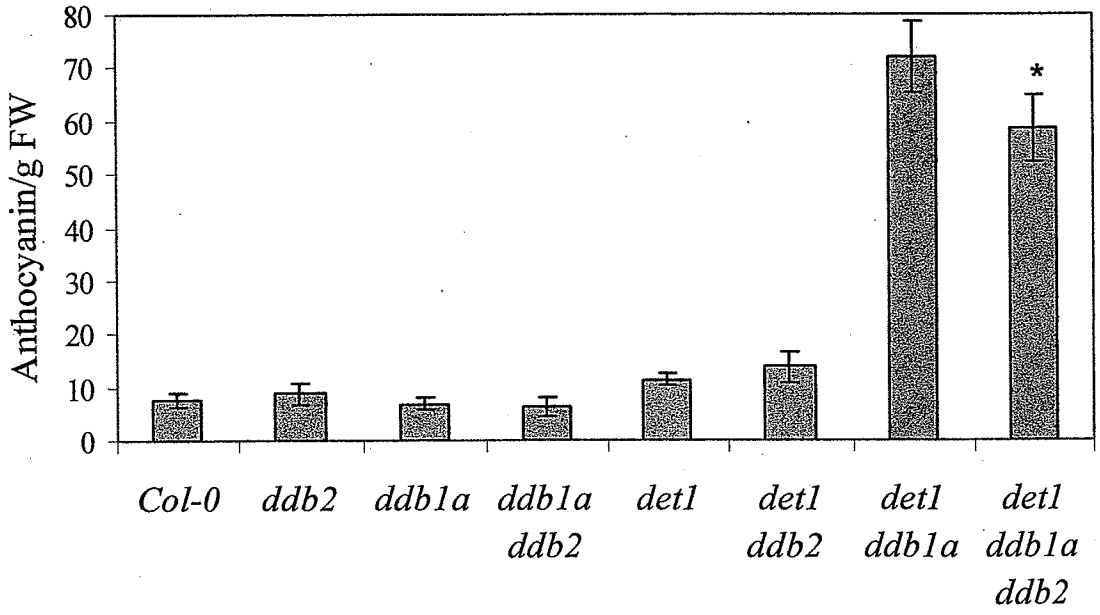


Figure 2.3 Phenotypic analysis of 7-day-old long day grown seedlings.
A: From left to right Col-0, *ddb2*, *ddb1a*, *ddb1a ddb2*, *det1*, *det1 ddb2* and *det1 ddb1a*, *det1 ddb1a ddb2*. **B**: Hypocotyl length. Error bars indicate 95% C. I.

C



D

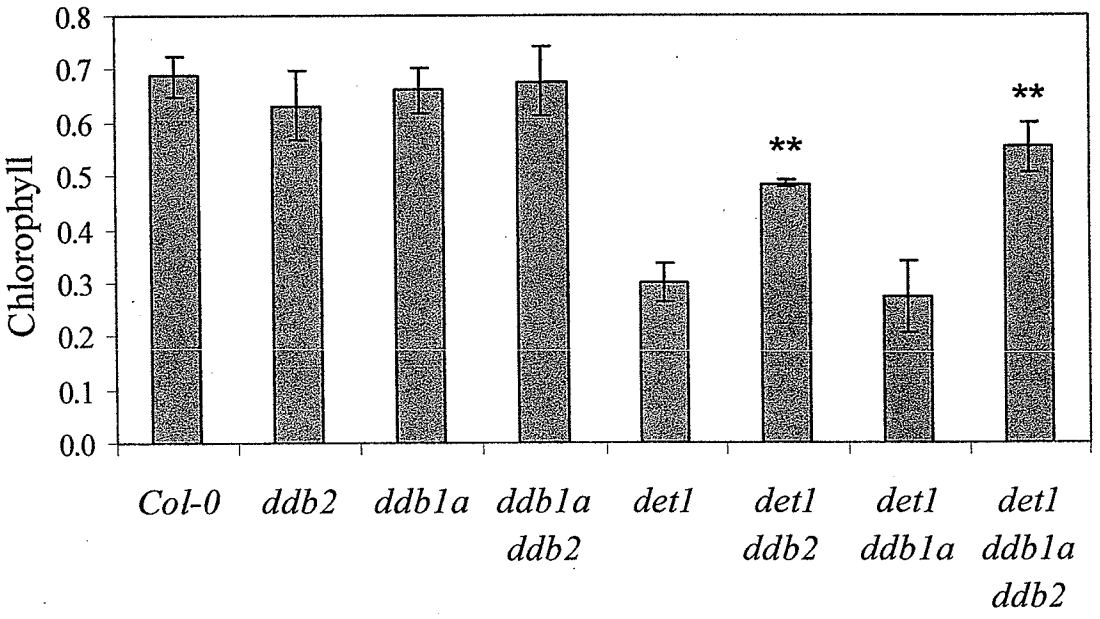


Figure 2.3 Phenotypic analysis of 7-day-old long day grown seedlings (cont.). C: Anthocyanin content. **D:** Chlorophyll content (μg Chlorophyll/mg fresh weight). Error bars indicate 95% C. I. *, **=P≤0.05 or P≤0.01, respectively, relative to *DDB2* wildtype control.

A

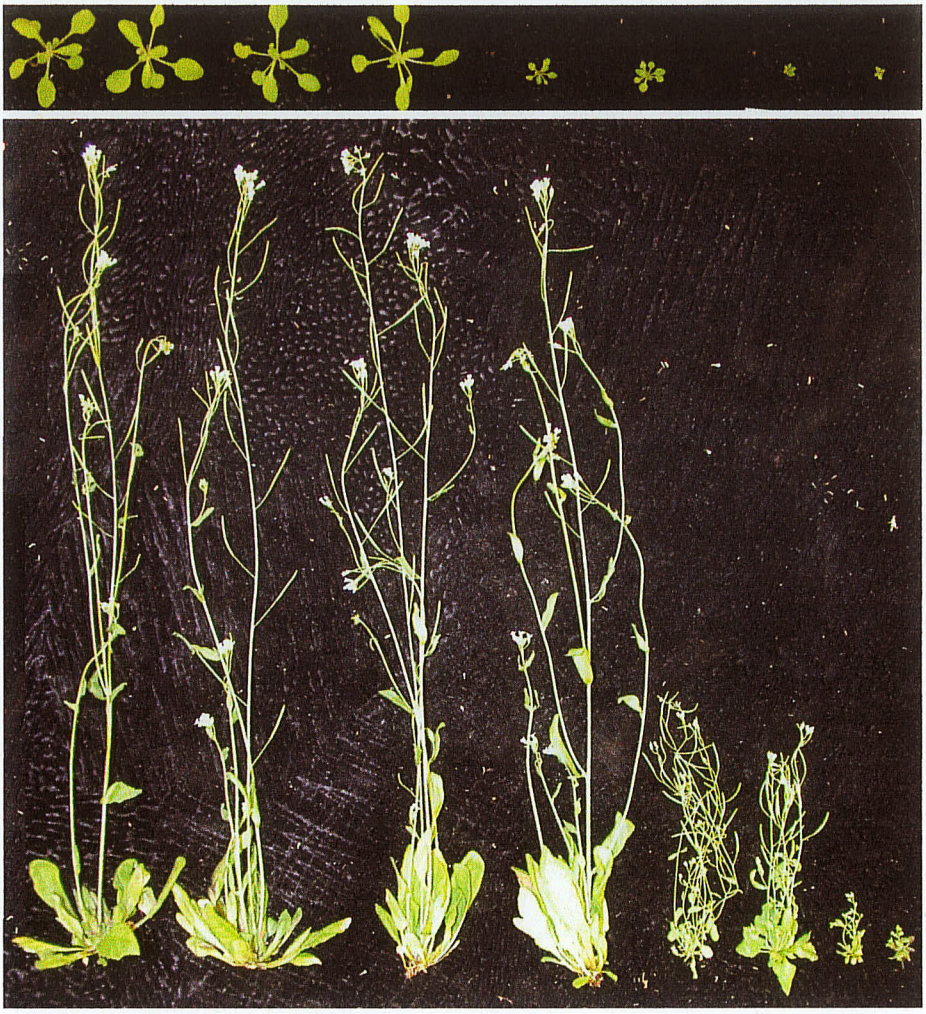
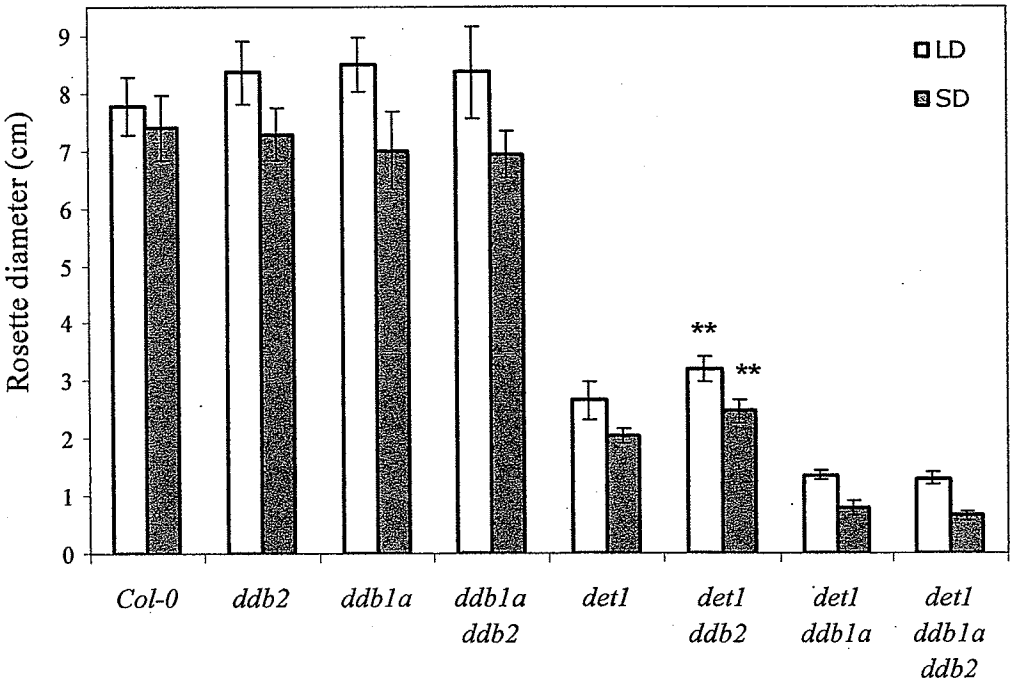


Figure 2.4 Adult phenotypes
A: From left to right Col-0, *ddb2*, *ddb1a*, *ddb1a ddb2*, *det1*, *det1 ddb2*, *det1 ddb1a* and *det1 ddb1a ddb2* grown in long day conditions. Upper panel: rosette diameter at 4 weeks. Lower panel: Height at 6 weeks.

B



C

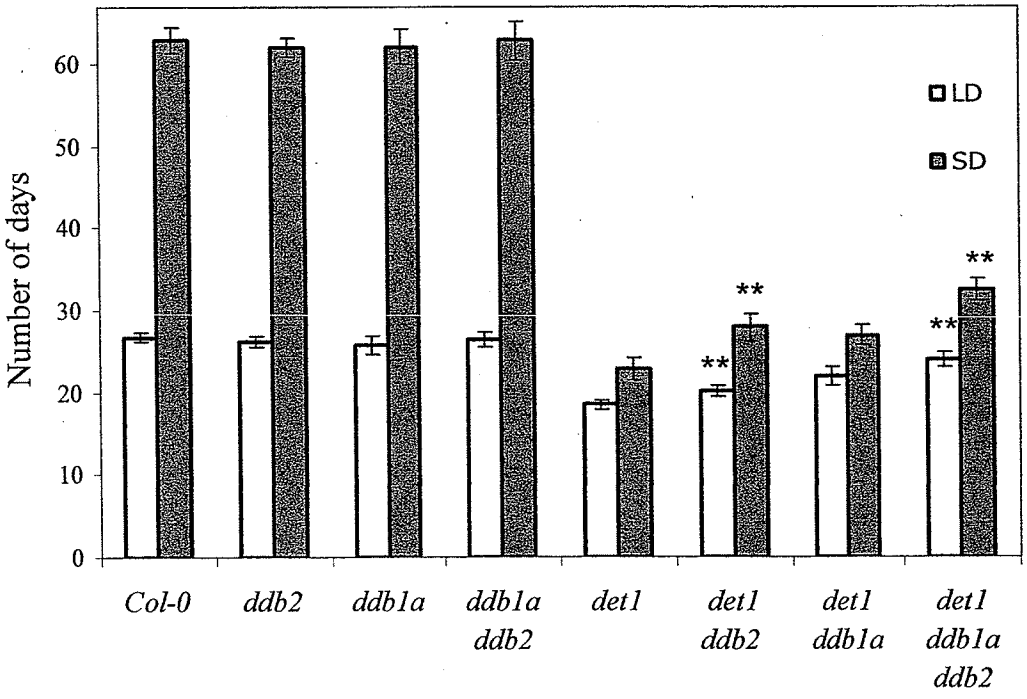


Figure 2.4 Adult phenotypes (cont.). B: Rosette diameter of 4-week-old plants. **C**: Flowering time (in days). Error bars indicate 95% C. I. **= P≤0.01 relative to *DDB2* wildtype control.

D

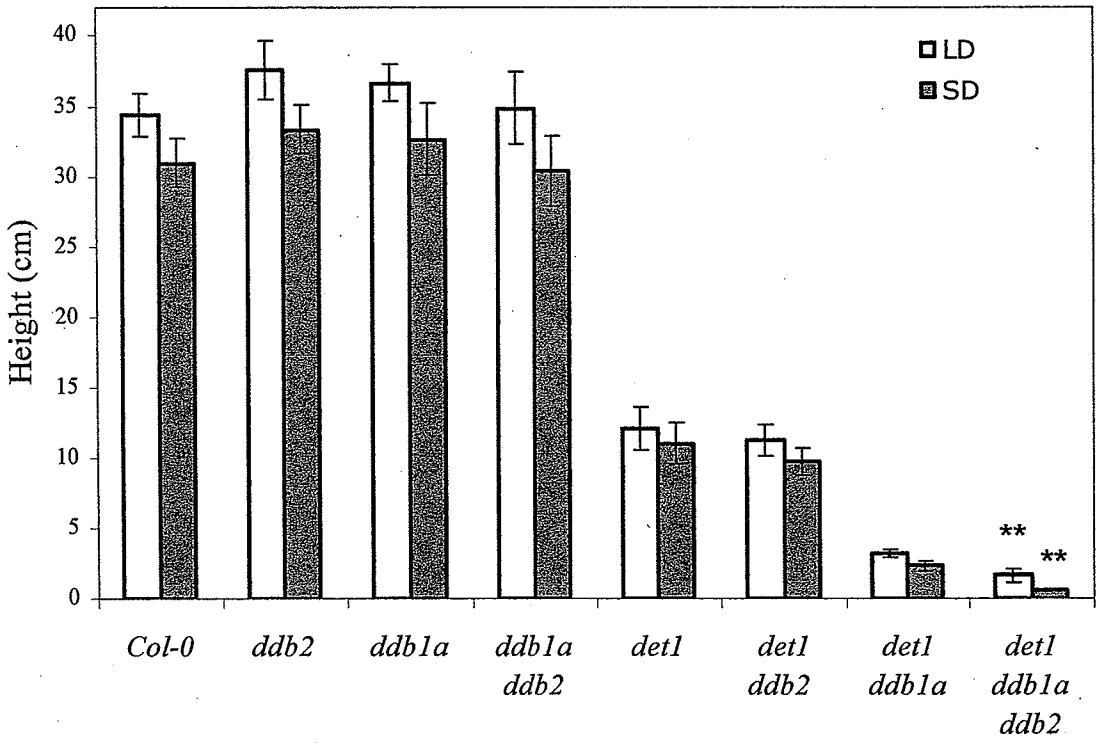
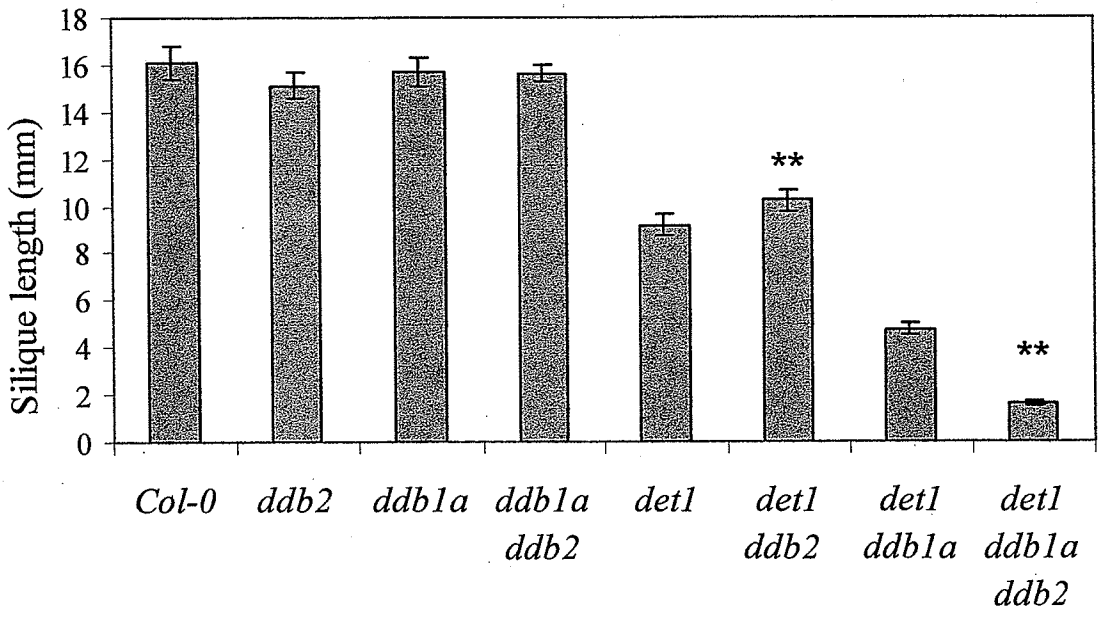


Figure 2.4 (cont.) D: Height (cm) of adult plants. Error bars indicate 95% C. I. *, **=P≤0.05 or P≤0.01, respectively relative to *DDB2* wildtype control.

A



B

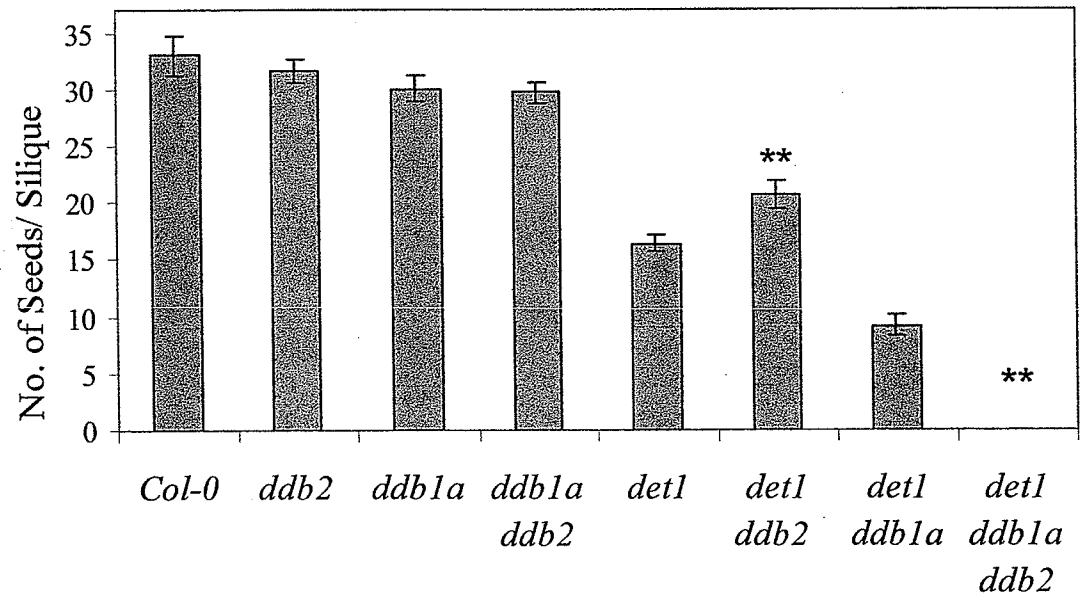


Figure 2.5 Floral and fruit morphology.
A: Silique length (mm). **B**: Number of seeds/half silique (n=6). Error bars indicate 95% C. I. *, **=P≤0.05 or P≤0.01, respectively relative to *DDB2* wildtype control.

C

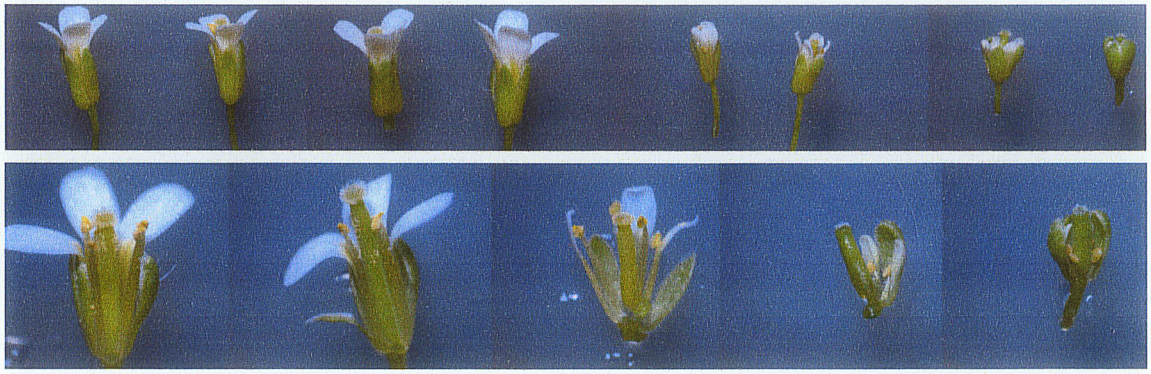


Figure 2.5 Floral and fruit morphology (cont.). C: Top panel: Flower characteristics (From left to right) Col-0, *ddb2*, *ddb1a*, *ddb1a ddb2*, *det1*, *det1 ddb2*, *det1 ddb1a* and *det1 ddb1a ddb2*. Bottom panel: Dissected flower (from left to right) of Col-0, *det1*, *det1 ddb2*, *det1 ddb1a* and *det1 ddb1a ddb2*.

A

B

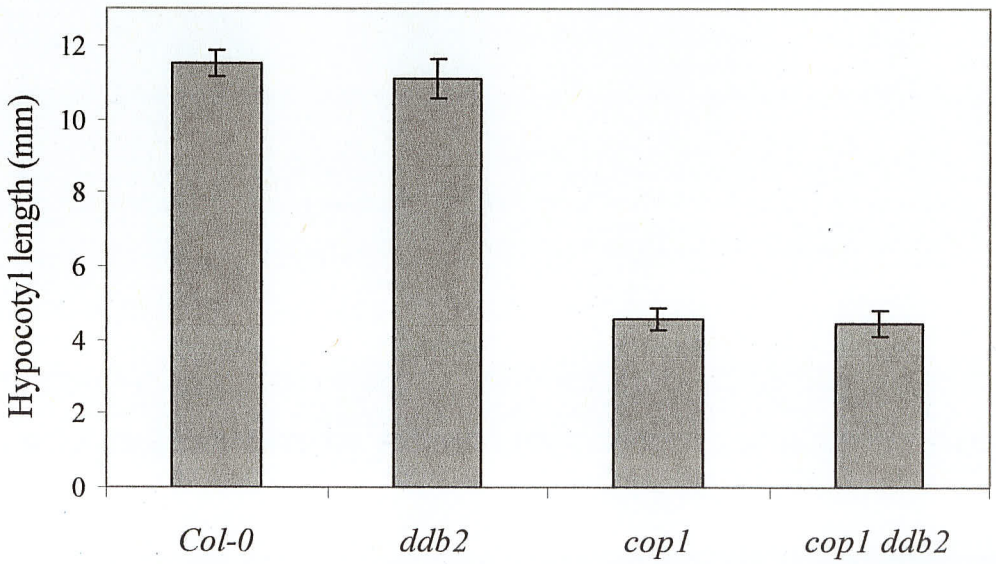


Figure 2.6 **A**: 7-day-old seedlings of wild type, *ddb2, cop1-4* and *cop1-4 ddb2* grown under dark conditions. **B**: Hypocotyl length of 7-day-old dark grown seedlings. Error bars indicate 95% C. I.

2.6 SUPPLEMENTARY DATA

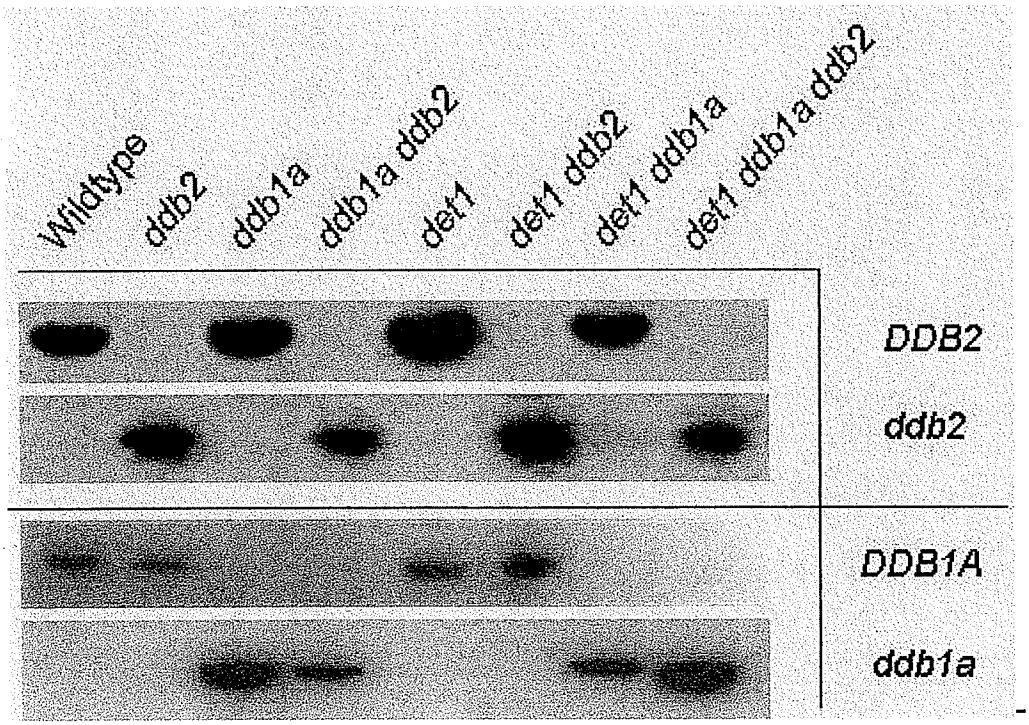


Figure S2.1 T-DNA genotyping of *ddb1a* and *ddb2* mutants. Genotype is indicated at the top and respective PCR product on the right.

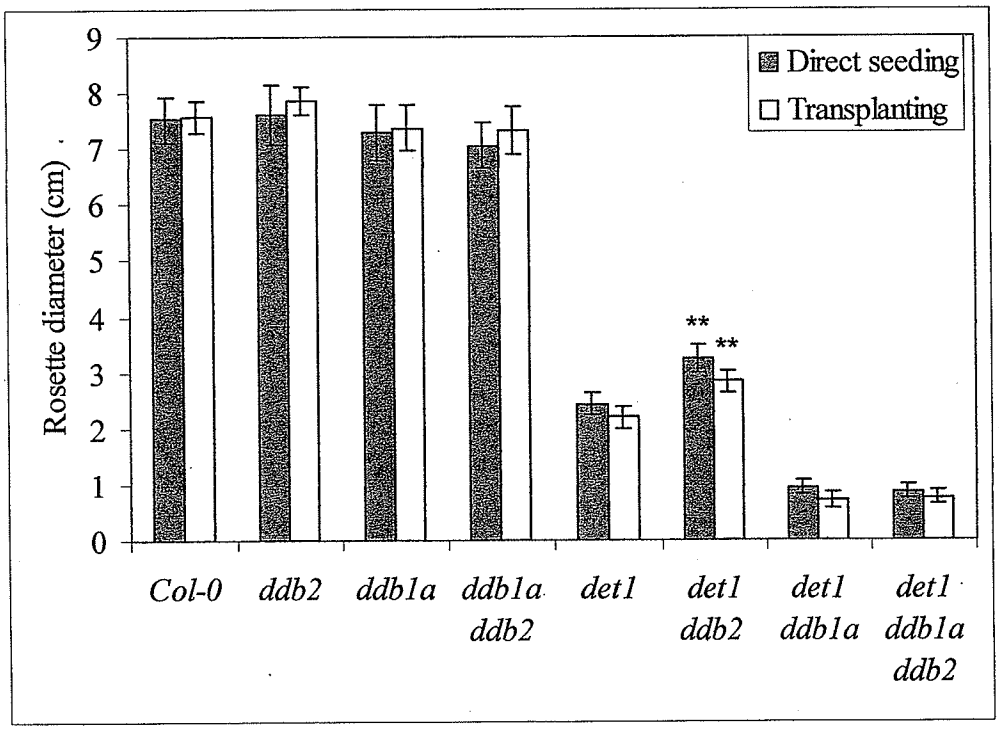


Figure S2.2 Rosette diameter of 4-week-old Col-0, *ddb2*, *ddb1a*, *ddb1a ddb2*, *det1*, *det1 ddb2*, *det1 ddb1a* and *det1 ddb1a ddb2* grown in long day conditions. Seeds were sown directly into soil (direct seeding) or plated and grown on MS media (No sucrose) for 2 weeks then transplanted to soil (transplanting). Error bars indicate 95% C. I. **=P≤0.01 relative to *DDB2* wildtype control

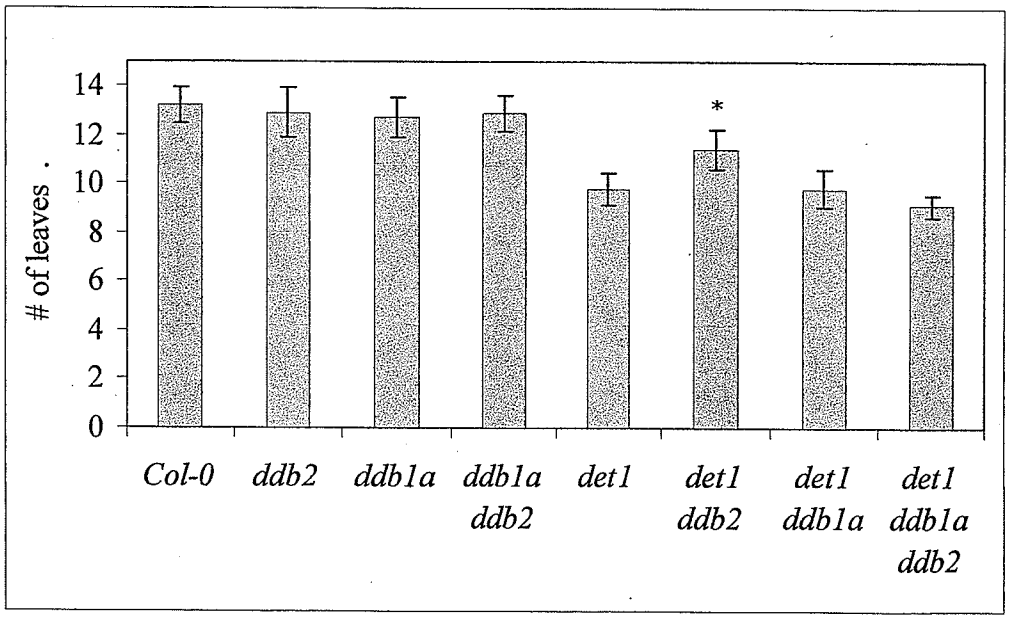


Figure S2.3 Flowering time (number of leaves) of Col-0, *ddb2*, *ddb1a*, *ddb1a ddb2*, *det1*, *det1 ddb2*, *det1 ddb1a* and *det1 ddb1a ddb2* grown in long day conditions. Error bars indicate 95% C. I. * =P≤0.05 relative to *DDB2* wildtype control.

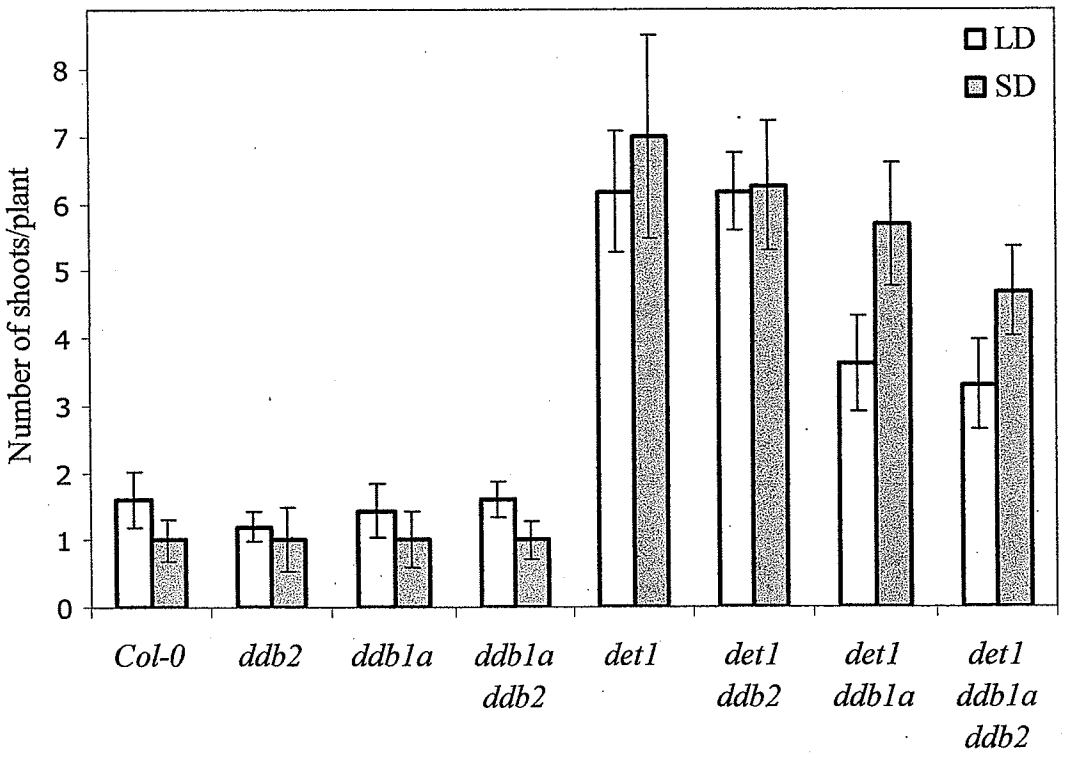


Figure S2.4 Number of shoots per plant of 6-week-old Col-0, *ddb2*, *ddb1a*, *ddb1a ddb2*, *det1*, *det1 ddb2*, *det1 ddb1a* and *det1 ddb1a ddb2* grown in long or short day conditions. Error bars indicate 95% C. I.

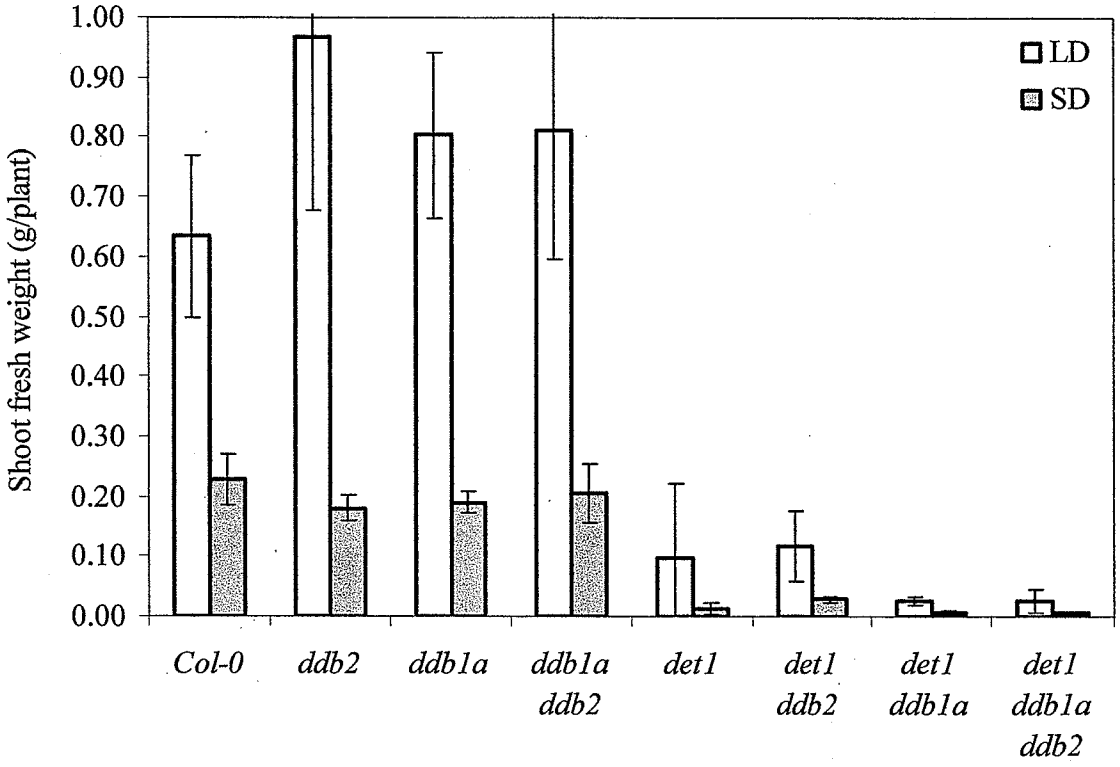
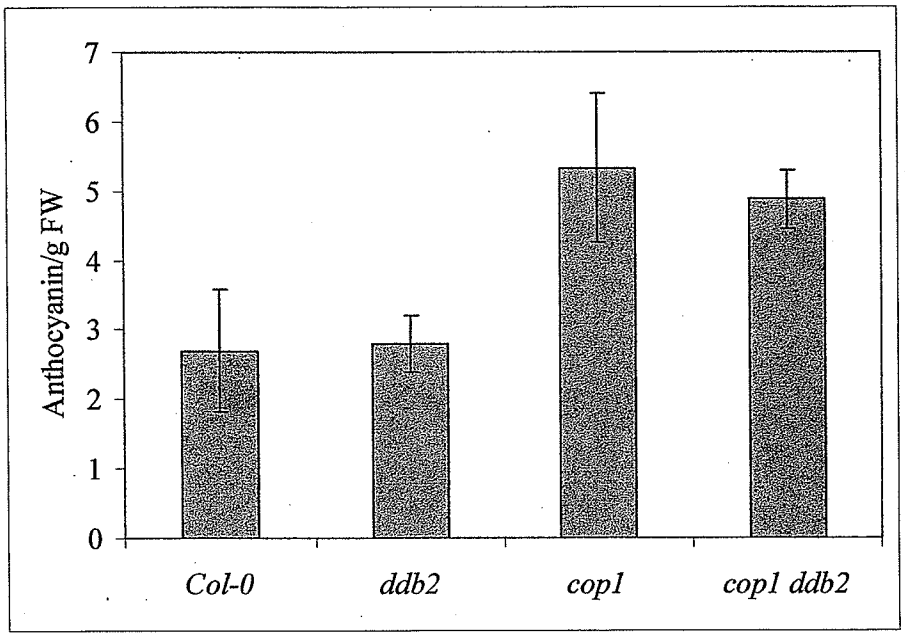


Figure S2.5 Shoot fresh weight of 4-week-old Col-0, *ddb2*, *ddb1a*, *ddb1a ddb2*, *det1*, *det1 ddb2*, *det1 ddb1a* and *det1 ddb1a ddb2* grown in long or short day conditions

A



B

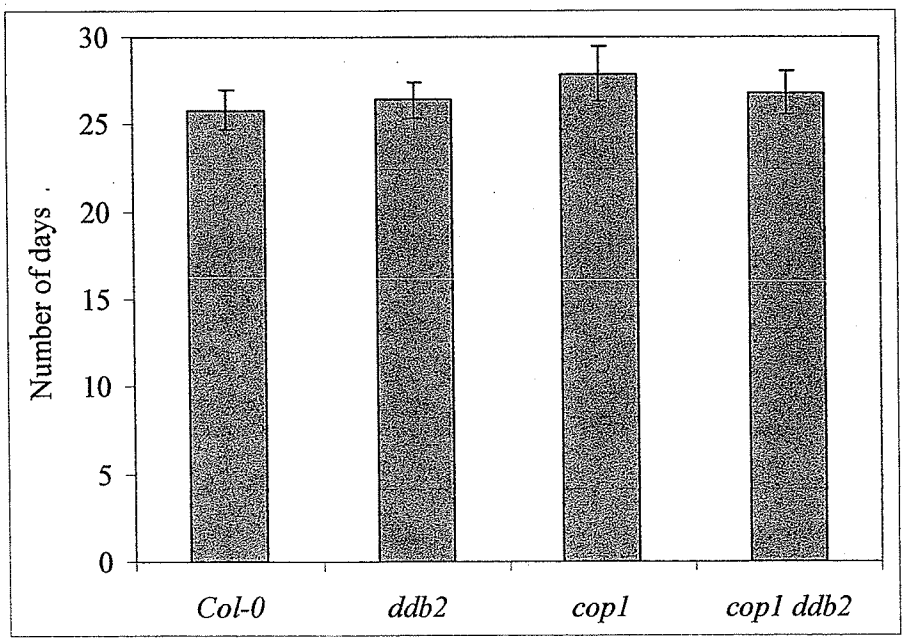
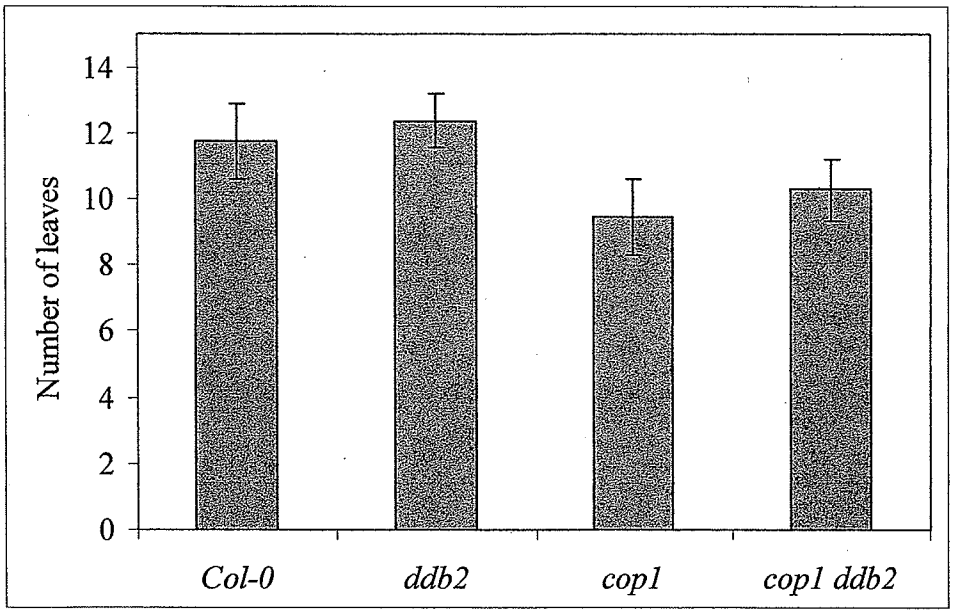


Figure S2.6 Analysis of wild type, *ddb2*, *cop1-4* and *cop1-4 ddb2*.
A: Anthocyanin content of 7-day long day grown seedlings. **B**: Flowering time (in days). Error bars indicate 95% C. I.

C



D

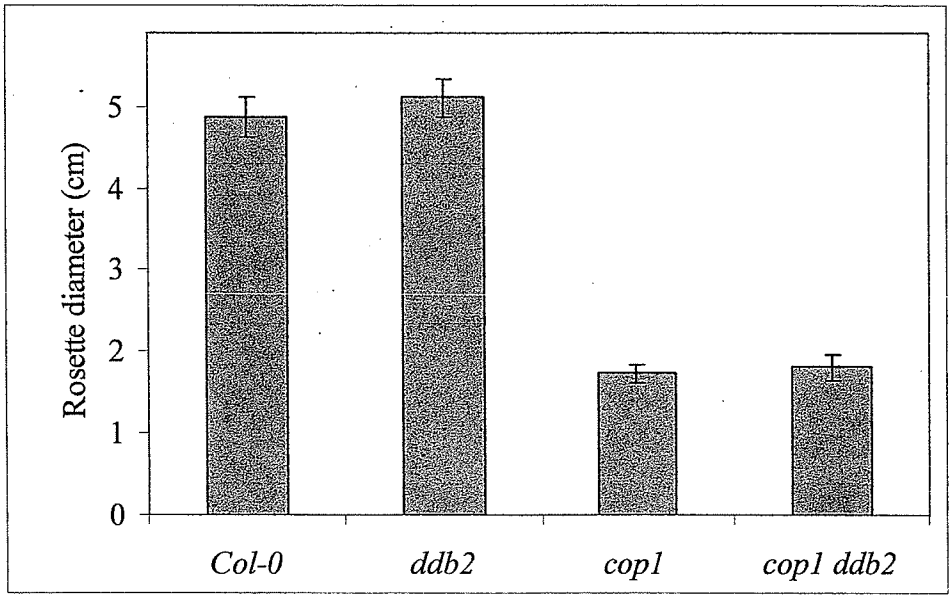
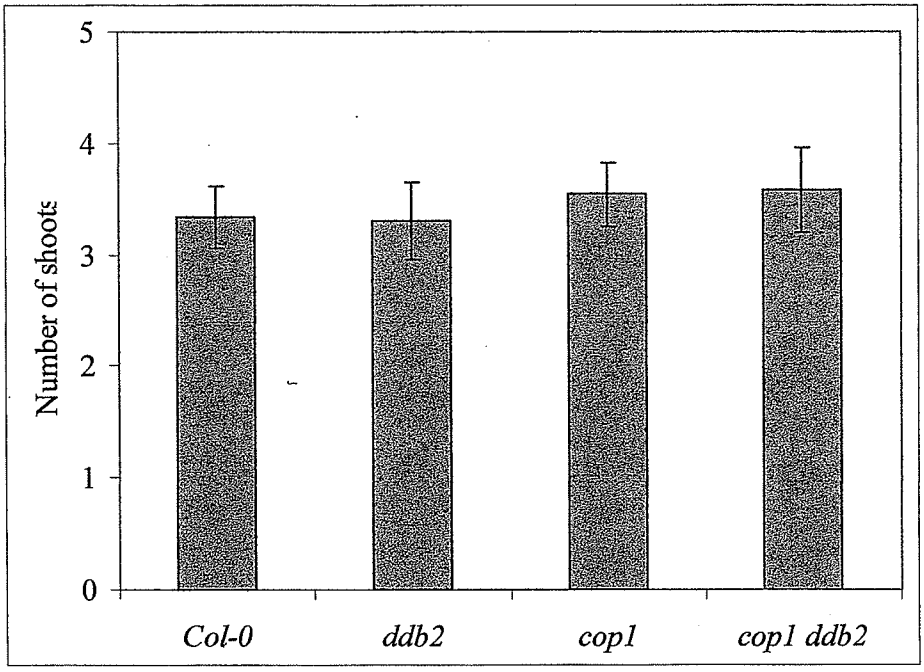


Figure S2.6 Analysis of wild type, *ddb2*, *cop1-4* and *cop1-4 ddb2* (cont.). C: Flowering time (in # of leaves). **D**: Rosette diameter of 4-week-old plants. Error bars indicate 95% C. I.

E



F

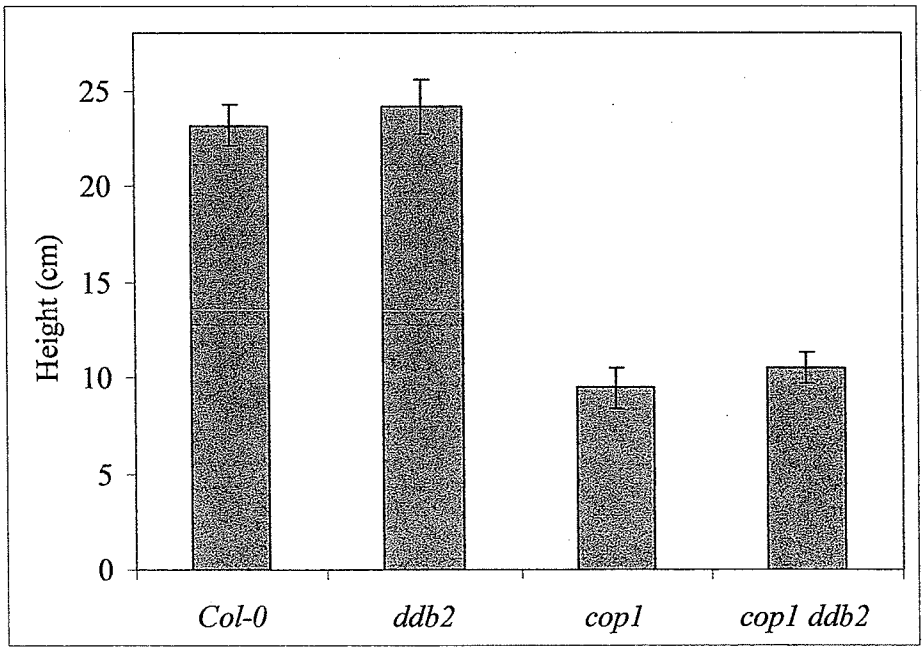


Figure S2.6 Analysis of wild type, *ddb2*, *cop1-4* and *cop1-4 ddb2* (cont.). **E**: Number of shoots (6-week-old plants). **F**: Height (cm) of adult plants (6-week-old plants). Error bars indicate 95% C. I.

G

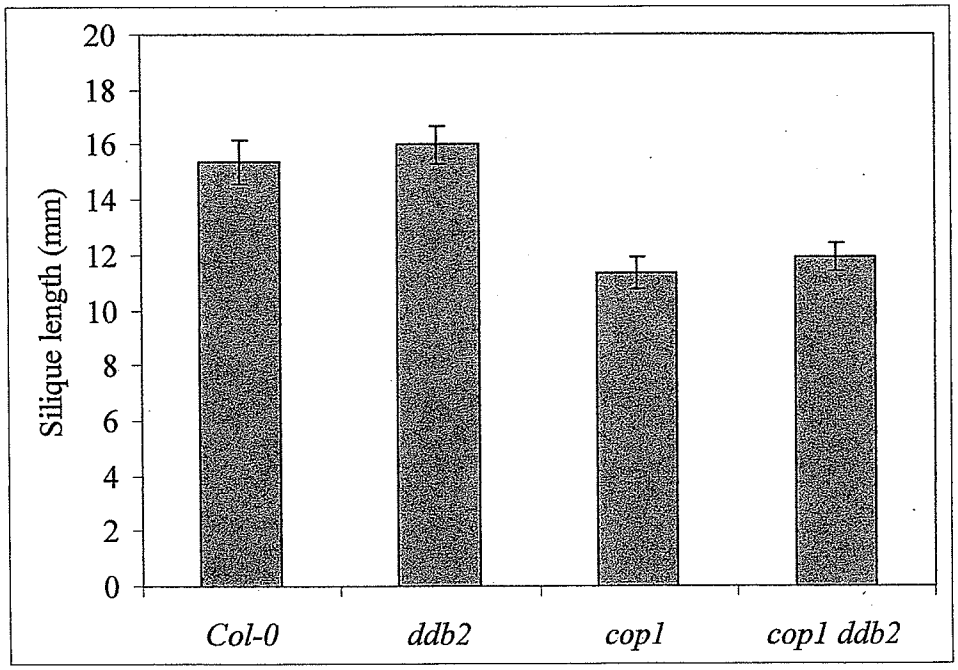


Figure S2.6 Analysis of wild type, *ddb2, cop1-4* and *cop1-4 ddb2* (cont.). G: Silique length (mm). Error bars indicate 95% C. I.

CHAPTER 3: THE EFFECT OF *DDB1A* OVEREXPRESSION ON *ARABIDOPSIS* GROWTH AND DEVELOPMENT

3. THE EFFECT OF *DDB1A* OVEREXPRESSION ON *ARABIDOPSIS* GROWTH AND DEVELOPMENT

3.1 ABSTRACT

Three independent *DDB1A* overexpression lines were used in this study to examine the effect of *DDB1A* overexpression on *Arabidopsis* development. No significant difference was observed between any of the overexpression lines and wildtype at the seedling or adult stage.

3.2 INTRODUCTION

Damaged DNA-Binding protein 1 (DDB1) is a component of the Damaged DNA-binding protein complex (DDB) which also contains DDB2. DDB plays an important role in damage recognition and initiation of nucleotide excision repair (NER).

DDB1 is a conserved protein. DDB1 homologues have been found in yeast, mammals, worms, insects and plants. In contrast, DDB2 homologues have been identified only in mammals and plants (Zolezzi and Linn, 2000). DDB1 is not only important in NER but also in the ubiquitin-proteasome pathway. It forms an E3 ubiquitin ligase complex with Cul4A, Roc1, and substrate receptors. It has been shown that many candidates can act as receptor proteins for their targets, these include DDB2 as a receptor for XPC, Cockayne syndrome A (CSA) as a receptor for its partner CSB and COP1 as a receptor for c-jun in humans (Lee and Zhou, 2007). This suggests that DDB1 regulates many proteins in a wide range of pathways.

Several studies have shown that mutation of *DDB1* can cause many developmental changes in various organisms. *Schizosaccharomyces pombe DDB1* mutants exhibit long cells, slow growth rate and unusual nuclei (Zolezzi *et al.,* 2002). Drosophila *DDB1* null alleles are lethal (Takata *et al.,* 2004). Similarly, conditional mutation of *DDB1* in mouse was shown to be lethal during early stages of embryo development (Cang *et al.,* 2006). In *C. elegans,* mutants of *DDB1* generated by RNAi showed embryonic and larval lethality (Kim and Kipreos, 2006). Recently, it has been shown that mutation of *DDB1* in chicken and human cells severely affects the cell cycle as well as cell viability (Wakasugi *et al.,* 2007).

Arabidopsis DDB1A and *DDB1B* are 91% identical. *ddb1a* mutants are similar to wildtype plants when grown in long or short day conditions. In contrast, the *ddb1b* mutant appears to be lethal (Schroeder *et al.,* 2002; Al Khateeb and Schroeder, 2007). It has been shown that more than 80% of *Arabidopsis* genes are duplicated (Briggs *et al.,* 2006). The redundancy of DDB1 in *Arabidopsis* plus the lethal phenotype of *ddb1b* mutant limits the understanding of the role of *DDB1A* in *Arabidopsis* growth and development.

Another way to study the role of *DDB1A* in *Arabidopsis* development is by examining the effect of *DDB1A* overexpression. Three independent *DDB1A* overexpression lines were used in this study. Lines were examined at the seedling and adult stages for protein characteristics and growth parameters.

3.3 MATERIALS AND METHODS

3.3.1 Growth conditions

Seedlings were grown in a growth chamber at 20° and 50% relative humidity under long day conditions (16 h light and 8 h dark). Light was provided by fluorescent bulbs (100 μmol photons. m^{-2}. s^{-1}). Plants were grown in Sunshine mix number 1 (SunGro, Bellevue, WA).

3.3.2 Generation of DDB1A-HA overexpression lines

The full length *DDB1A* coding sequence was amplified from Kazusa clone RZL 02f07 (Kazusa DNA Research Institute, Japan) and cloned into binary vector pCHF3, a pPZP211-based plant expression vector carrying the cauliflower mosaic virus 35S promoter and a pea ribulose 1,5-bisphosphate carboxylase/oxygenase terminator (C Fankhauser, K Hanson, and J Chory, unpublished data), along with a C-terminal 3xHA tag amplified from pMPY-3xHA (Schneider *et al.,* 1995). Wild-type Col-0 was transformed via standard *Agrobacterium*-mediated techniques (Weigel and Glazebrook, 2002). Single insertion lines were identified and analyzed via anti-HA western blot to determine DDB1A-HA level. Homozygous lines were used for all analysis.

3.3.3 Protein analysis

For seedling analysis, five 7-day-old seedlings grown under either long day or dark conditions were used. For adult plant analysis, leaf samples (0.5 cm^2) were taken from 4-week old plants grown under long-day conditions. Tissues were frozen in liquid

nitrogen then ground in 50 μL protein loading buffer and analyzed via western blot (Schroeder *et al.,* 2002) using anti-HA antibody (12CA5, Roche).

3.3.4 Growth parameters

Hypocotyl, pigment and other growth parameter measurements were as described in Chapter 2.

3.3.5 Statistical Analysis

All experiments were repeated at least three times. Results presented are means ± SE. Means were compared by student *t* test. Probabilities of 0.05 or less were considered to be statistically significant.

3.4 RESULTS

3.4.1 Light grown seedlings

All DDB1A overexpression lines exhibit a similar approximately 8-fold increase in *DDB1A* mRNA level in 7-day long day grown seedlings (Figure 3.1 A). In contrast, the overexpression line 5-1 shows the highest DDB1A-HA abundance compared to the other two lines. In addition, 10-5 shows more DDB1A-HA than 7-16 (Figure 3.1 B). Anthocyanin and chlorophyll content of seven-day old seedlings grown under long day

conditions was studied. No significant differences were observed between wildtype and all *DDB1A* overexpression lines (Figure 3.1 C, D).

3.4.2 Dark grown seedlings

To test the effect of dark conditions on the abundance of DDB1A-HA in 5-1, 10-5 and 7-16, seedlings were grown for 7 days under dark conditions then analyzed using anti-HA western blot. DDB1A-HA was detected in all lines (data not shown) but at lower levels/seedling than observed in long day grown seedlings. This experiment was repeated three times.

To examine if *DDB1A* is still overexpressed under dark conditions, total RNA was extracted from 7-day old dark grown seedlings. RT-PCR analysis was used to examine *DDB1A* mRNA level in the wildtype and the overexpression line 5-1. In agreement with light grown seedlings, dark grown 5-1 seedlings showed more *DDB1A* mRNA than wildtype (data not shown).

We studied hypocotyl elongation and anthocyanin content of 7-day old dark grown seedlings. No significant differences were observed between wildtype and all *DDB1A* overexpression lines (Figure 3.2 A,B).

3.4.3 Adult plants

DDB1A-HA level in adult plants (1 month old) was also examined. In contrast to seedlings, 10-5 adult plants exhibit the highest DDB1A-HA level followed by 5-1 then 7-16 (Figure 3.3 A).

Flowering time, rosette diameter, plant height, apical dominance and silique length of wildtype and all *DDB1A* overexpression lines were also examined. No significant differences were observed in any of the above mentioned phenotypes between the wildtype and any of the *DDB1A* overexpression lines (Figure 3.3 B, C, D, E, F).

3.5 DISCUSSION

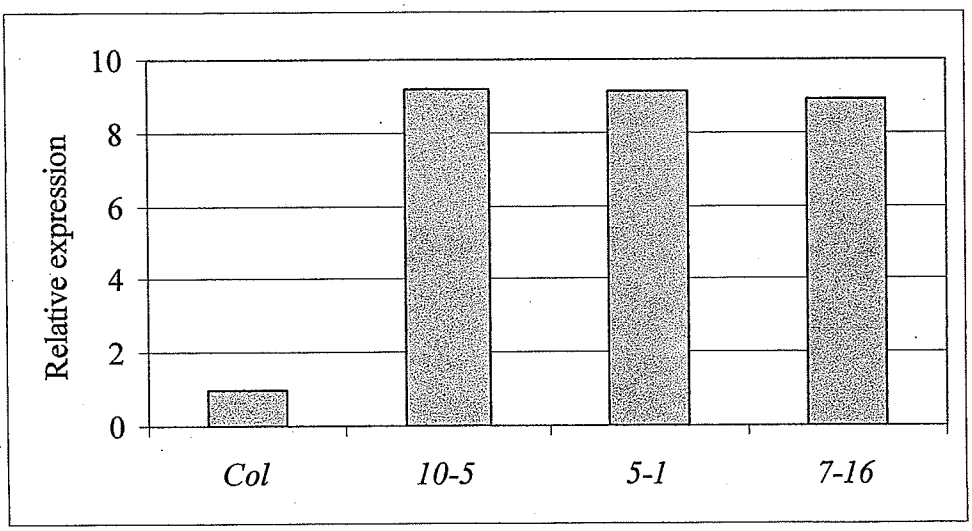
For all growth parameters mentioned above, no significant differences were observed between wildtype and *DDB1A* overexpression lines either at the seedling stage or for adults. Similarly, it has been shown previously that *ddb1a* mutation also exhibits wildtype phenotypes in the above mentioned growth parameters (Schroeder *et al.,* 2002).

The result of western blot analysis for DDB1A-HA abundance in dark grown seedlings showed that it is not highly abundant/seedling. One possible reason for this could be that dark grown seedlings are not photosynthetically active, so as a result total protein is less in these seedlings which subsequently affects DDB1A-HA abundance as well as level of other proteins. Estimates of total protein content, as measured by Ponceaus Staining, showed that the total protein content of light grown seedlings is more than those grown under dark conditions. Therefore, relative to total protein, DDB1A-HA content was found more to be similar in light and dark grown seedlings (data not shown).

However, the change in DDB1A-HA abundance between seedlings and adult plants suggests that DDB1A-HA stability may be developmentally regulated.

DDB1 is a component of many complexes. In combination with CUL4A, ROC1, and the specific substrate receptors, DDB1 forms E3 ubiquitin ligase complexes which are important regulators of protein degradation in many organisms (Groisman *et al.,* 2003). Since our overexpression lines do not exhibit any phenotypes, it seems that DDB1A is not the limiting factor in these complexes during *Arabidopsis* development. When we overexpressed *DDB1A*, we did not observe changes in *Arabidopsis* development so perhaps there is a specific DDB1A threshold that is sufficient for ubiquitination inside the cell. On the other hand, when we knockout *DDB1A*, again there is no observed phenotype (Schroeder *et al.,* 2002; Al Khateeb and Schroeder, 2007), this could be due to the high similarity between *DDB1A* and *DDB1B*.

A



B

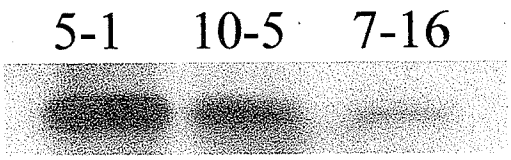
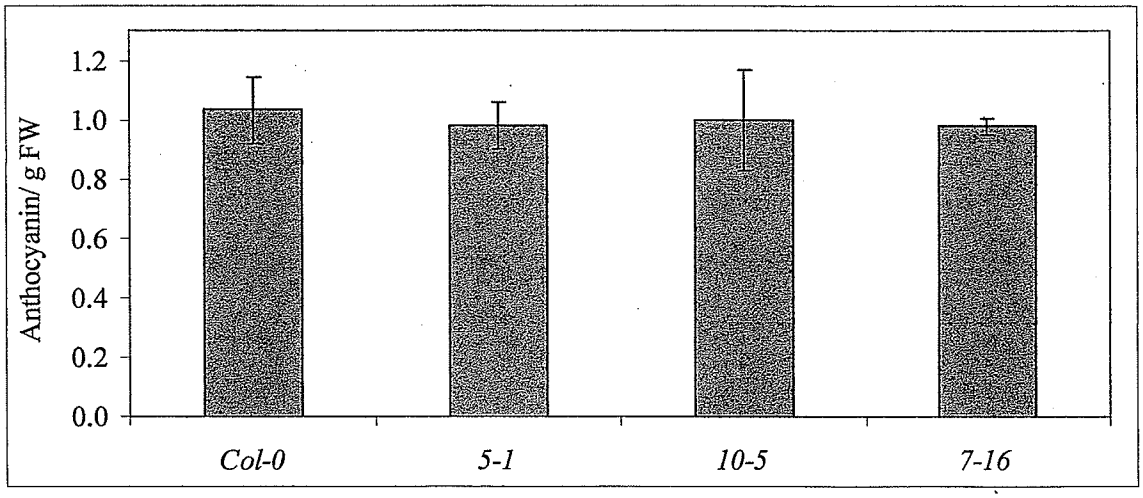


Figure 3.1 Analysis of 7-day-old long day grown seedlings.
A: *DDB1A* mRNA level in wildtype and three *DDB1A* overexpression lines corrected for *UBQ10*. **B:** Abundance of DDB1A-HA protein in 7-day-old long day grown overexpression lines as detected by anti-HA western blot.

C



D

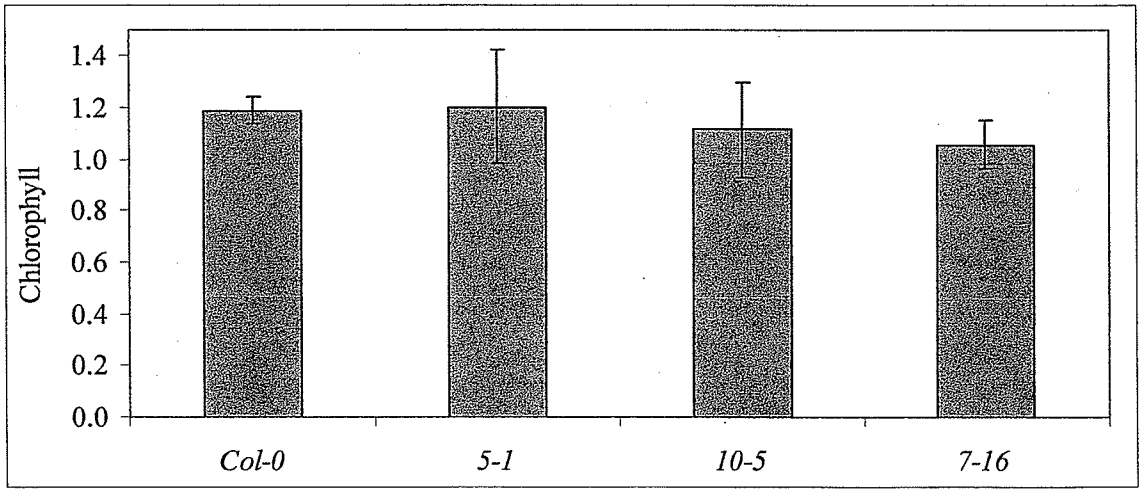
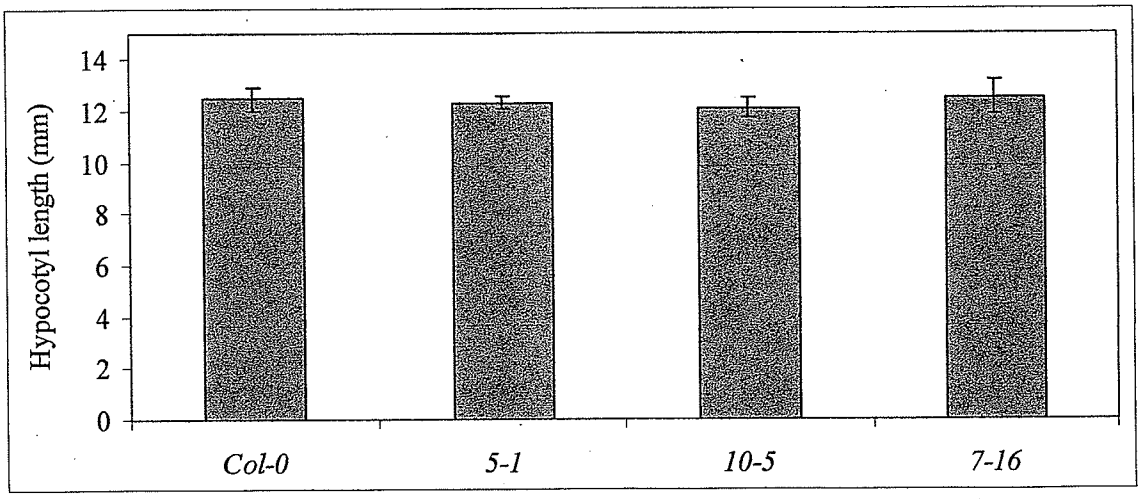


Figure 3.1 Analysis of 7-day-old long day grown seedlings (cont.). C: Anthocyanin content (n=4) **D:** Chlorophyll content (µg Chlorophyll/mg fresh weight) (n=4). Error bars indicate ± SE.

A



B

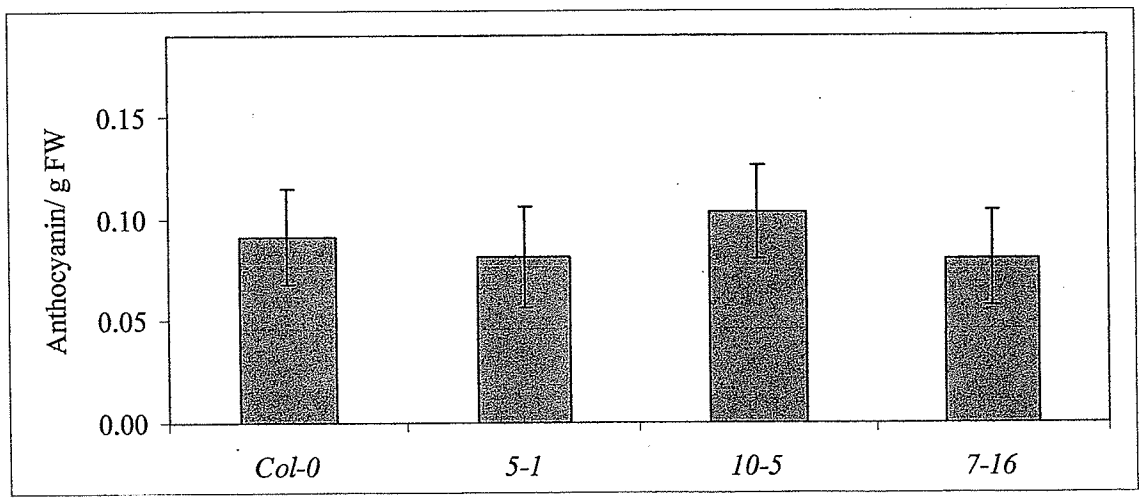
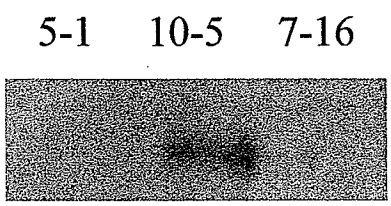
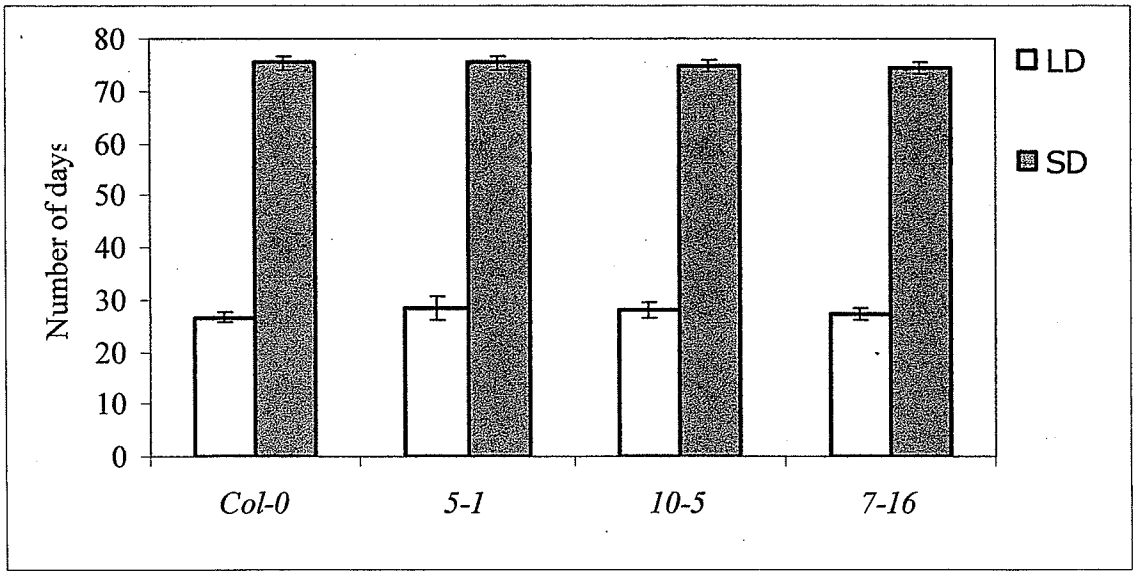


Figure 3.2 Phenotypic analysis of 7-day-old dark grown seedlings. A: Hypocotyl length (n=15). **B**: Anthocyanin content ($A_{530} - A_{657}$/g fresh weight) (n=4). Error bars indicate ± SE.

A



B



C

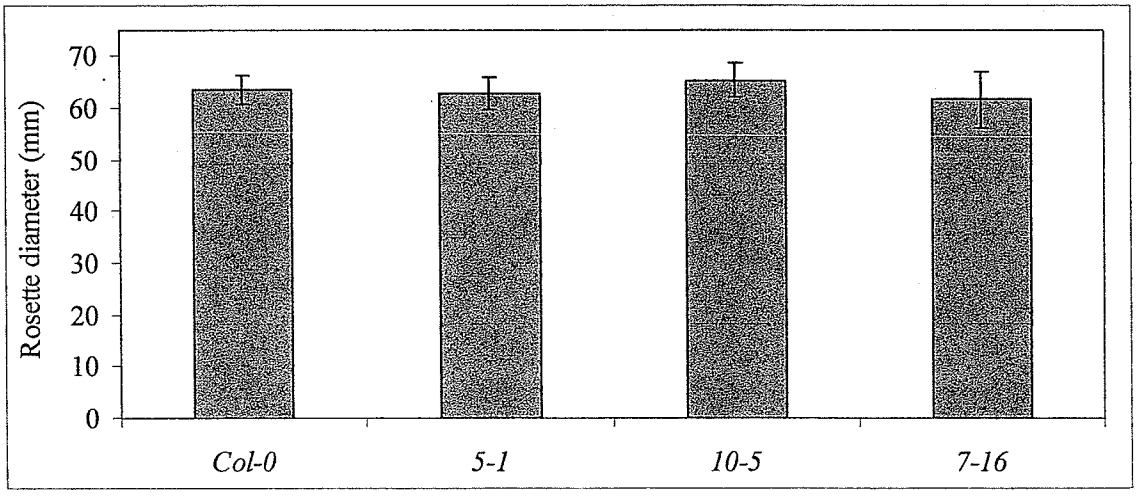
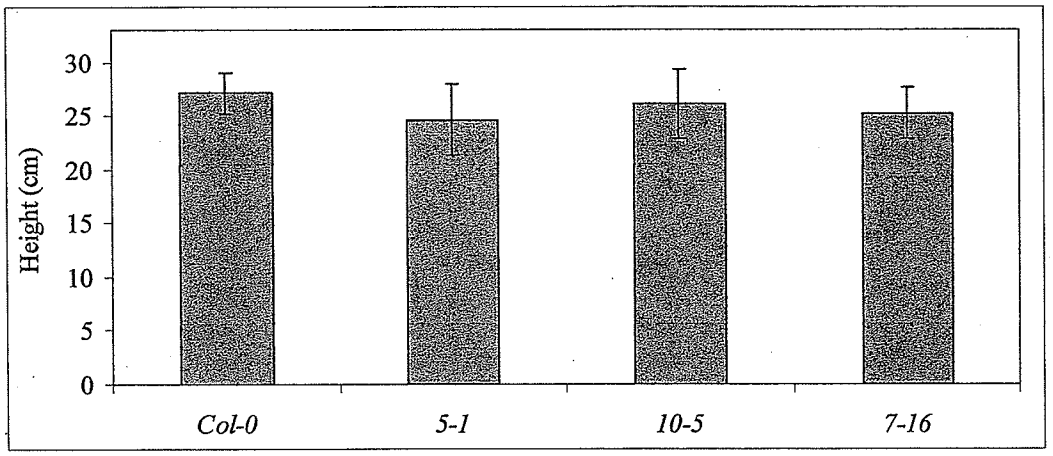
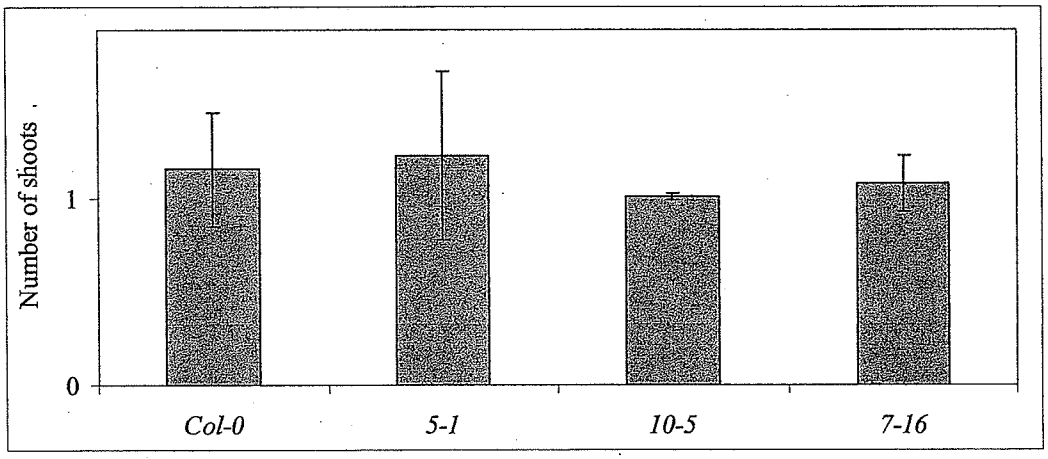


Figure 3.3 Adult plant analysis.
A: Abundance of DDB1A-HA protein in 1 month-old long day grown overexpression lines. **B**: Flowering time (in days) for long or short day grown plants. **C**: Rosette diameter of 4-week-old long day grown plants. Error bars indicate 95% C. I.

D



E



F

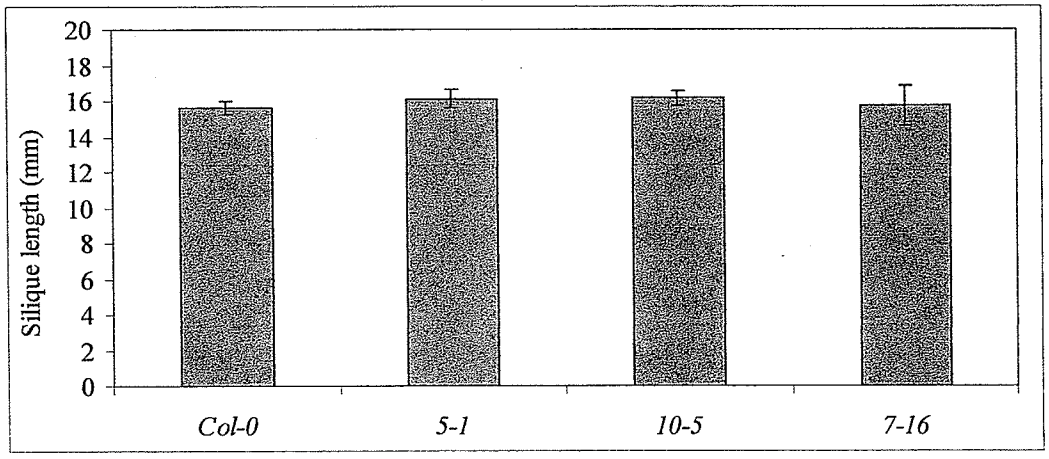


Figure 3.3 Adult plant analysis (cont.). D: Height (cm) of adult plants. **E**: Apical dominance. **F**: Silique length (mm). Error bars indicate 95% C. I.

CHAPTER 4: OVEREXPRESSION OF *ARABIDOPSIS* DAMAGED DNA BINDING PROTEIN 1A (DDB1A) ENHANCES DNA REPAIR

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This study has been submitted to The Plant Journal

4. OVEREXPRESSION OF *ARABIDOPSIS* DAMAGED DNA BINDING PROTEIN 1A (DDB1A) ENHANCES DNA REPAIR

4.1 ABSTRACT

DDB1 is a component of multiple complexes involved in genome stability, cell cycle regulation, histone modification, DNA replication and repair. *Arabidopsis* has two homologues of DDB1: DDB1A and DDB1B. In this study we examine the role of DDB1A in *Arabidopsis* DNA repair using a *DDB1A* null mutant (*ddb1a*) and overexpression lines generated using the CaMV 35S promoter. While UV tolerance assays showed no significant difference between wildtype plants and *ddb1a* mutants, a slight delay was detected in *ddb1a* mutants in photoproducts repair. *DDB1A* overexpression lines, however, showed higher levels of UV-resistance as well as faster DNA repair than wildtype. Upon UV exposure, *DDB1A* mRNA levels were shown to increase in wildtype and overexpression lines. *DDB1B* and *DDB2* mRNA levels also increased after UV exposure in wildtype, but induction was not observed in the *DDB1A* loss of function background. In conclusion, these results indicate that *DDB1A* plays an important role in *Arabidopsis* DNA repair.

4.2 INTRODUCTION

Plants must adapt to environmental stresses in order to perform optimally. Ultraviolet radiation (UV) is a component of the sunlight that arrives at the earth's surface. High levels of UV radiation induce a series of morphological changes in plants. These include changes in plant height, leaf thickening, cotyledon curling, stem elongation, leaf expansion, axillary branching and root/shoot ratio (Caldwell *et al.,* 2007; Frohnmeyer and Staiger, 2003). In addition, UV irradiation can damage plant membranes, proteins and DNA. This damage can result in toxic or mutagenic effects (Bray and West, 2005). UV damage to DNA generates lesions called photoproducts. UV photoproducts consist primarily of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidinone dimers (6-4PPs). It has been shown that UV photoproducts have the ability to inhibit DNA replication and transcription (Britt, 1995).

Plants use several mechanisms to protect themselves from UV irradiation including UV-absorbing pigments (e.g. flavonoids) and DNA repair mechanisms. Repair pathways of the damaged DNA in plants include photoreactivation (light-dependent repair), which uses UV-A or blue light to revert the damaged DNA to a normal configuration through the action of an enzyme called photolyase. The second pathway is the light-independent repair mechanism, which does not directly reverse the DNA damage, but instead replaces it with new, undamaged nucleotides. This process is known as nucleotide excision repair (NER) (Kunz *et al.,* 2006). In humans, *Xeroderma pigmentosum* (*XP*) is a rare disease caused by deficiency in nucleotide excision repair factors (*XPA-XPG*) characterized by increased sensitivity to sunlight with the

development of carcinomas at an early age (Shuck *et al.*, 2008).

NER involves several distinct steps. DNA is unwound by two DNA helicases, called XPB and XPD. Excision of the damaged DNA requires two nucleases. The first nuclease XPF: ERCC1 makes a cut at the 5' end of the DNA lesion. At the 3' end of the DNA lesion another nuclease called XPG makes the cut. Finally gap filling and strand ligation is achieved by a variety of enzymes including DNA polymerase (Shuck *et al.*, 2008).

The initiation of this pathway varies with the location of the damage. If the DNA lesions are throughout the genome, the NER subpathway called global genomic repair (GGR) can repair the damage. If the DNA lesions are in the transcribed strand of active genes, another subpathway called transcription-coupled repair (TCR) can repair the damage. These two pathways differ in the damage recognition step. In TCR, the stalled RNA polymerase II recruits CSA and CSB and initiates NER (Cleaver, 2005).

Several proteins are involved in damage recognition in GGR, such as XPC and the damaged DNA binding protein complex (DDB) (Shuck *et al.*, 2008). DDB may function to alter chromatin structure and recruit nucleotide excision repair factors to DNA damage sites (Gillet and Scharer, 2006). The DDB complex can distinguish between many DNA lesions which are induced by treatment with DNA-damaging factors (Fujiwara *et al.*, 1999). In humans, DDB consists of a 127 kDa subunit (DDB1) and a 48 kDa subunit (DDB2). The two DDB subunits have a higher affinity for DNA lesions than other damage recognition factors (Wakasugi *et al.*, 2007).

In rice (*Oryza sativa* cv. Nipponbare), DDB homologues have been cloned and found to encode a protein with a molecular mass of 122 kDa (DDB1) and another with a

molecular mass of 65 kDa (DDB2). The rice DDB1 has 78.0%, 53.0%, 53.2%, and 32.3% amino acid sequence identity with *Arabidopsis thaliana*, *Homo sapiens*, *Chlorocebus aethiops* and *Caenorhabditis elegans* DDB1, respectively. On the other hand, rice DDB2 shows only 62.6% and 23.0% identity with *A. thaliana* and *H. sapiens* DDB2, respectively (Ishibashi *et al.,* 2003).

Proteins have been found to interact with DDB1 and form a variety of complexes. DDB1 is a component of the newly identified multisubunit complexes that contain cullin-4 and other proteins. These complexes display an E3 ubiquitin ligase activity that recognizes specific substrates and mediates their degradation (Lee and Zhou, 2007). DDB1 has been shown to act as a linker to recruit WD40 receptor proteins via a conserved protein motif (the DWD box) to the E3 ligase machinery (He *et al.,* 2006). The DDB1–CUL4A ligase has been shown to target a variety of substrates such as the DNA replication licensing factor CDT1 (Hu *et al.,* 2004) and several histones (Kapetanaki *et al.,* 2006). In humans, in addition to DDB1, the CUL4A complex can contain DET1, a highly conserved protein. This complex is involved in ubiquitination and degradation of the proto-oncogenic transcription factor c-Jun (Wertz *et al.,* 2004). Also, DDB1 is integrated into nearly identical complexes with DDB2 and CSA with important roles in global genomic repair (GGR) and transcription coupled repair (TCR), respectively (Groisman *et al.,* 2003). Furthermore, DDB1 (and its partner DDB2) interact *in vivo* and *in vitro* with p300 which has histone acetyltransferase (HAT) activity (Datta *et al.,* 2001). Another study (Rapic-Otrin *et al.,* 2002) showed that DDB1 – p300 interaction is DDB1-dependent but not DDB2-dependent. DDB1 may form another complex with SPT3-$TAF_{II}31$-GCN5L acetylase (STAGA). This complex may be involved in the NER

pathway by facilitating the assembly of the repair machinery on the nucleosome by chromatin unfolding (Martinez *et al.,* 2001).

Schroeder *et al.* (2002) found that tobacco DDB1 forms an approximately 350 kDa complex with DET1. Similarly, it has been shown that *Arabidopsis* DDB1A, CUL4 and RBX1 are associated with DET1 (Bernhardt *et al.,* 2006; Chen *et al.,* 2006), or DDB2 (Bernhardt *et al.,* 2006), or PRL (Lee *et al.,* 2008). *Arabidopsis* has two homologues of DDB1: DDB1A and DDB1B. These two homologues are almost identical at the amino acid level (91%). *DDB1A* and *DDB1B* are expressed throughout *Arabidopsis* development (Al Khateeb and Schroeder, 2007). *Arabidopsis ddb1a* mutants show no obvious difference from wildtype plants. In contrast, *ddb1b* mutants appear to be lethal (Schroeder *et al.,* 2002).

Here, we use a *ddb1a* T-DNA mutant and *DDB1A* overexpression lines as genetic tools to investigate the role of *DDB1A* in *Arabidopsis* DNA damage repair. We find that overexpression of *DDB1A* enhances UV resistance. In addition, we examine the response of *DDB1A*, *DDB1B* and *DDB2* mRNA to UV light and the role of *DDB1A* in *DDB1B* and *DDB2* regulation. Furthermore, we use an epitope-tagged version of DDB1A (DDB1A-HA) to study the behavior and abundance of this protein under UV light. Using this genetic and molecular analysis we conclude that *DDB1A* plays a major role in *Arabidopsis* DNA damage repair.

4.3 MATERIALS AND METHODS

4.3.1 Plant materials and growth conditions

Throughout this study, the Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used as the wild-type plant. *ddb1a* T-DNA mutants were described previously (Schroeder *et al.,* 2002), and the *uvh1* mutant line (TAIR # CS3819) (Harlow *et al.,* 1994) was obtained from the *Arabidopsis* Stock Center (http://www.arabidopsis.org/). Seeds were grown on plates containing Murashige and Skoog (MS) media (1X MS salts, 0.8% phytoblend (Caisson Laboratories Inc., USA), 2% sucrose), stratified at 4 °C for two days then transferred to a growth chamber at 20 ±1 °C and 50% relative humidity. Light was supplied by cool white fluorescent bulbs in a photoperiod of 16 h light (100 µmol photons. m^{-2}. s^{-1}). Plants were grown in Sunshine mix number 1 (SunGro, Bellevue, WA).

4.3.2 Generation of DDB1A-HA overexpression lines

The full length *DDB1A* coding sequence was amplified from Kazusa clone RZL 02f07 (Kazusa DNA Research Institute, Japan) and cloned into binary vector pCHF3, a pPZP211-based plant expression vector carrying the cauliflower mosaic virus 35S promoter and a pea ribulose 1,5-bisphosphate carboxylase/oxygenase terminator (C Fankhauser, K Hanson, and J Chory, unpublished data), along with a C terminal 3xHA tag amplified from pMPY-3xHA (Schneider *et al.,* 1995). Wild-type Col-0 was transformed via standard *Agrobacterium*-mediated techniques (Weigel and Glazebrook, 2002). Single insertion lines were identified and analyzed via anti-HA western blot to determine DDB1A-HA level. Homozygous lines were used for all analysis.

4.3.3 UV sensitivity assays

Shoot assay: 21-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} measured with a UV meter (UVX-Radiometer, UVP, Upland, CA), and then incubated under dark conditions for 3 days to avoid photoreactivation. Plants were then transferred to standard growth conditions for three days where sensitivity was assessed by leaf yellowing and tissue death. The whole experiment was repeated three times.

Root assay: Seeds were grown on vertically oriented plates for 3 days under the same growth conditions mentioned above. 3-day-old seedlings were irradiated with UV-C for a final dose of 0, 500, 1000, and 1500 J. m^{-2}. Plates were rotated by 90° and incubated under dark conditions for 3 days. New root growth was measured using NIH ImageJ 1.36b software. The whole experiment was repeated three times.

4.3.4 DNA damage analysis

The amount of DNA damage in the samples was quantified by ELISA using TDM-2 and 64M-2 monoclonal antibodies (MBL, Naka-Ku Nagoya, Japan), which recognize CPDs and 6–4 photoproducts, respectively. The protocol used was based on Takeuchi *et al.,* (1996) and the manufacture's instructions (with some modifications as follows). 7-day-old seedlings were irradiated with UV-C (450 J. m^{-2}) and incubated under dark conditions. Samples were harvested at different time intervals after irradiation (0, 4, and 24h) under non-photoreactive red light conditions. DNA was extracted using the

quick DNA prep protocol (Weigel and Glazebrook, 2002). DNA concentration was measured by spectrophotometer at 260 nm absorbance. DNA samples were heat-denatured and plated onto microtiter 96-well plates and allowed to dry at 37°C. To remove unbound DNA, microtiter plates were washed 5 times with PBS-T (0.05% Tween-20 in Phosphate buffered saline (PBS), 137 mM NaCl, 10 mM phosphate, pH 7.4), and then blocked with fat-free skim milk solution (5% w/v) for 1h. Plates were washed with PBS-T three times, followed by first antibody (TDM-2 or 64M-2) incubation under dark conditions for 60 min. Plates were then washed 5 times with PBS-T followed by addition of the secondary antibody (biotinylated goat anti-mouse, Molecular Probes, Inc, Eugene, OR) and incubated under dark conditions for 60 min. Plates were then washed 5 times with PBS-T and streptavidin-linked horseradish peroxidase (Molecular Probes, Inc, Eugene, OR) was added. After 60 min of incubation under the same conditions, plates were washed with PBS-T three times followed by a single wash with citrate-phosphate buffer (0.05 M, pH 5.0). *O*-phenylenediamine (1, 2-benzenediamine) was used for the final color development. To stop the reaction, sulfuric acid (2 M) was used by adding 50 µl /well. Using a microplate reader (OPSYS MR, Dynex) plates were read at 492 nm to quantify the colorometric reaction. Four wells were used per sample and the whole experiment was repeated 3 times.

4.3.5 RNA extraction and semi-quantitative RT-PCR

Extraction of total RNA from 7-day-old long day grown seedlings was carried out by following the manufacturer's instruction using an RNeasy plant minikit (Qiagen,

Valencia, CA). Quality and quantity of isolated RNA was checked by denaturing gel electrophoresis and spectrophotometric analysis. The one-step RT-PCR system (Access RT-PCR, Promega, Madison, WI) was used to study expression in wildtype and transformed plants. For RT-PCR analysis, the following specific primers were used: *DDB1A*, 5'-TAAAGAAGTTAGTCATATGTGCCCT-3' and 5'-AGGAGCTGTTTATTC TCTCAAT -3', *DDB1B*, 5'-CACGAAACCAACAATTGCAG-3' and 5'-TTCCATCACA AAAGCATATG-3', *DDB2*, 5'- ACAGCCTGGCCATGAAGCTGGA-3' and 5'-CCTGC CATCCATCAGGGTTGAG-3'. Endogenous *DDB1A*, 5'-GAGAAAAGAACCGCGGA AGC-3' and 5'-GGGACCCAGAAGACGTC-3', transgenic *DDB1A*, 5'-GAGAAAAGAA CCGCGGAAGC-3' and 5'-CCCCCGGGTCAGCGGCCGCACTGAGCAGCGTA-3'. The *UBQ10* gene of *Arabidopsis* was used as a control (Al Khateeb and Schroeder, 2007). To detect relative differences in transcript levels, amplification was performed when the PCR product was accumulating exponentially with respect to cycle number for each gene. PCR products were separated on 1% (w/v) agarose gels and the intensities of ethidium bromide-stained bands were determined using ImageJ software (1.36b National Institutes of Health, USA). Band intensities of *DDB1A*, *DDB1B* and *DDB2* were corrected according to the relative quantity of *UBQ10* product. Each experiment was repeated 2-3 times (Appendix).

4.3.6 mRNA stability analysis

mRNA stability was examined as described by Gutierrez *et al.* (2002) with some modifications. Seedlings were grown on MS plates for 7 days using the same conditions

described above, then transferred to a flask containing incubation buffer (10 mM Sodium phosphate buffer, pH 7) for 60 minutes. The transcription inhibitor (Cordycepin, Sigma) was added to a final concentration of 600 μM and tissues were incubated under dark conditions with gentle agitation. Samples were taken at 2 and 4 hrs and frozen in liquid nitrogen. Total RNA extraction and RT-PCR was used as described above using the following primers: At1g72450, 5'-GAGATGTAGTCTGCTCAGC-3' and 5'-CTGGGC AGCAACATCAGG-3', *S18*, 5'-AAACGGCTACCACATCCAAG-3' and 5'-ACCCATC CCAAGGTTCAACT-3'. Band intensities of *DDB1A* and the unstable gene (At1g72450) were corrected according to the relative quantity of *S18* product. The whole experiment was repeated two times.

4.3.7 Protein analysis

Five 7-day-old seedlings were frozen in liquid nitrogen and then ground in 50 μL protein loading buffer and analyzed via western blot using anti-HA antibody (12CA5, Roche). For gel filtration analysis, protein was extracted as described in Schroeder *et al.* (2002) with the addition of 10 mM MG132 (EMD Biosciences Inc. San Diego, CA) to the extraction buffer and then fractionated on a Superdex 200 10/300 GL high performance column (Amersham). Fractions were collected and used in western blot analysis as described above (Schroeder *et al.*, 2002). ImageJ software (1.36b National Institutes of Health, USA) was used to measure band intensities.

4.4 RESULTS

4.4.1 Molecular analysis of *ddb1a* mutant and *DDB1A* overexpression lines in *Arabidopsis thaliana*

The transcript levels of *DDB1A*, *DDB1B* and *DDB2* in various *DDB1A* backgrounds were examined (Figure 4.1 A,B). The previously described T-DNA insertion in the *DDB1A* gene (Schroeder *et al.*, 2002) was found to result in a null mutation. The overexpression line 5-1 showed approximately 8-fold *DDB1A* transcript level compared to wildtype (Figure 4.1 A,B). Knockout of *DDB1A* resulted in no detectable effect on *DDB1B* expression, whereas overexpression of *DDB1A* increased *DDB1B* expression by 43% (Figure 4.1 A,B). *DDB2* levels were similar in wildtype, *ddb1a* and the *DDB1A* overexpression line.

We analyzed three independent *DDB1A* overexpression lines (5-1, 10-5 and 7-16), and found that all lines exhibit similar *DDB1A* mRNA levels (Chapter 3, Figure 3.1B). However, when we examined the level of DDB1A-HA protein using western blot analysis, differences were observed, with 5-1 showing the highest abundance, followed by 10-5 (about 60% of 5-1), and 7-16 exhibiting the lowest abundance with only 35% of 5-1 levels (Figure 4.1 C,D).

4.4.2 Overexpression of *DDB1A* enhances UV tolerance

The range of DDB1A-HA abundance among overexpression lines gave us the opportunity to examine the effect of DDB1A level on UV tolerance. We tested this effect with three assays; root, shoot and photoproduct repair. Seedlings of wildtype, *ddb1a*

mutant, and three *DDB1A* overexpression lines (namely; 5-1, 10-5 and 7-16) were examined. A mutant in *Arabidopsis UVH1* (XPF homologue) was used as the negative control (Harlow *et al.*, 1994).

For the shoot assay, 3-week-old plants were irradiated with 450 J. m^{-2} UV-C then incubated for 3 days under dark conditions to eliminate photoreactivation, then returned to long day conditions to assess phenotypes. As expected, all *uvh1* plants were severely affected or dead (Figure 4.2 A). Leaves of wildtype, *ddb1a* and the overexpression line 7-16 displayed yellow to brown lesions after UV irradiation. In contrast, the overexpression lines with the highest DDB1A content (5-1 and 10-5) exhibited a UV resistant phenotype, with normal growth until senescence. Thus, as the level of DDB1A increased, the seedlings exhibit more UV-resistance.

To better quantify these phenotypes we performed root growth analysis. Seedlings were irradiated with 0, 500, 1000 and 1500 J. m^{-2} UV-C and new root growth was measured. Figure 4.2 B shows the relative root growth of all genotypes compared to the average root growth in the respective unirradiated control (100% root growth). As expected, root growth of the *uvh1* mutant was severely affected by UV-C, with the lowest dose of UV-C resulting in more than 70% inhibition of root growth. No new growth was observed at 1000 and 1500 J. m^{-2}. No significant difference was observed between wildtype and *ddb1a* at all UV-C doses tested. The dose required for 50% inhibition of root growth in wildtype and *ddb1a* was more than 1500 J. m^{-2} (Figure S4.1). All the *DDB1A* overexpression lines showed more root growth than the wildtype, *ddb1a* and *uvh1* seedlings. Both 5-1 and 10-5 seedlings exhibit a similar response to UV, with less than 20% inhibition of root growth even with the highest dose of UV-C (1500 J. m^{-2}).

Interestingly, the 7-16 seedlings, which have lower abundance of DDB1A than the other overexpression lines (Figure 4.1 D), showed more inhibition of root growth than 5-1 and 10-5, but still show more growth than wildtype (Figure 4.2 B). Thus, the level of UV-C tolerance in *Arabidopsis* roots is proportional to DDB1A level.

We next examined the effect of other DNA damaging agents on wildtype, *ddb1a* and the *DDB1A* overexpression line 5-1. Seeds were plated with various concentrations of hydrogen peroxide and MMC for 3 weeks under both dark and light conditions. We did not observe any differences between lines (Figure S4.2 and S4.3 and data not shown).

UV light induces photoproducts in DNA. The DDB complex has been shown to be involved in dark repair of DNA photoproducts (Kimura and Sakaguchi, 2006). To examine the DNA repair ability of *ddb1a* and one of the overexpression lines (5-1) after UV irradiation, 7-day-old seedlings were exposed to 450 J. m^2 UV-C and DNA was extracted from whole seedlings after various time intervals. An ELISA test was carried out to measure the repair rate of both kinds of photoproducts (6-4PPs and CPDs). In general, all lines showed more efficient repair of CPDs than 6-4PPs 24 hours after irradiation. *uvh1* mutant seedlings showed minimal reduction in the amount of 6-4PPs and CPDs after UV exposure, with 88% and 82% remaining 24 hours after irradiation, respectively (Figure 4.3 A,B).

With respect to repair of 6-4 photoproducts, a significant difference ($P \leq 0.01$) was observed between *ddb1a* and wildtype 4 hours after irradiation (93% vs. 67% remaining, respectively). By 24 hours after irradiation, this difference was smaller (77% vs. 67% remaining, respectively). For CPDs, *ddb1a* exhibited lower levels of repair than wildtype at both time points, but this difference was only significant at 24 hours (46% vs.

35% remaining, respectively, $P \leq 0.05$). In contrast, the *DDB1A* overexpression line 5-1 exhibited higher levels of repair than wildtype at all time points with significant differences ($P \leq 0.05$) observed at 4 and 24 hours for 6-4 photoproducts and at 4 hr for CPDs (Figure 4.3 A,B). Thus, rate of photoproduct repair correlates with DDB1A level.

4.4.3 *DDB1A*, *DDB1B* and *DDB2* mRNA levels are affected by UV light

In order to further examine the role of *DDB1A* in UV response, semi-quantitative RT-PCR analysis was used to study *DDB1A*, *DDB1B* and *DDB2* mRNA levels following UV irradiation of wildtype, *ddb1a* and the overexpression lines. In wildtype, *DDB1A* transcript level was found to increase to more than three fold the original level 3 hours after UV-C exposure (Figure 4.4 A). Later, *DDB1A* level in wildtype seedlings decreased gradually until it reached the level in non-irradiated seedlings. *UBQ10* was used as the loading control for this experiment, but a similar result was obtained if *Actin* or *S18* were used (Table 4.1). *DDB1A* level in seedlings incubated in the dark in the absence of UV treatment was unchanged (Table 4.1). Thus, the increase in *DDB1A* level is the result of UV treatment.

For the *DDB1A* overexpression line 5-1, *DDB1A* level also increased to more than three fold the original level 3 hours after UV-C exposure then dropped by 6 and 24 hours after UV exposure but still showed higher mRNA levels than the non-irradiated seedlings (Figure 4.4 A). To determine if the increase in *DDB1A* mRNA level after irradiation in the overexpression line is due to the effect of UV on the endogenous *DDB1A* gene or the transgenic version, we used specific primers to distinguish between the endogenous and

the transgenic message. We found that *DDB1A* mRNA level of both versions (endogenous and transgenic) increased after UV irradiation (Table 4.1). An approximate 2 to 3-fold induction in total *DDB1A* was also obtained when the 10-5 and 7-16 lines were UV treated (Table 4.1). Thus, *DDB1A* is also UV induced in the *DDB1A* overexpression lines.

In wildtype seedlings, *DDB1B* mRNA level also increased 3 hours after UV exposure (Figure 4.4 B), although not to the degree observed for *DDB1A*. In *ddb1a* mutant seedlings, however, no change in *DDB1B* mRNA level was observed following UV irradiation. Thus, UV induction of *DDB1B* requires *DDB1A*. Irradiation of the *DDB1A* overexpression line 5-1 enhanced *DDB1B* mRNA level (20%) starting from 3 hours after exposure until 24 hours (Figure 4.4 B). Since the level of *DDB1B* is higher in the overexpression line under normal conditions (43%) (Figure 4.1 B), this suggests that the overall level of *DDB1B* in wildtype and the overexpression line 3 hours after UV exposure is similar (around 1.7-fold the untreated wildtype level).

UV-C treatment did not change *DDB2* mRNA level in wildtype or the overexpression line 5-1 in the first 6 hours after exposure (Figure 4.4 C). However, 24 hours after exposure a 20-25% increase in mRNA level was detected in both lines compared to the non-irradiated seedlings. In contrast, *ddb1a* seedlings exhibited a dramatic reduction in *DDB2* levels following UV-C irradiation, with approximately 31, 53 and 70 % reduction in mRNA 3, 6 and 24 hours after irradiation, respectively (Figure 4.4 C). Thus, *DDB1A* is also required for *DDB2* transcript induction.

4.4.4 *DDB1A* induction

Although *DDB1A* in the overexpression line has a different promoter (CaMV 35S) than the endogenous gene, RT-PCR analysis showed that *DDB1A* levels were increased by UV in both lines (Figure 4.4 A). One possible explanation for this result is that UV affects the 35S promoter. To assess this possibility we examined the effect of UV on the mRNA level of another 35S driven gene, *Myc-DET1* (Schroeder *et al.,* 2002). No change in the mRNA level of 35S *Myc-DET1* was observed following UV-C exposure (Table 4.1). Therefore UV does not appear to affect the 35S promoter.

A second explanation is that UV affects *DDB1A* mRNA stability. To assess this possibility, we inhibited transcription with cordycepin to examine the effect of UV on *DDB1A* mRNA stability. Two unstable genes (*At1g72450* and *NIA2*) were used as controls (Gutierrez *et al.,* 2002; Lidder *et al.,* 2005). Because the *UBQ10* transcript is unstable in the presence of the transcription inhibitor (Table 4.2), we used *S18* as a loading control.

In wildtype seedlings, as expected, both *At1g72450* and *NIA2* were unstable and start to degrade shortly after the addition of the transcription inhibitor (Figure 4.5 and Table 4.2). Only minor differences were observed between UV treated and untreated seedlings in terms of mRNA stability. In contrast, the *DDB1A* message was relatively stable over the time intervals studied (Figure 4.5). This is consistent with previous results which suggest that the majority of *Arabidopsis* transcripts are stable (Gutierrez *et al.,* 2002). Similar *DDB1A* mRNA levels were detected between irradiated and non-irradiated seedlings. Minor differences were insufficient to account for the 3-fold induction observed. In the overexpression line 5-1, UV also did not significantly affect the stability

of total *DDB1A* (Table data 4.2). When the transgenic and endogenous versions of *DDB1A* were examined separately in this background, again no major differences in stability were detected (Table 4.2). Thus, UV irradiation did not appear to dramatically change RNA stability in either background, therefore, the change in *DDB1A* mRNA level is not due to UV regulation of mRNA stability. Since the increase in *DDB1A* mRNA level (3-fold) was not observed in the presence of the transcription inhibitor, transcription must be required for this effect, but the basis is unclear.

4.4.5 Effect of UV on DDB1A-HA abundance and complex size

We were also interested in studying the behavior of DDB1A protein after UV exposure. We used an epitope-tagging approach to investigate DDB1A-HA abundance and complex formation before and after UV irradiation. Western blot analysis was carried out using anti-HA antibody. Three hours after UV exposure, DDB1A-HA abundance increased by more than 50% (Figure 4.6 A). This increase in protein abundance is in agreement with the increase in mRNA level after 3 hours but to a lesser extent (Figure 4.4 A). Six hours after irradiation, DDB1A-HA level decreased to less than 50% of the non-irradiated seedlings. After 6 hours, DDB1A-HA level started to increase again.

DDB1 can be in many forms in the cell, as a monomer or forming many different complexes with other proteins. *Arabidopsis* DDB1A forms a variety of CUL4/RBX1 E3 ligase complexes (Bernhardt *et al.,* 2006; Chen *et al.,* 2006; Lee *et al.,* 2008) including COP10 and DET1 (Schroeder *et al.,* 2002; Yanagawa *et al.,* 2004), or DDB2 (Bernhardt *et al.,* 2006; Chen *et al.,* 2006), or other proteins (Lee *et al.,* 2008). The change in

DDB1A abundance after UV irradiation encouraged us to determine if UV has an effect on DDB1A complex size. Complex formation in UV-irradiated and non-irradiated seedlings was examined using gel filtration. As described previously (Yanagawa *et al.*, 2004), a broad profile was observed in the control sample. After UV exposure we often saw a relative decrease in fractions 9, 10 and 11. These fractions correspond approximately to molecular weights between 160 and 370 kD (Figure 4.6 B,C). This decrease may be due to degradation of complexes in this range or due to recruitment or dissociation of these complexes into other forms.

4.5 DISCUSSION

DNA repair mechanisms are highly conserved among organisms, and *Arabidopsis* has homologues of human NER factors (Kimura and Sakaguchi, 2006). In the present study, we used an *Arabidopsis ddb1a* null allele to examine the effect UV on *Arabidopsis* DNA repair. We also generated *DDB1A* overexpression lines to test the effect of enhanced expression of *DDB1A* on UV treated plants.

4.5.1 NER mutants in *Arabidopsis*

UV tolerance assays (shoot and root) showed no difference between *ddb1a* mutant and wildtype plants. In contrast, a significant difference in 6-4 photoproduct repair (4 hours after UV exposure) and CPD repair (24 hours after UV exposure) was observed between *ddb1a* and wildtype. One possible explanation for this discrepancy is

since phenotypic analysis was not performed until 3 days after UV treatment, subtle differences in initial repair rate were not detected at this point.

Arabidopsis has two homologues of Damaged DNA Binding protein 1: DDB1A and DDB1B. *ddb1b* mutants appear to be lethal (Schroeder *et al.,* 2002), so we are unable to examine the effect of UV on *ddb1b* mutant seedlings. Due to the high sequence similarity between the two homologues, perhaps DDB1B alone is sufficient for minimal NER, even though *DDB1B* is neither upregulated nor UV-induced in *ddb1a* plants.

The role of other *Arabidopsis* NER proteins has also been examined. A T-DNA insertion allele of *DDB2*, the other component of the DDB complex, exhibits increased sensitivity to DNA damaging agents (UV-B, methyl methanesulfonate and hydrogen peroxide) (Koga *et al.,* 2006). Knockouts of *Atxpb1*, one of the *Arabidopsis* homologues of XPB/RAD25, exhibit no phenotypic difference from wildtype when exposed to a wide range of UV-C irradiation. *Arabidopsis* has two XPB homologues that are 95% identical at the amino acid level (Costa *et al.,* 2001). The *uvh6* mutant, defective in the *Arabidopsis* homologue of XPD, resulted in severe browning and death 3 days after UV-C irradiation (Liu *et al.,* 2003). Mutation of *UVR1* in *Arabidopsis* (the XPG homologue), resulted in reduction of root growth and a sensitive shoot phenotype after UV-B irradiation (Britt *et al.,* 1993; Liu *et al.,* 2001). The *Arabidopsis* homologue of the 5' endonuclease (XPF) is UVH1. *uvh1* seedlings showed sensitive phenotypes (shoot and root) after UV exposure (Fidantsef *et al.,* 2000). Mutants in *Arabidopsis ERCC1* are found to be severely affected by UV-B and mitomycin C (Hefner *et al.,* 2003). C*entrin2* is a component of NER that interacts with *AtXPC* and *AtRAD4* early during the damage recognition step. C*entrin2* mutants showed UV-C sensitive phenotypes (Molinier *et al.,*

2004). These UV-sensitive phenotypes in *Arabidopsis* due to mutation in NER factors indicate that the components of this pathway are conserved among a variety of organisms.

4.5.2 UV-tolerant phenotypes

DDB1A overexpression lines showed enhanced UV resistance phenotypes. The overexpression line 5-1 exhibited healthier plants in the shoot assay, as well as more relative root growth and faster photoproduct repair rates than wildtype. The variation in DDB1A abundance among the overexpression lines, with highest abundance in 5-1 and the lowest in 7-16, coincides with root and shoot assays results. 5-1 shows the best relative growth, while 7-16 was lower than the other overexpression lines. This suggests that UV tolerance is DDB1A dose dependent.

Other *Arabidopsis* UV resistant mutants include the following. UV *insensitive1* (*uvi1*) was isolated as a single recessive mutation resistant to UV-B with higher fresh weight, less inhibition of root growth and faster photoproduct repair than wildtype after UV exposure (Tanaka *et al.,* 2002). Similarly, *uvi4* showed increased plant fresh weight after UV-B exposure compared to wildtype, but no significant difference was observed in UV-B absorbing compounds and CPD repair rate (Hase *et al.,* 2006). Another study found a dominant mutation in single gene (*uvt1*) showed a UV-B tolerance phenotype. This increase in UV-B tolerance is due to an increase in accumulation of UV absorbing compounds that can protect *Arabidopsis* leaves from UV exposure (Bieza and Lois, 2001). In order to determine if the increase in UV tolerance in our *DDB1A*

overexpression lines is at the UV protection level or the DNA repair level, we assessed anthocyanin accumulation in both wildtype and the overexpression line 5-1. Both lines exhibit similar anthocyanin content (chapter 3 Figure 3.2B). This further confirms that DDB1A increases UV tolerance via enhancement of DNA repair.

4.5.3 Overexpression of NER factors

Overexpression lines of *Arabidopsis Centrin2* exhibit enhancement in DNA repair (Liang *et al.,* 2006). While few overexpression lines are available in *Arabidopsis* for NER factors, the following results were obtained from other organisms. The response of *DDB2* overexpression rice lines to high levels of UV-B was assessed in callus and seedlings. *DDB2* overexpression lines demonstrated larger calli and increased UV-tolerance in seedlings (Ishibashi *et al.,* 2006). In mouse, an ectopically overexpressing *DDB2* line exhibited a delay in squamous cell carcinoma and fewer tumors after exposure to UV-B (Alekseev *et al.,* 2005). Similarly, overexpression of *DDB2* in human cells reduced UV-induced apoptosis (Sun and Chao, 2005). When *DDB2*-overexpressing cell lines of hamster were exposed to UV irradiation, more than 50% of the photoproducts were removed within 12 h in *DDB2*-overexpressing cells compared to no repair in control cells (Sun *et al.,* 2002).

4.5.4 Effect of UV on *DDB1* and *DDB2* mRNA level

We previously reported that *DDB1A* is more abundant (> 2-fold) than *DDB1B* in most *Arabidopsis* tissues (Al Khateeb and Schroeder, 2007). Here, we examined the effect of UV-C irradiation on mRNA levels of these two genes and its partner (*DDB2*) in the DDB complex. Our mRNA analysis showed that *DDB1A* levels increase and reached a maximum 3 hours after UV-C exposure in wildtype and the overexpression lines. In rice, *OsUV-DDB1* mRNA level increased and reaches a maximum 4 to 7 hours after UV-B irradiation (Ishibashi *et al.,* 2003). Recently, the effect of different DNA damaging agents on the *Aspergillus nidulans* DNA damage binding protein 1 homologue (DdbA) were examined (Lima *et al.,* 2008). *DdbA* mRNA level increased significantly 3-fold 2.5 hours after UV irradiation (500 J. m^{-2}). In contrast to these findings, human *DDB1* mRNA level was not affected by UV irradiation (Rapic-Otrin *et al.,* 2002).

We found that *DDB2* mRNA level increased in wildtype and the *DDB1A* overexpression line 24 hours after UV irradiation. Similarly, human *DDB2* mRNA increases significantly 24 hours after irradiation and reaches a maximum after 48 hours (Rapic-Otrin *et al.,* 2002). In other studies, UV irradiation of normal human cells showed an increase (2 to 3 -fold) in *DDB2* mRNA level 38 hours after irradiation, then mRNA level dropped to the normal levels (Itoh *et al.,* 2001; Nichols *et al.,* 2000). In rice, *OsUV-DDB2* mRNA increased with UV and reached the maximum level 7 to 10 hours after irradiation (Ishibashi *et al.,* 2003).

In contrast to wildtype and the overexpression line, the mRNA level of *DDB2* in *ddb1a* seedlings decreased after UV exposure (Figure 4.4 C). It has been shown in human cells that knockdown of *DDB1* by siRNA impaired DDB2 degradation after UV exposure

(Li *et al.,* 2006). Perhaps cells try to maintain DDB2 at a certain level by suppression of transcription.

Arabidopsis XPB1 and *XPB2* showed significant decreases in transcript levels 24 hours after UV-B exposure (Morgante *et al.,* 2005). mRNA levels of the other DNA helicase in *Arabidopsis* (*AtXPD*) was not affected by UV exposure up to 9 hours post irradiation (Vonarx *et al.,* 2006). It has been found that mutation of one component of the NER pathway can affect (upregulation or downregulation) the expression profile of other components. For example, mutation of *Arabidopsis* C*entrin* downregulated *XPC* by 60% 2 hours after irradiation but upregulated *XPB*, *XPD* and *UVH1* (Molinier *et al.,* 2004).

4.5.5 Effect of UV on DDB1 and DDB2 Abundance

At the protein level, we found that the abundance of the tagged version of DDB1A increased 3 hours after UV irradiation then dropped below the normal level. Human DDB2 levels drop during the first hour after UV exposure, then are restored to normal level at 24 hours and subsequently increased to reach the maximum (10 to12-fold) 48 hours after UV exposure (Rapic-Otrin *et al.,* 2002). In a study where the effect of UV irradiation on tagged DDB2 concentration was examined, a similar pattern was observed (Alekseev *et al.,* 2005).

DDB1 is present in many forms, such as a monomer with a molecular weight of 120 kD, or associated with its partner DDB2 with a total of ~185 kD, or with DDB2, CUL4, and RBX1 (~285 kD), or in the CDD complex (~200 kD), or with CUL4, RBX1 and CDD (~300 kD), or with many others (Bernhardt *et al.,* 2006; Chen *et al.,* 2004; Lee

et al., 2008; Schroeder *et al.,* 2002). The change in complex size after UV exposure in fractions 9, 10 and 11 corresponds to molecular weights between 160 kD and 370 kD, which encompasses many of the above complexes. Our results suggest that UV can modulate DDB1A complex formation.

4.5.6 mRNA stability

RNA stability is important in gene expression regulation in eukaryotic cells (Meyer *et al.,* 2004). It has been shown that unstable transcripts have sequence elements and transacting factors that affect the half-life of the RNA (such as AU-rich elements) (Gutierrez *et al.,* 2002). In *Arabidopsis*, some transcripts exhibit a change in stability level under specific environmental conditions. For example, *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) transcript was found to be degraded under light conditions (Yakir *et al.,* 2007). Light-Harvesting chlorophyll-binding protein transcripts are degraded after exposure to blue light (Folta and Kaufman, 2003). Also, UV irradiation can stimulate rapid cytoplasmic mRNA degradation (Revenkova *et al.,* 1999). We found that the *DDB1A* transcript is stable with or without UV exposure compared to the unstable control genes we analyzed. This means the increase in *DDB1A* transcript level after UV exposure is not apparently due to transcript stabilization.

4.5.7 Suggested roles for DDB in DNA repair

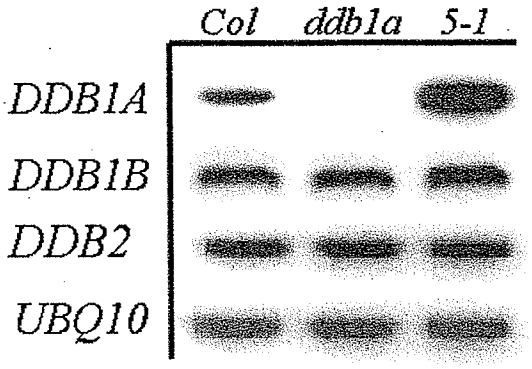
The initial step in GGR of the major UV-induced photoproducts, cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts (6–4PPs) is damage recognition. The available literature suggests that proteins such as XPC and/or the DDB complex carry out this function. The DDB1-DDB2 complex can bind not only to UV induced photoproducts but also to DNA containing small base pair mismatches (2 – 3 bps), suggesting that this binding acts as a sensor for structural change in the DNA (Wittschieben *et al.,* 2005). DDB2-XPC interaction is also important in damage recognition. DDB2 recruits XPC to DNA damage sites and increases cyclobutane pyrimidine dimer repair (Wang *et al.,* 2004). The association of DDB with CBP/p300 proteins that have histone acetyltransferase activities may act at the chromatin remodeling level so will recruit other repair complexes to the damage site (Rapic-Otrin *et al.,* 2002). Li *et al.,* (2006) suggested that DDB1 acts in NER through recruiting essential factors from different complexes to the damaged chromatin to open the condensed chromatin and allow NER factors to initiate the repair process.

In addition to its role in GGR, DDB1 is also important in TCR. It has been shown that CSA is part of the E3-ubiquitin (Ub) ligase complex that contains DDB1, Cullin 4A, and ROC1/Rbx1 (Groisman *et al.,* 2003). After UV exposure, CSB recruits the CSA-DDB1 E3-Ub ligase/CSN complex to the damage site (Fousteri and Mullenders, 2008). Further analysis is required to distinguish between the role of *Arabidopsis* DDB1A in GGR and TCR.

In summary, we have found that mutation of *ddb1a* showed a phenotype in 6-4 photoproduct repair. In addition, *DDB1A* overexpression lines exhibit dose-dependent

phenotypes including healthier plants in shoot assay, as well as more relative root growth and faster photoproduct repair after UV irradiation. Our findings, together with recent results from other systems, suggest that DDB1 is an important factor in the NER pathway.

A



B

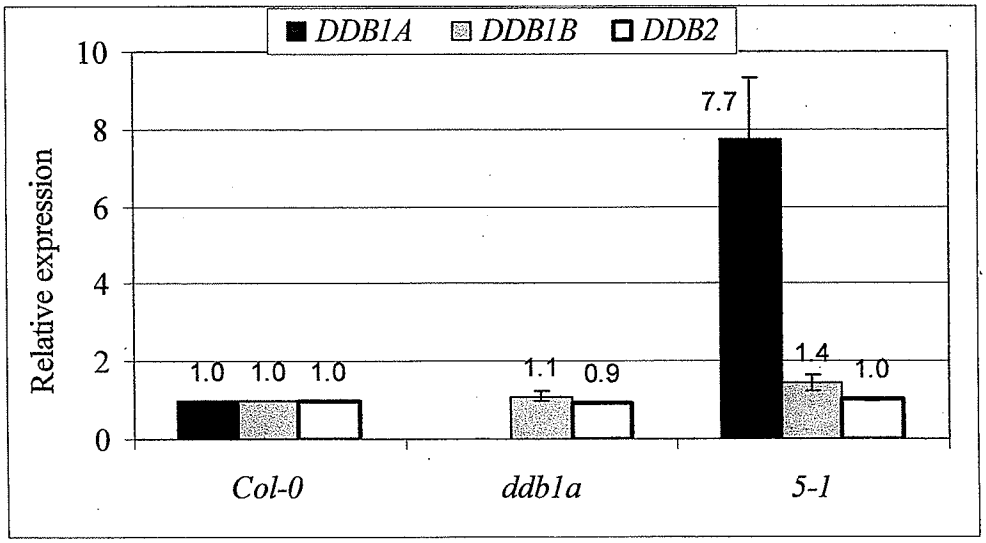
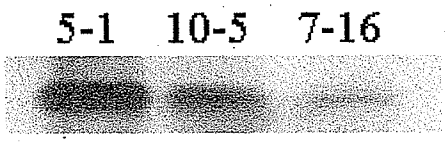


Figure 4.1 Characterization of mRNA and protein levels in *DDB1A* mutant and overexpression lines.
A: mRNA level of *DDB1A*, *DDB1B*, *DDB2* and *UBQ10* in 7-day-old *Arabidopsis* wildtype, *ddb1a* and *DDB1A* overexpression line 5-1 seedlings as measured by semi-quantitative RT-PCR. **B:** Quantification of *DDB1A*, *DDB1B*, *DDB2* mRNA level (normalized to *UBQ10* levels). Data are shown as the means ± SE (n=3). Numbers above error bars indicate mean expression relative to wildtype.

C



D

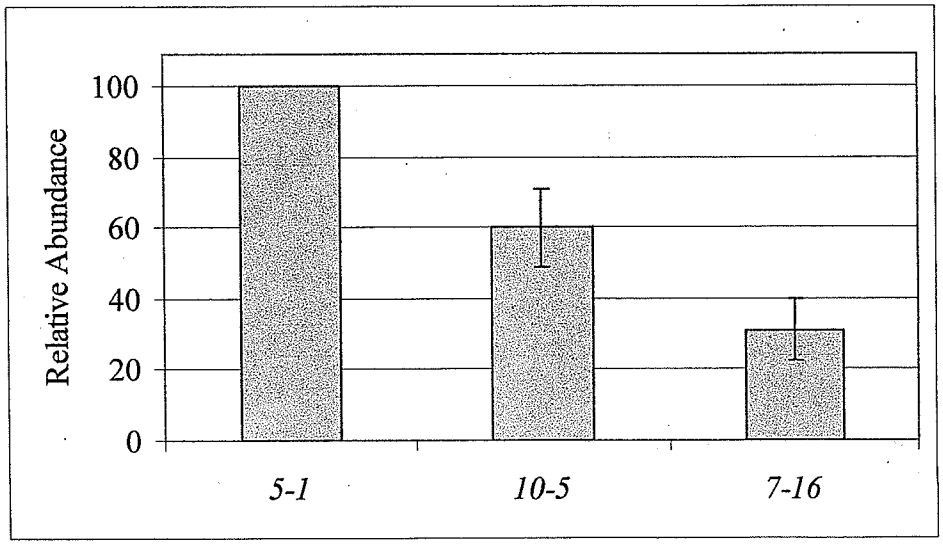


Figure 4.1 Characterization of mRNA and protein levels in *DDB1A* mutant and overexpression lines (cont.). C: Abundance of DDB1A-HA protein in 7-day-old long day grown overexpression lines. **D:** Quantification of DDB1A-HA abundance in overexpression lines relative to the line showing the highest abundance (5-1). Error bars indicate ± SE (n=3).

A

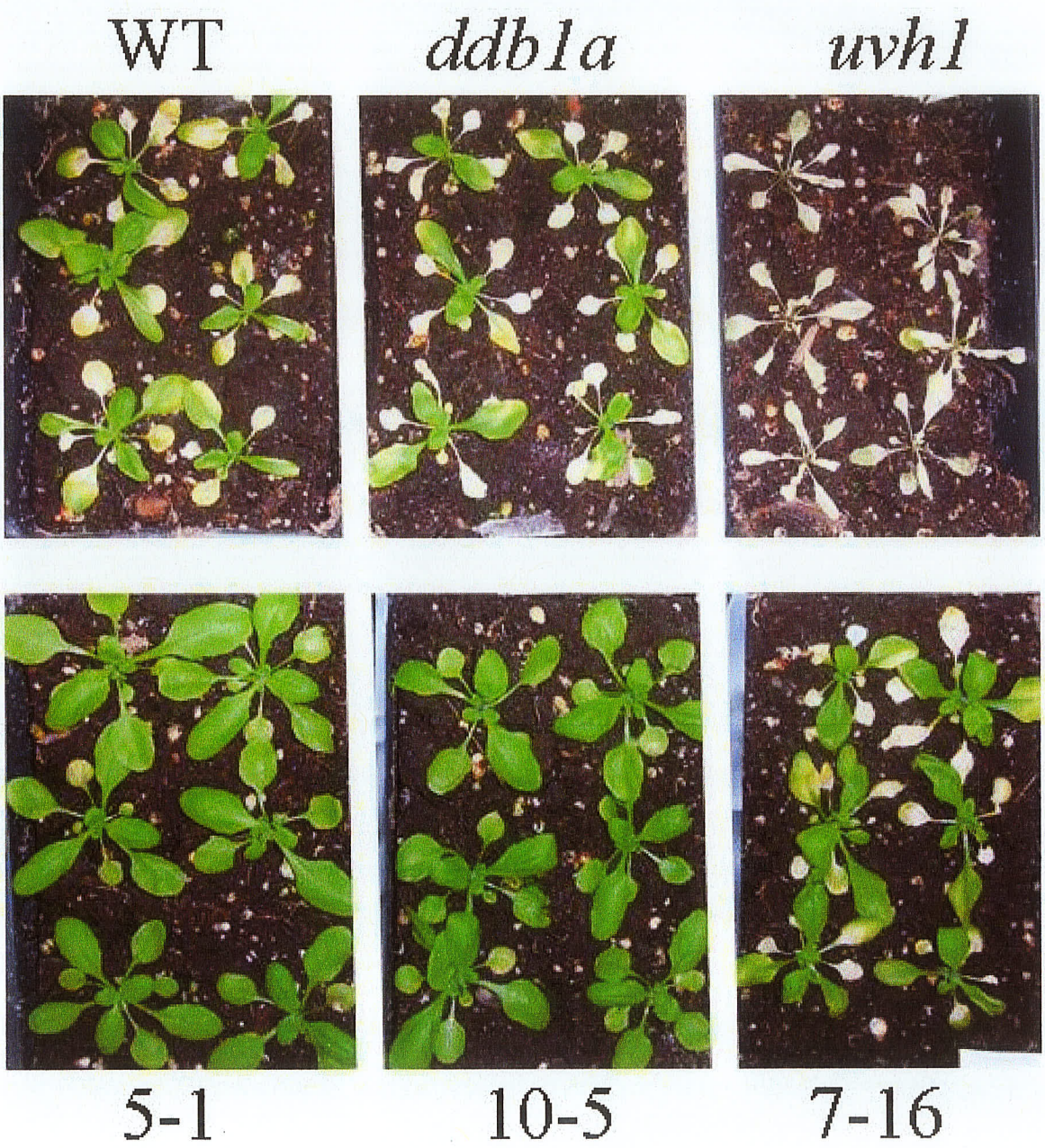


Figure 4.2 UV tolerance assays
A: Phenotypes of wildtype, *ddb1a*, *uvh1* and *DDB1A* overexpression lines (5-1, 10-5 and 7-16) after UV-C irradiation (450 J. m^{-2}).

B

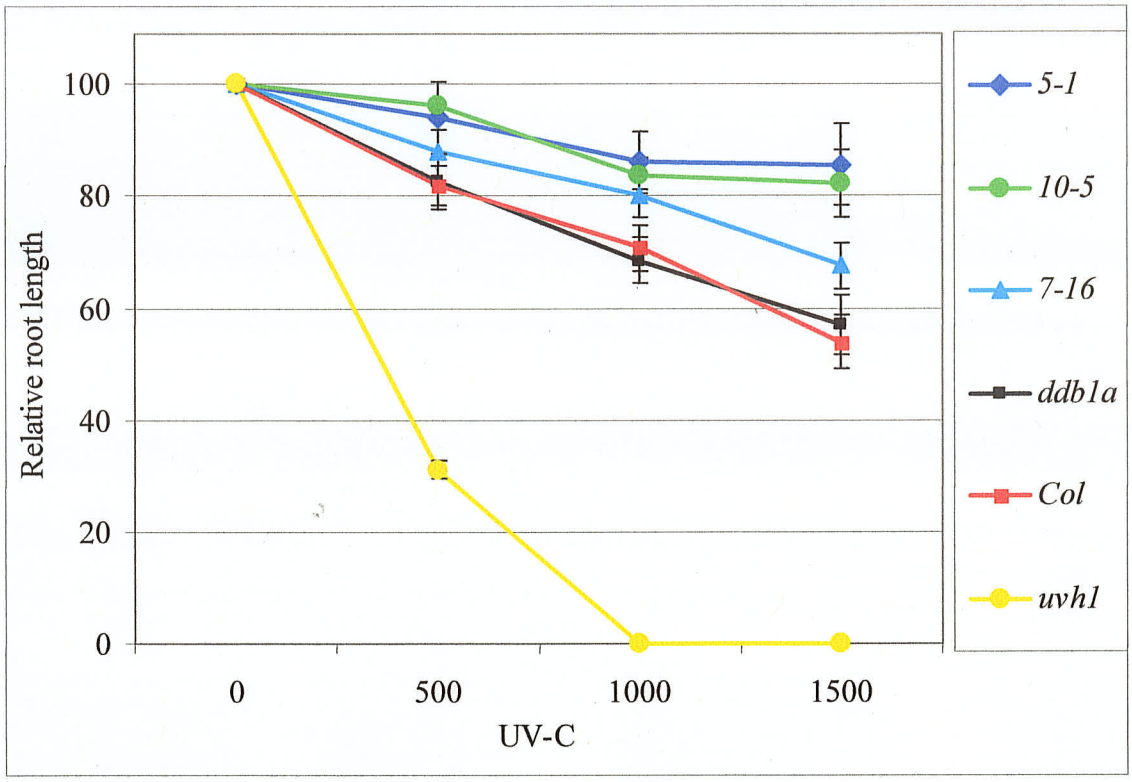
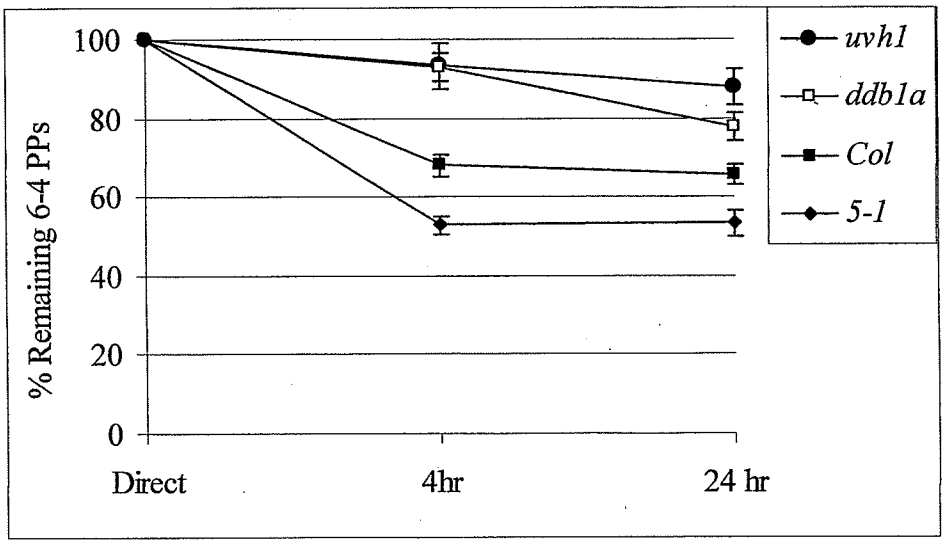


Figure 4.2 UV tolerance assays (cont.). B: Root bending assay of wildtype, *ddb1a*, *uvh1* and *DDB1A* overexpression lines (5-1, 10-5 and 7-16) after exposure to 0, 500, 1000 and 1500 J. m^{-2} UV-C irradiation. Measurements were taken after 3 days of dark incubation. Error bars indicate ±SE (n=20).

A



B

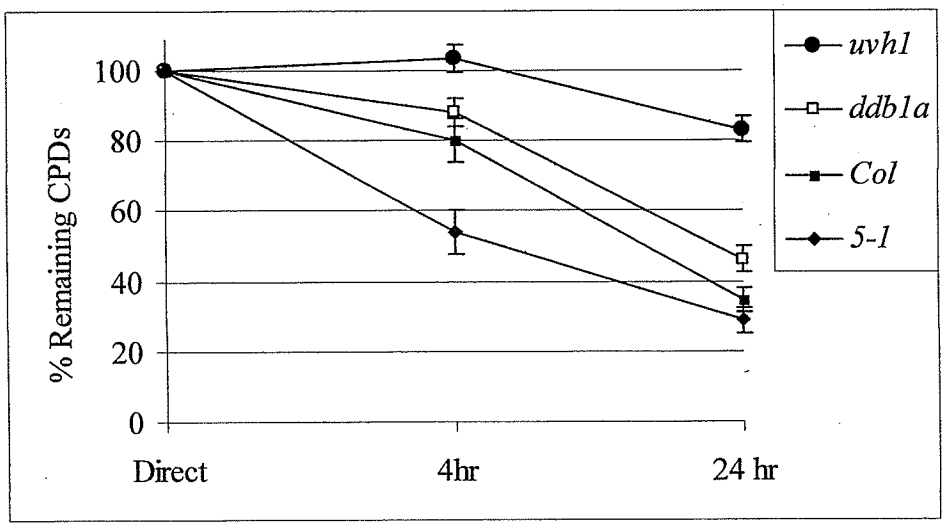


Figure 4.3 DNA photoproduct repair in wildtype, *ddb1a*, *uvh1* and a *DDB1A* overexpression line (5-1) after UV-C irradiation.
A: Pyrimidine (6-4) pyrimidinone dimers (6-4 PPs) and **B:** Cyclobutane pyrimidine dimers (CPDs) levels directly, 4 and 24 hours after irradiation. Data are shown as the means ± SE (n=4).

A

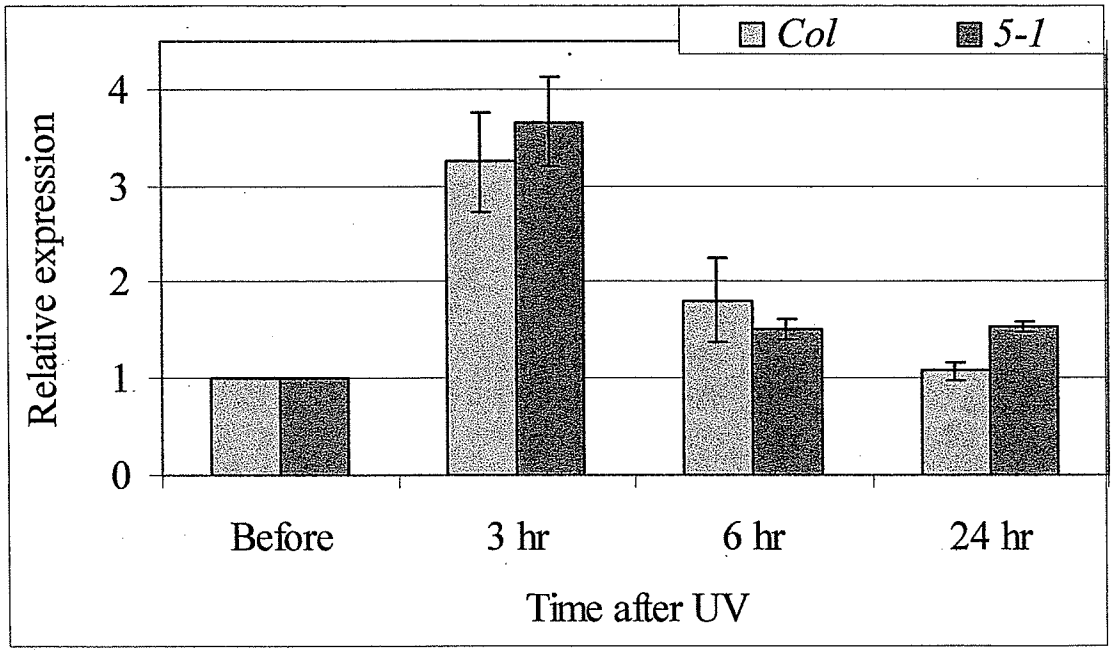


Figure 4.4 Effect of UV irradiation on mRNA level of *DDB1A*, *DDB1B* and *DDB2*
A: Relative *DDB1A* mRNA levels before or 3, 6 and 24 hours after UV-C exposure in 7-day-old wildtype and overexpression line 5-1 seedlings. Error bars indicate ± SE (n=4). mRNA level in non-irradiated samples (before) was designated 1. *UBQ10* was used to normalize mRNA level.

B

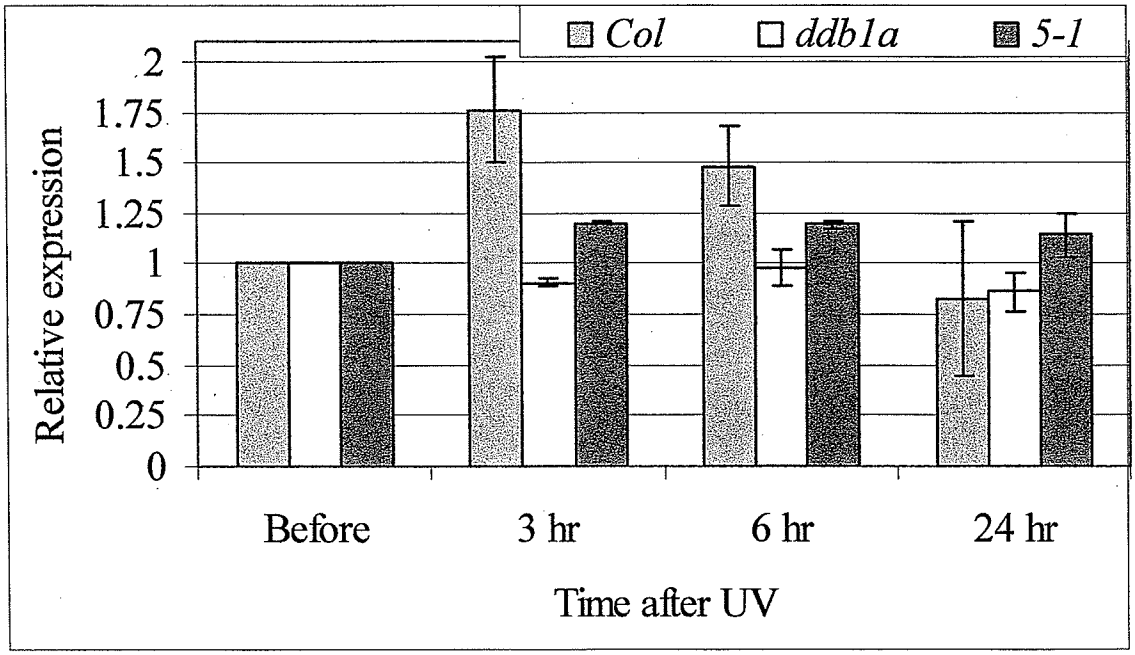


Figure 4.4 Effect of UV irradiation on mRNA level of *DDB1A*, *DDB1B* and *DDB2* (cont.). B: Relative *DDB1B* mRNA levels before or 3, 6 and 24 hours after UV-C exposure in 7-day-old wildtype, *ddb1a* and overexpression line 5-1 seedlings. Error bars indicate ± SE (n=4). mRNA level in non-irradiated samples (before) was designated 1. *UBQ10* was used to normalize mRNA level.

C

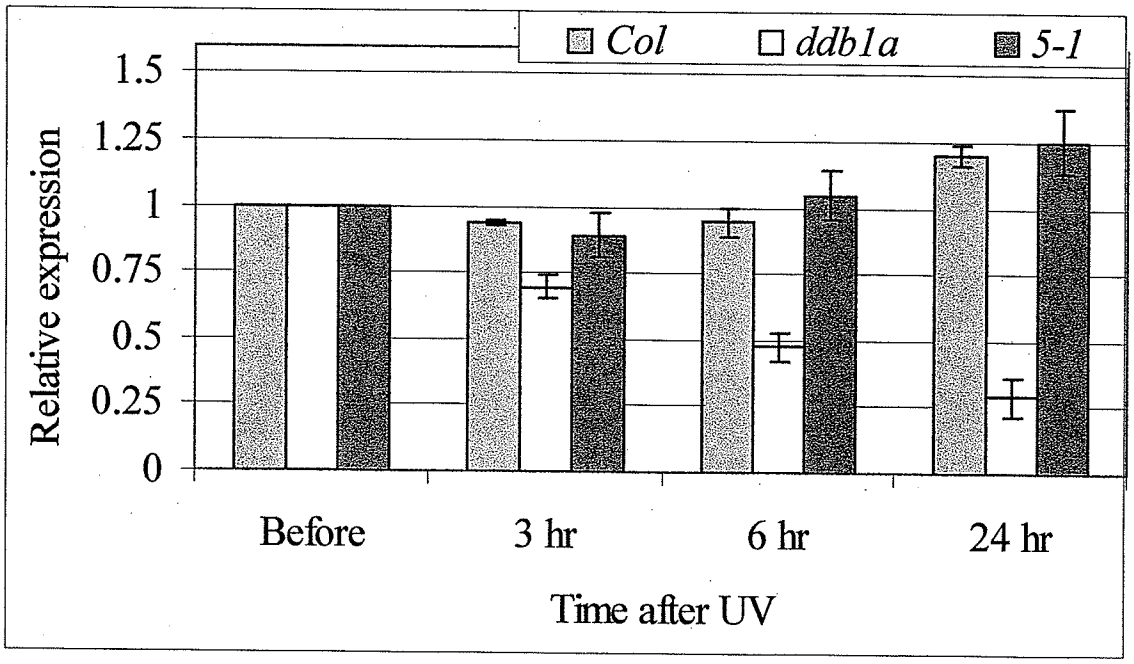


Figure 4.4 Effect of UV irradiation on mRNA level of *DDB1A*, *DDB1B* and *DDB2* (cont.). C: Relative *DDB2* mRNA levels before or 3, 6 and 24 hours after UV-C exposure in 7-day-old wildtype, *ddb1a* and overexpression line 5-1 seedlings. Error bars indicate ± SE (n=4). mRNA level in non-irradiated samples (before) was designated 1. *UBQ10* was used to normalize mRNA level.

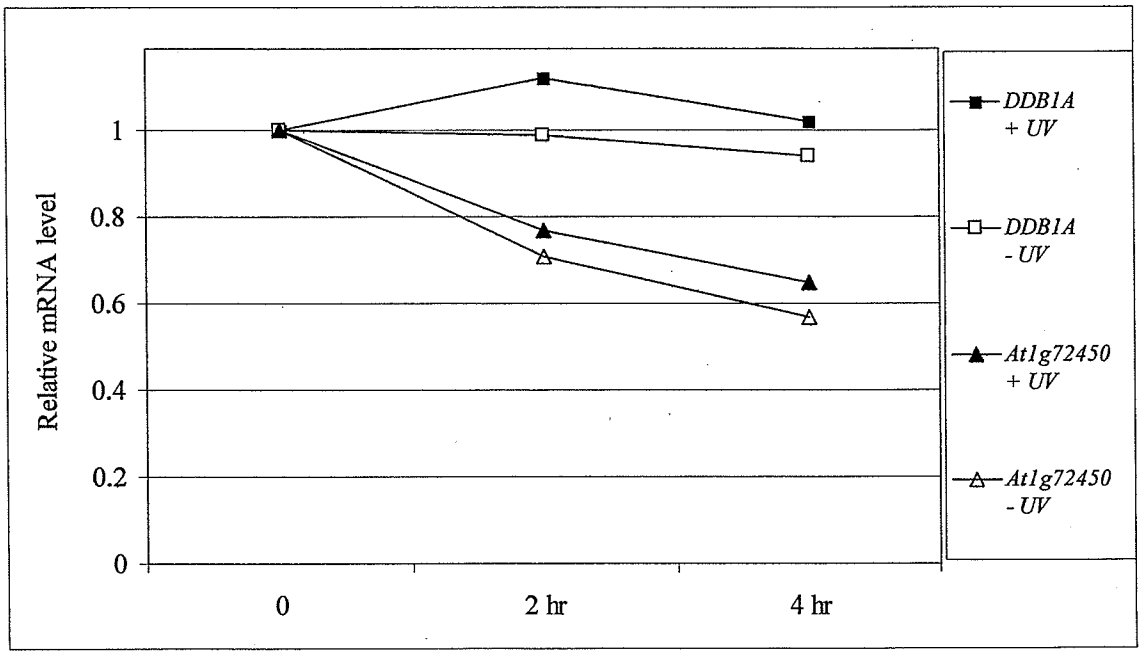


Figure 4.5 Effect of UV irradiation on mRNA stability
Relative mRNA level of *DDB1A* and an unstable gene (At1g72450) in wildtype seedlings treated with the transcription inhibitor (cordycepin) with or without UV exposure. mRNA level in samples taken directly after adding the transcription inhibitor (0) was designated 1. *S18* was used to normalize mRNA level.

A

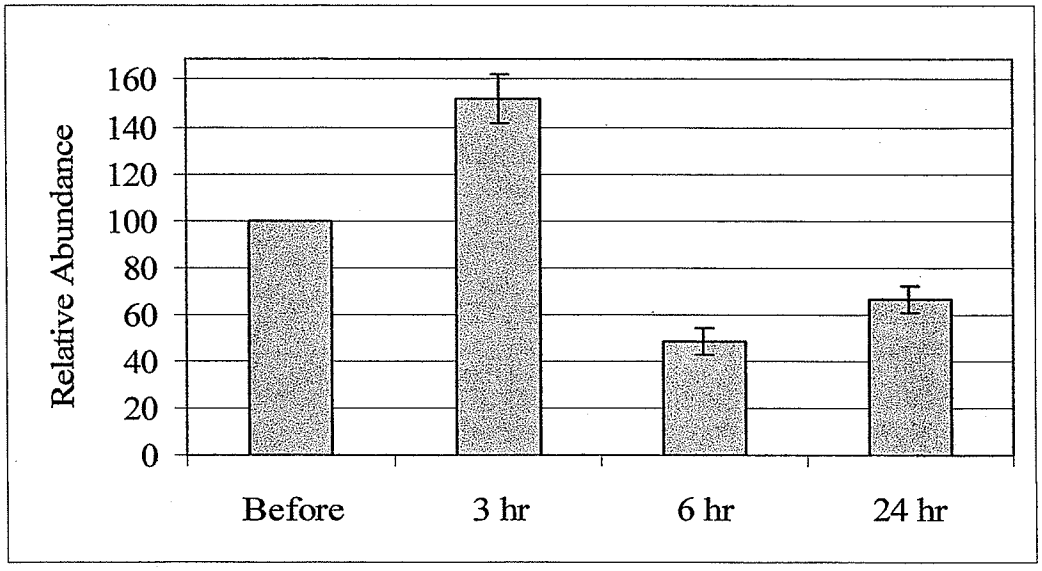
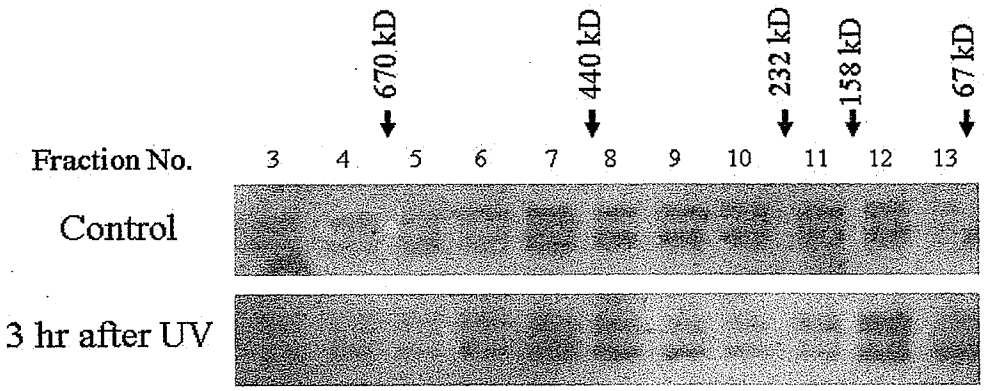


Figure 4.6 Effect of UV irradiation on DDB1A-HA Protein.
A: Relative DDB1A-HA protein level before, 3, 6 and 24 hours after UV exposure in the overexpression line 5-1. Error bars indicate ± SE (n=3). DDB1A-HA protein level in non-irradiated samples (before) was designated 100%.

B



C

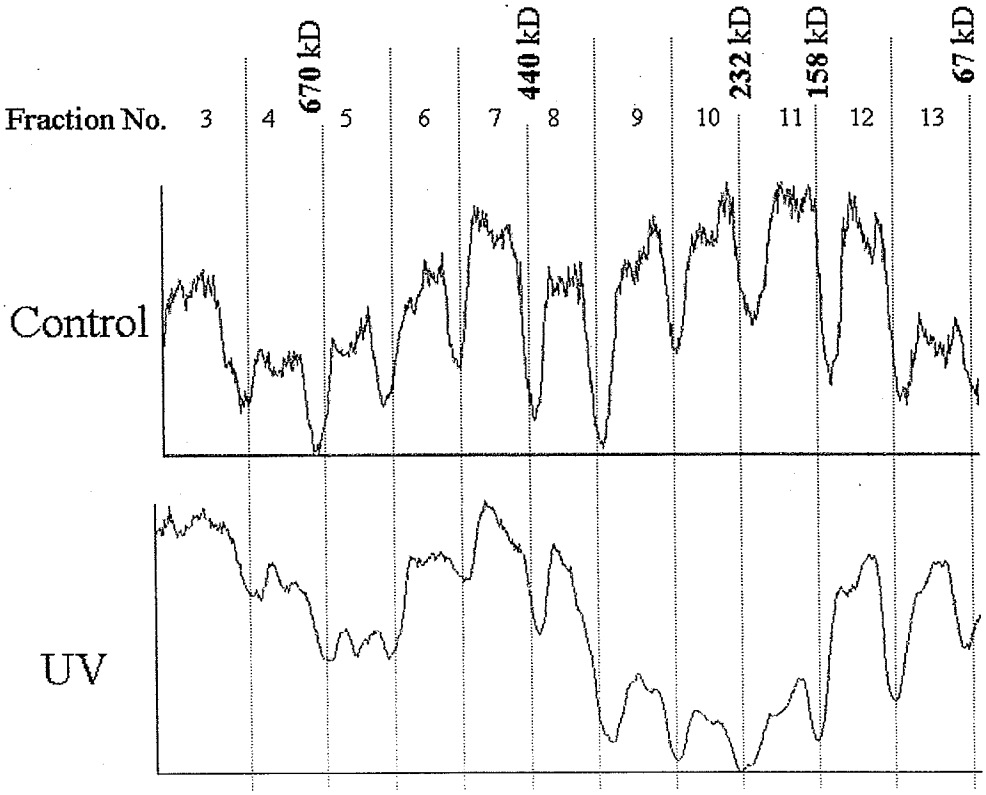


Figure 4.6 Effect of UV irradiation on DDB1A-HA Protein (cont.) B: Effect of UV-C on DDB1A-HA complex size. Total protein of 7-day light grown seedlings (with or without UV exposure) was extracted, separated by gel filtration and analyzed via anti-HA western blot. **C:** Quantification of DDB1A complex size. ImagJ software was used to quantify the intensity of each band in **(B)**. Fraction numbers and molecular size standards are indicated at the top.

4.6 SUPPLEMENTARY DATA

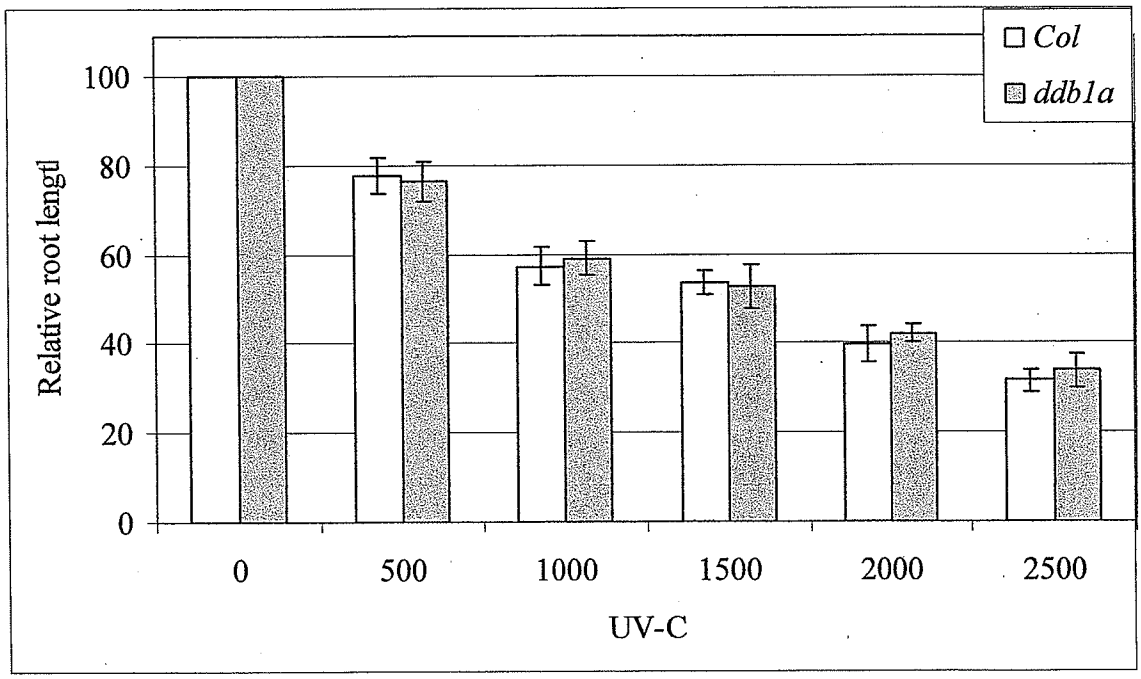


Figure S4.1 Root bending assay of wildtype and *ddb1a* after exposure to 0, 500, 1000, 1500, 2000 and 2500 J. m^{-2} UV-C irradiation. Measurements were taken after 3 days of dark incubation. Error bars indicate ±SE (n=20).

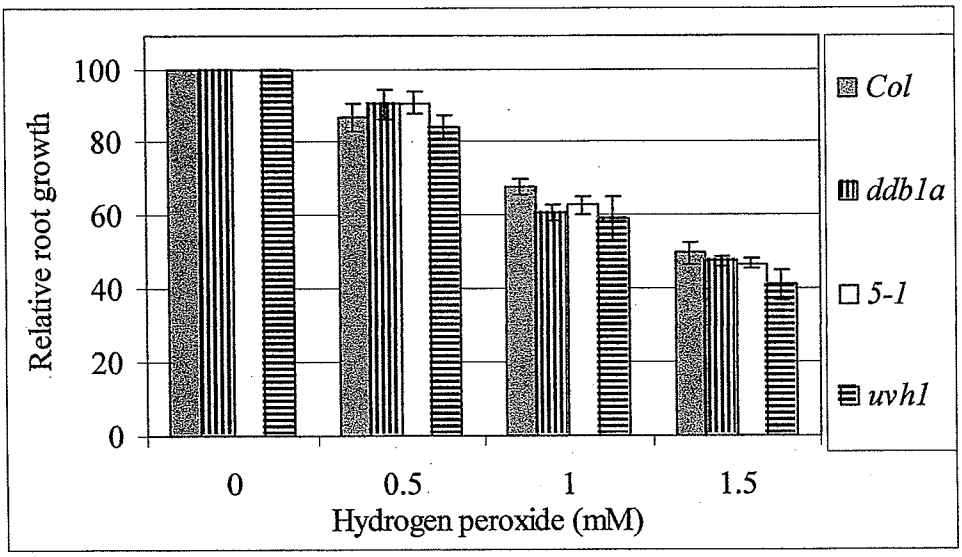


Figure S4.2 Effect of Hydrogen peroxide on root growth of 3-week old long day grown seedlings (wildtype, *ddb1a*, *uvh1* and the *DDB1A* overexpression line (5-1)). Error bars indicate ±SE (n=15).

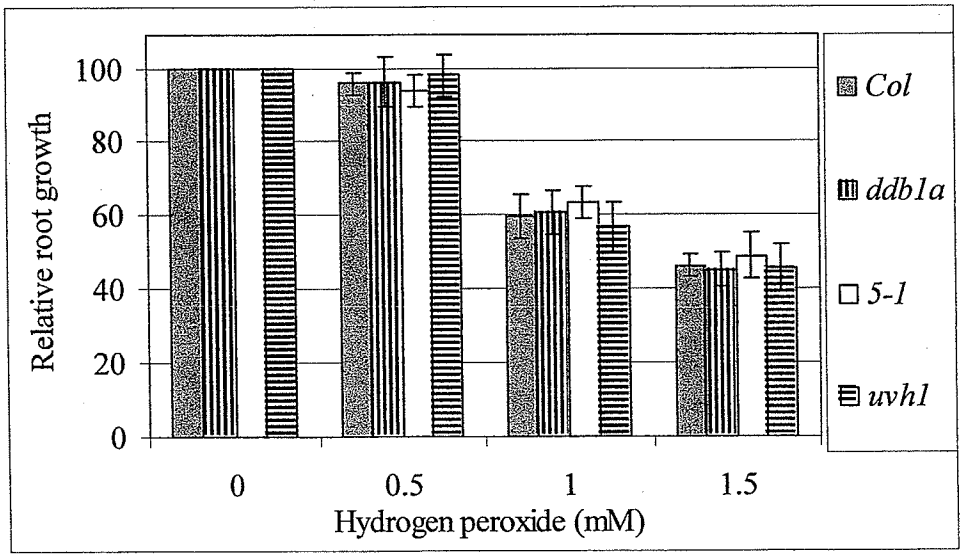


Figure S4.3 Effect of Hydrogen peroxide on root growth of 7-day old dark grown seedlings (wildtype, *ddb1a*, *uvh1* and the *DDB1A* overexpression line (5-1)). Error bars indicate ±SE (n=15).

Table S4.1 Effect of dark or UV-C on *DET1* and *DDB1A* mRNA levels. mRNA level before treatment was always designated 1. Values normalized to *UBQ10* unless indicated otherwise.

Gene	Background	Treatment	Before	3 hr
DDB1A relative to *UBQ10*	Col	UV-C	1	3.3
DDB1A relative to *Actin*	Col	UV-C	1	3.0
DDB1A relative to *S18*	Col	UV-C	1	3.5
DDB1A	Col	Dark	1	1
Endogenous *DDB1A*	5-1	UV-C	1	1.8
Transgenic *DDB1A*	5-1	UV-C	1	2.2
DDB1A	10-5	UV-C	1	3.1
DDB1A	7-16	UV-C	1	2
DET1	Col	UV-C	1	1
DET1	Myc-DET1	UV-C	1	1.1

Table S4.2 Effect of UV-C on mRNA level of *UBQ10*, *Nia2* and *DDB1A*. Seedlings were treated with cordycepin to inhibit transcription. mRNA level in samples taken directly after adding the transcription inhibitor (0) was designated 1. Values normalized to S18.

Gene	Background	UV	Cordycepin	0	2 hr	4 hr
UBQ10	Col	-	+	1	0.2	0.15
NIA2	Col	-	+	1	0.33	0.19
NIA2	Col	+	+	1	0.30	0.17
DDB1A	5-1	-	+	1	0.67	0.41
DDB1A	5-1	+	+	1	0.60	0.40
Endogenous *DDB1A*	5-1	-	+	1	0.51	0.20
Endogenous *DDB1A*	5-1	+	+	1	0.50	0.46
Transgenic *DDB1A*	5-1	-	+	1	0.85	0.60
Transgenic *DDB1A*	5-1	+	+	1	0.63	0.60

CHAPTER 5: *DDB1A-DDB2* INTERACTION IN *ARABIDOPSIS* DNA DAMAGE REPAIR

5. *DDB1A-DDB2* INTERACTION IN *ARABIDOPSIS* DNA DAMAGE REPAIR

5.1 ABSTRACT

In this study we examined the role of *DDB1A* and *DDB2* in *Arabidopsis* DNA damage repair using a reverse genetics approach. No visible phenotype (shoot or root) was found in *ddb1a*, *ddb2* or the double mutant *ddb1a ddb2* after irradiation with various doses of UV-C. However, 6-4PP repair analysis indicates that DDB1A-DDB2 is important in 6-4PP damage recognition 4 hours after UV exposure. CPD repair 24 hours after UV exposure indicates an important role for the DDB1A/B-DDB2 complex. In addition, RT-PCR analysis showed that *DDB2* is important for *DDB1A* and *DDB1B* induction following UV exposure. In conclusion, a subtle interaction was observed between *DDB1A*, *DDB1B* and *DDB2* in DNA repair and UV response.

5.2 INTRODUCTION

The Damaged DNA Binding protein complex (DDB) consists of two subunits, DDB1 and DDB2. It has been shown in many organisms that this complex is important in damaged DNA repair and other developmental pathways. Due to the fact that no differences were observed between wildtype, *ddb1a*, *ddb2* and the double mutant *ddb1a ddb2* in all parameters mentioned in chapter 2 (e.g. hypocotyl length, chlorophyll content) during *Arabidopsis* development under normal conditions (no stress), It was the

objective of this chapter to examining the role of *DDB1A* and *DDB2* in *Arabidopsis* DNA damage repair.

5.3 MATERIALS AND METHODS

Plant material was as described in chapter 2. UV sensitivity assays, RNA extraction and RT-PCR analysis were as described in chapter 4.

5.4 RESULTS

5.4.1 The effect of UV on *ddb1a*, *ddb2* and the double mutant *ddb1a ddb2*

To examine the role of *DDB1A* and *DDB2* in *Arabidopsis* DNA damage repair, the *ddb1a* null mutant (chapter 4), the *ddb2* partial loss of function mutant (chapter 2) and the double mutant *ddb1a ddb2* (chapter 2) were used to examine the response of *Arabidopsis* to UV-C in the absence of these two genes. The same approaches were used to examine UV sensitivity and repair rate in these lines as in chapter 4.

For the shoot assay, 3-week-old plants were irradiated with 450 J. m^{-2} UV-C, and then incubated for 3 days under dark conditions to prevent photoreactivation. No significant differences were observed between wildtype, *ddb1a*, *ddb2* and the *ddb1a ddb2* double mutant (Figure 5.1 A).

Similarly, in the root assay, no significant differences were observed between wildtype, *ddb1a*, *ddb2* and the *ddb1a ddb2* double mutant at any of the UV-C doses used in this experiment (Figure 5.1 B).

5.4.2 Photoproduct analysis

As described previously in chapter 4, an ELISA test was used to measure the amount of 6-4PPs and CPDs after irradiation with 450 J. m^{-2} UV-C. A significant difference ($P \leq 0.05$) was observed between *ddb2* and wildtype in 6-4PP repair 4 hours after irradiation (82% vs. 68% remaining, respectively). By 24 hours after irradiation, both lines exhibit similar 6-4PP repair rates (~ 68% remaining). *ddb1a* mutants showed 87% and 82% remaining 6-4PPs 4 and 24 hr after irradiation, respectively. The double mutant *ddb1a ddb2* shows similar repair rates to *ddb1a* 4 and 24 hr after irradiation (Figure 5.2 A).

With regard to CPD repair, wildtype and *ddb2* mutants behave similarly 4 and 24 hr after irradiation (~ 82% and 45% remaining, respectively). *ddb1a* exhibits a clear difference in CPD repair from wildtype and *ddb2* 24 hr after irradiation with more than 56% CPDs remaining. In contrast to 6-4PP repair, mutation of *ddb2* in the *ddb1a* background reduces CPD repair rate (69% remaining) (Figure 5.2 B). This indicates that the DDB complex is important in the repair of both types of photoproducts.

5.4.3 Effect of *ddb2* mutation on *DDB1A* and *DDB1B* expression with UV

As mentioned previously in chapter 4, *DDB1A* and *DDB1B* mRNA levels increase 3 hours after irradiation with 450 J. m^{-2} UV-C. To examine the response of these two genes to UV-C in the *ddb2* mutant, *ddb2* seedlings were grown under long day conditions for 7 days then irradiated with 450 J. m^{-2} UV-C. RNA extraction and RT-PCR methods were used

as described in chapter 4. No differences in *DDB1A* and *DDB1B* mRNA levels were observed between non-irradiated and irradiated (after 3 and 24 hours) seedlings (Figure 5.3). Therefore, UV induction of *DDB1A* and *DDB1B* require *DDB2*.

5.5 DISCUSSION

Xeroderma pigmentosum (XP) is a rare autosomal disease. XP patients suffer from ultraviolet light (UV) hypersensitivity. Seven complementation groups (A–G) were assigned for XP patients (Cleaver, 2005). *Xeroderma pigmentosum* group E (*ddb2* loss of function) showed a mild phenotype after exposure to UV, with NER activity close to normal cells (Itoh *et al.,* 2004).

In this study, UV tolerance assays (root and shoot) showed that *ddb1a* and *ddb2* and *ddb1a ddb2* mutants exhibit wildtype phenotypes. In photoproduct repair assays, differences were observed between *ddb2* and wildtype (6-4PPs) as well as *ddb1a ddb2* and all other lines (CPDs). This could be explained as follows. In shoot and root assays, plants were irradiated with UV-C then incubated for 3 days under dark conditions to eliminate photoreactivation. Then, plants were assessed. In contrast, for the photoproduct repair assays phenotypes were measured a maximum of one day after irradiation. This suggests that mutation of *ddb1a* or *ddb2* affects the rate of DNA repair, but plants can overcome this effect after longer periods (at the time of shoot and root assay).

In another study (Koga *et al.,* 2006), *Arabidopsis ddb2* null mutants showed sensitive phenotypes (root and shoot) after exposure to 30 and 40 KJ. m^{-2} UV-B. As shown in chapter 2, the *ddb2* mutant line that I am using in this study is a partial loss of function

mutant resulting from same T-DNA insertion in the *DDB2* gene. This could explain the difference in UV response between the two experiments.

Arabidopsis has two copies of *DDB1*: *DDB1A* and *DDB1B*. It is still unclear whether DDB1A, DDB1B or both associate with DDB2 in photoproduct damage recognition. Also we do not know if there is a limiting factor in each complex, or if other NER factors are involved in the damage recognition step in addition to DDB complex.

The 6-4PP repair assay showed that loss of function of *ddb1a* or *ddb2* results in decreased 6-4PP repair 4 hours after UV exposure. Also at 4 hours, loss of function of *ddb2* in the *ddb1a* background resulted in a similar amount of remaining 6-4PPs that were observed in the *ddb1a* single mutant. This indicates that DDB2 repair of 6-4PPs 4 hours after UV exposure is DDB1A-dependent. That is the DDB1B-DDB2 complex is not active at this stage. These conclusions are consistent with the induction of *DDB1A* mRNA and protein levels 3 hours after UV exposure.

While 6-4PP repair analysis revealed that DDB1B-DDB2 complex is not involved in 6-4PP damage recognition 4 hours after UV exposure, CPD repair 24 hours after UV exposure shows an important role of this complex. Partial loss of function of *ddb2* showed similar amount of CPDs as wildtype 24 hours after UV exposure. On the other hand, *ddb1a* loss of function had less CPD repair than the wildtype and *ddb2*. Mutation of *ddb2* in the *ddb1a* background enhanced the *ddb1a* phenotype. This suggests that the DDB1B-DDB2 complex is active at this point.

The reasons for this difference in activity of DDB1B-DDB2 over time and/or photoproduct type could be due to many reasons. Although *DDB1A* and *DDB1B* show high homology, there are still some differences at the amino acid level between them.

These differences could generate photoproduct specificity. Another reason could be due to the availability of DDB1A or DDB1B at that time point. Also, we do not know if other NER factors are involved in the damage recognition step. The interaction between DDB1A / DDB1B and these factors could be time dependent or damage type dependent.

For *DDB1A* and *DDB1B* mRNA levels, no significant differences were observed between samples before, 3 hr and 24 hrs after irradiation in *ddb2* mutants. In contrast, wildtype seedlings showed an increase in *DDB1A* and *DDB1B* mRNA level 3 hours after irradiation. This suggests that *DDB2* is important for *DDB1A* and *DDB1B* induction in response to UV. Similarly, we had previously shown that *DDB1A* is required for UV induction of *DDB1B* and *DDB2*. Therefore, this data revealed a subtle interaction between *DDB1A*, *DDB1B* and *DDB2* in DNA repair and UV response.

A

B

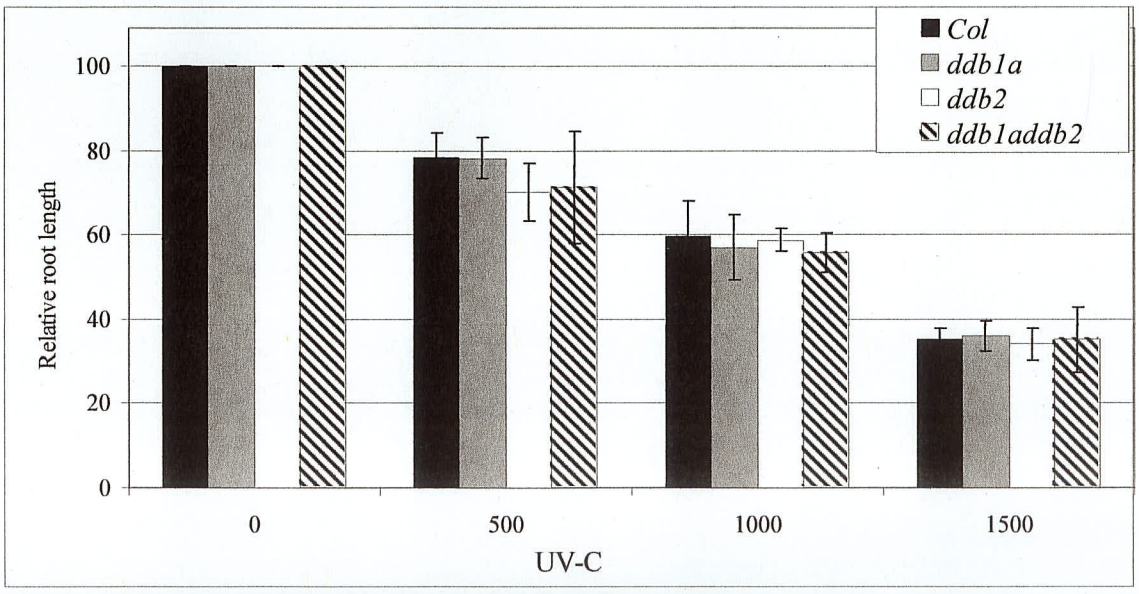
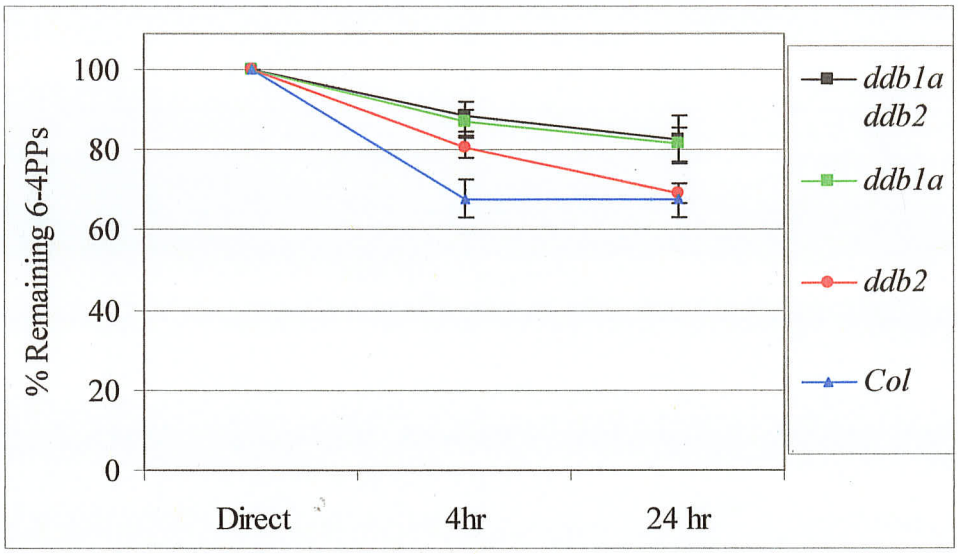


Figure 5.1 UV tolerance assays
A: Phenotypes of wildtype, *ddb1a*, *ddb2* and the *ddb1a ddb2* double mutant after UV-C irradiation (450 J. m^{-2}). **B:** Root bending assay of wildtype, *ddb1a*, *ddb2* and the *ddb1a ddb2* double mutant after exposure to 0, 500, 1000 and 1500 J. m^{-2} UV-C irradiation. Measurements were taken after 3 days of dark incubation. Error bars indicate ±SE (n=20).

A



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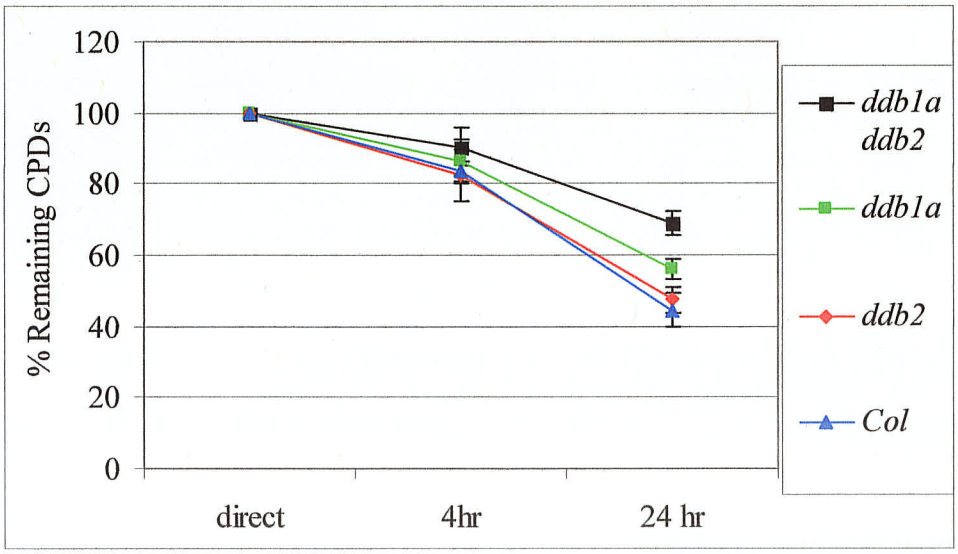
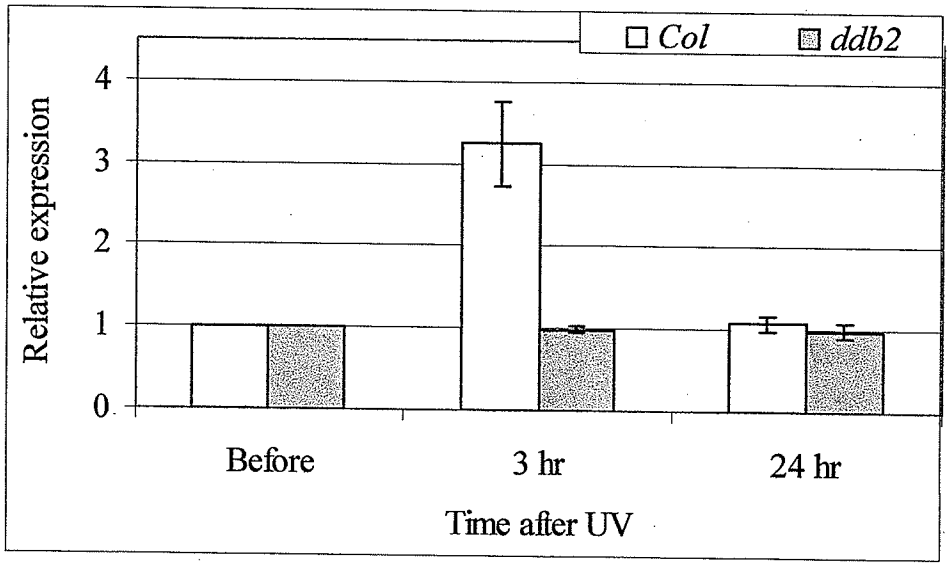


Figure 5.2 DNA photoproduct repair in wildtype, *ddb1a*, *ddb2* and the *ddb1a ddb2* double mutant after UV-C irradiation.
A: Pyrimidine (6-4) pyrimidinone dimers (6-4 PPs) and **B:** Cyclobutane pyrimidine dimers (CPDs) levels directly, 4 and 24 hours after irradiation. Data are shown as the means ± SE (n=4).

A



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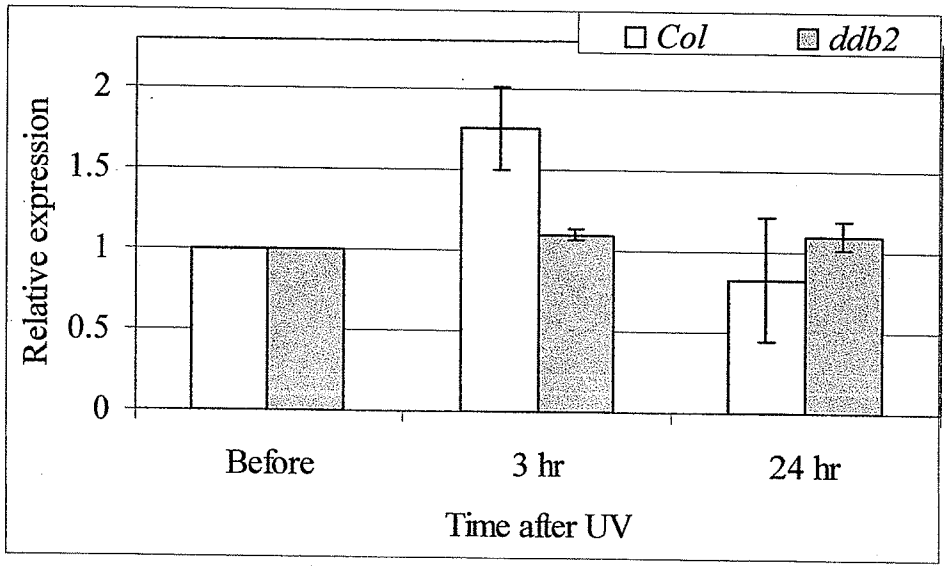


Figure 5.3 Effect of UV irradiation on mRNA level of *DDB1A* and *DDB1B*.
Relative *DDB1A* (**A**) and *DDB1B* (**B**) mRNA levels before or 3 and 24 hours after UV-C exposure in 7-day-old wildtype and *ddb2* seedlings. Error bars indicate ± SE (n=3). mRNA level in non-irradiated samples (before) was designated 1. *UBQ10* was used to normalize mRNA level.

CHAPTER 6: *DET1-DDB2* GENETIC INTERACTION IN *ARABIDOPSIS* DNA DAMAGE REPAIR.

CHAPTER 6: *DET1-DDB2* GENETIC INTERACTION IN *ARABIDOPSIS* DNA DAMAGE REPAIR.

6.1 ABSTRACT

DET1 and *DDB2* interact genetically in *Arabidopsis*. Previous studies showed that mutation of *ddb2* in the *det1* background modulated *det1* seedling phenotypes. The primary objective of this study was to examine if mutation of *det1* in the *ddb2* partial loss of function background affects *ddb2* UV tolerance phenotypes. Both *ddb2* and *det1 ddb2* showed similar photoproduct repair rates 4 and 24 hours after irradiation. Similarly, no significant effect of UV was observed on Myc-DET1 abundance in wildtype or the *ddb2* background. Thus, while *ddb2* modifies *det1* phenotypes under visible light, *det1* does not appear to modify *ddb2* UV phenotypes.

6.2 INTRODUCTION

It has been shown previously that the DDB1-CUL4A ligase requires a receptor protein in order to target the designated substrate for degradation (He *et al.,* 2006). Schroeder *et al.* (2002) showed that tobacco DDB1 associates with DET1 in a 350 kD complex. In *Arabidopsis*, DDB1A, CUL4A, COP10, RBX1 and DET1 form one complex (Chen *et al.,* 2006, Bernhardt *et al.,* 2006). In humans, the DDB1- CUL4A complex also can contain DET1 (Wertz *et al.,* 2004). A second candidate that associates with the DDB1- CUL4A complex and works as a receptor protein is DDB2. It has been shown

that *Arabidopsis* DDB1A-CUL4A-RBX1 and DDB2 are in one complex (Bernhardt *et al.*, 2006).

As shown in chapter 2 and other studies (Chory *et al.*, 1989; Schroeder *et al.*, 2002), *det1-1* mutants exhibit short hypocotyls, open cotyledons, and high anthocyanin content under dark conditions. When grown under light conditions, they are smaller and paler than wild type plants with reduced apical dominance and day-length-insensitive flowering. *ddb2* mutants on the other hand, show wildtype phenotypes under dark or light conditions (chapter 2). As shown in chapter 2, mutation of *ddb2* in the *det1* background modulated *det1* phenotypes (hypocotyl elongation, anthocyanin content, chlorophyll content, flowering time (days), and rosette diameter).

This indicates that *DET1* genetically interacts with *DDB2* in *Arabidopsis* under visible light conditions. Our model is that the interaction between *DET1* and *DDB2* is through *DDB1A/B*, and that there is competition between DET1 and DDB2 for DDB1A/B. *ddb2* mutation liberates DDB1A/B and it become more available for the DET1 complex. Conversely, in the case of *det1* mutation, DDB1A/B may be are more abundant for DDB2 interaction (Figure 6.1)

In this chapter, we tested this model by examining the genetic interaction between *DET1* and *DDB2* under UV light.

6.3 MATERIALS AND METHODS

6.3.1 Plant materials

Col was used as a wildtype in this study. *ddb2*, *det1* and *det1 ddb2* mutants were described in chapter 2. Myc-DET1 and GFP-DET1 transgenic plants were as described in Schroeder *et al.* (2002). The double mutant Myc-DET1 *ddb2* was generated by crossing Myc-DET1 and *ddb2* lines. F_1 plants were genotyped using PCR for the *ddb2* insertion and western bloted for Myc-DET1 expression. Selected plants were self crossed to get the F_2 generation. F_2 plants were genotyped again for *ddb2* insertion and Myc-DET1 expression. Putative homozygous double mutants in the F_2 generation were selected and used.

6.3.2 Microscopy

For GFP-DET1 localization, 5-day old light grown seedlings were irradiated with 450 J. m^{-2} UV-C. Samples were taken directly, 1, 2 and 4 hours after irradiation. Hypocotyl was examined under the fluorescent microscope as described in Schroeder *et al.* (2002).

6.3.3 DNA damage analysis, RNA extraction, protein extraction, western blot and gel filtration

As described in chapter 4. For Myc-DET1 western blot, anti-Myc antibody was used (sc-789, Santa Cruz Biotechnology, Inc).

6.3.4 RT-PCR

For RT-PCR analysis, two *DET1* specific primers were used, 5'-CACTCACATC GTCTCCTCC-3' and 5'-GCGAGGTAGAGGAGGTAGGG-3'. PCR conditions were as follows : 5 min at 94, 25 cycles (1 min at 94, 1 min at 58, 1 min at 72), 7 min at 72. PCR products were separated on 1% (w/v) agarose gels, and the intensities of ethidium bromide-stained bands were determined by ImageJ software (1.36b National Institutes of Health).

6.4 RESULTS

The objective of this study was to examine the genetic interaction between *DET1* and *DDB2* in response to UV exposure. To test this interaction, *det1*, *ddb2*, *det1 ddb2*, *Myc-DET1* and *Myc-DET1 ddb2* lines were used. At the beginining of this experiment, shoot and root analysis were conducted to compare the degree of damage between *det1* and *det1 ddb2* mutants. These two assays required a period of dark incubation after UV exposure in order to eliminate photoreactivation. Unfortunately, even non-treated *det1* and *det1 ddb2* plants showed abnormal phenotypes. In the shoot assay, most of the leaves were dead. It has been shown that *det1* mutants exhibit acclimation defects (Walters *et al.,* 1999). In addition, in the root assay, *det1* and *det1 ddb2* seedlings show gravitropism defects. Gravitropism is a light regulated phenotype (Fankhauser and Casal, 2004), and because DET1 is important in light responses, hence mutation of *det1* appears to affect gravitropism. Because the gravitropism response that allow us to measure root bending. So we did not carry out the assay using these mutants. Thus we were unable to carry out

root and shoot assays using any line in the *det1* background. This means that the only UV tolerance assay that could be used to examine these lines is the photoproduct repair assay.

6.4.1 Effect of *det1* on *ddb2* photoproduct repair.

det1 mutants exhibits similar 6-4PP and CPD repair rates as wildtype seedlings 4 and 24 hours after UV exposure. Similarly, mutation of *det1* in the *ddb2* background did not alter repair rates (Figure 6.2 A,B). This indicates that *DET1* is not required for photoproduct repair and does not detectably modify the *ddb2* repair phenotype.

6.4.2 Effect of *ddb2* mutation on *DET1* expression with UV

DET1 mRNA level is not affected by UV exposure in *Arabidopsis* wildtype seedlings (Figure 6.3 and S4.4). Here, the effect of UV on *DET1* mRNA level in the *ddb2* mutant was examined. Using semi-quantitative RT-PCR, *DET1* transcript level was found to increase 3 and 24 hours after UV exposure (by more than 2 and 3-fold, respectively) (Figure 6.3). Therefore, wildtype DDB2 must prevent UV induction of *DET1* mRNA.

6.4.3 Effect of UV on DET1 localization (GFP-DET1)

Using fluorescence microscopy, it has been shown previously that GFP-DET1 is a nuclear protein (Schroeder *et al.*, 2002). In this chapter, the effect of UV-C on GFP-

DET1 localization was examined. After exposure, GFP-DET1 was found to be still primarily nuclear but with some weak cytoplasmic florescence. The extent and basis of this effect requires further characterization.

6.4.4 Effect of UV and *ddb2* on DET1 abundance (Myc-DET1)

Using western blot analysis, we found that mutation of *ddb2* decreased Myc-DET1 abundance by more than 50% (Figure 6.5 A). Then we examined the effect of UV on Myc-DET1 abundance. Similar Myc-DET1 abundance was observed before, 3 and 24 hours after UV exposure in the epitope tagged line *Myc-DET1*. Similarly, when the abundance of Myc-DET1 was examined in the double mutant *Myc-DET1 ddb2*, a similar result was obtained (Figure 6.5). Thus, the partial loss of function in *ddb2* does not alter the effect of UV exposure on DET1 abundance.

6.4.5 Effect of UV on DET1 complex size

It has been shown that DET1 forms an approximately 350 kD complex in *Arabidopsis* (Schroeder *et al.,* 2002). Here, the effect of UV exposure on DET1 complex formation in *Myc-DET1* was examined using gel filtration (Figure 6.6 A). A possible relative increase in fractions 6, 9, 10 and 11 were observed 3 hours after UV exposure. In contrast, Myc-DET1 complex formation in the *Myc-DET1 ddb2* background showed a decrease in fractions 10, 11, and 12 after UV exposure (Figure 6.6 B).

6.5 DISCUSSION

In chapter 2 we proposed that there is a competition between DET1 and DDB2 for the available DDB1A/B. Mutation of *ddb2* or *det1* liberates DDB1A/B from one complex and makes it more available for the other complex (Figure 6.1). Thus, the genetic interaction between *DET1* and *DDB2* under visible light condition is achieved through *DDB1A/B*.

In this study, we want to examine this model under UV light. Does *det1* mutation modify *ddb2* damaged DNA repair phenotypes? What is the effect of *ddb2* mutation on DET1? What is the effect of UV on DET1?

6.5.1 *det1* does not modify *ddb2* damaged DNA repair phenotypes

DET1 has an important role in visible light responses in *Arabidopsis*, on the other hand, the role of DDB2 in DNA repair has been extensively studied in humans. In chapter 2, we found that the partial loss of function of *ddb2* in the *det1* background significantly changed *det1* phenotypes which suggests a genetic interaction between these two genes under visible light (Al Khateeb and Schroeder, 2007). Here, in a parallel experiment, we are interested in knowing if loss of function of *det1* modifies *ddb2* phenotypes under UV light.

The photoproduct repair assay revealed that mutation of *det1* in the *ddb2* background did not affect 6-4PP and CPD repair rate at 4 and 24 hours after UV-C exposure. This means that *det1* does not modify *ddb2* damaged DNA repair phenotypes.

One possible explanation for this could be due to the kind of phenotypes we are using. For example, when we examined the effect of *ddb2* mutation in the *det1* background, we used various assays including phenotypic analysis of dark grown seedlings (hypocotyl elongation, anthocyanin content), light grown seedlings (hypocotyl elongation, anthocyanin content, chlorophyll content) and adult plants (height, flowering time, rosette diameter, fertility). Whereas, to examine the effect of *det1* mutation on *ddb2* phenotypes after UV, we could only use photoproduct repair assays.

Another reason for this discrepancy could be due to the weak phenotype of the *ddb2* mutant. It has been shown in humans that XP-E patients exhibit mild symptoms and their cells show relativily high levels (more than 50%) of nucleotide excision repair compared to other XP groups (Stary and Sarasin, 2002).

6.5.2 Effect of *ddb2* mutation on DET1

Based on our model and phenotypic data (Al Khateeb and Schroeder, 2007), we expect the DET1 complex to become more active in the absence of *DDB2*. However, western blot analysis of Myc-DET1 in the wildtype or *ddb2* background showed that Myc-DET1 is more abundant in the wildtype background. This inconsistency could be due to several reasons. This analysis uses the transgenic version of DET1, not the endogenous protein, and there could be differences in response between versions. Also, the Myc-DET1 line is also a *DET1* overexpression line, so we expect more *DET1* mRNA than the wildtype. Based on our model, we expect more DDB1A in the absence of *DDB2*, however it has been shown in Dr. Schroeder's lab that DET1 and DDB1A negatively

regulate each others abundance (Yu Zhang in preparation). Thus, perhaps the decrease of DET1 level in the *ddb2* background is a result of degradation. We also do not know the difference in *DET1* mRNA level between *Myc-DET1* and *Myc-DET1 ddb2*. It will be interesting to examine if *ddb2* mutation affects relative *DET1* level.

6.5.3 Effect of UV on DET1

At the beginning of this experiment, our hypothesis was that UV exposure will increase *DDB2* mRNA level. This increase in DDB2 will increase the activity of the DDB complex. This will make DDB1 more incorporated in the DDB complex than the DET1 complex. However, now we know that UV increases *DDB1A* mRNA level significantly, but only a minor and late (24 hours after UV exposure) increase in *DDB2* mRNA level was observed. This suggests that the increase in *DDB1A* mRNA level after UV exposure may reach a level that is enough for both complexes and there is no longer competition between DET1 and DDB2 for DDB1.

Using western blot, no detectable effect of UV on Myc-DET1 abundance in *Myc-DET1* was observed. Similarly, UV did not affect *DET1* mRNA level in wildtype. Only a minor change was observed in GFP-DET1 localization 2 h after UV exposure.

The change in Myc-DET1 complex formation after UV exposure in fractions 6, 9, 10 and 11 indicates that DET1 is associated with a broad range of complexes. This may be consistent with increased levels of DDB1A.

To determine the role of *DDB2* in DET1 UV response, we examined the effect of UV on DET1 mRNA levels, protein abundance and complex formation in the *ddb2*

background. While *DET1* mRNA level showed more than a 2-fold increase in the *ddb2* mutant 3 and 24 hours after UV exposure, Myc-DET1 abundance was still unchanged. Our gel filtration results show an increase in fractions 6, 9, 10 and 11 in *Myc-DET1*. In contrast, a decrease was observed in the *ddb2* background in these fractions (Figure 6.6). This suggests that these complexes require DDB2. We previously showed that UV increased *DDB1A* mRNA level in wildtype, but not in the *ddb2* background. Thus perhaps it is the absence of *DDB1A* induction, which alters complex formation in the *ddb2* background.

In conclusion, the results of this study indicate that *det1* does not appear to modify *ddb2* UV phenotypes. However, in some cases we noticed a weak effect of UV light on DET1 and this could be an indirect effect of DDB1A.

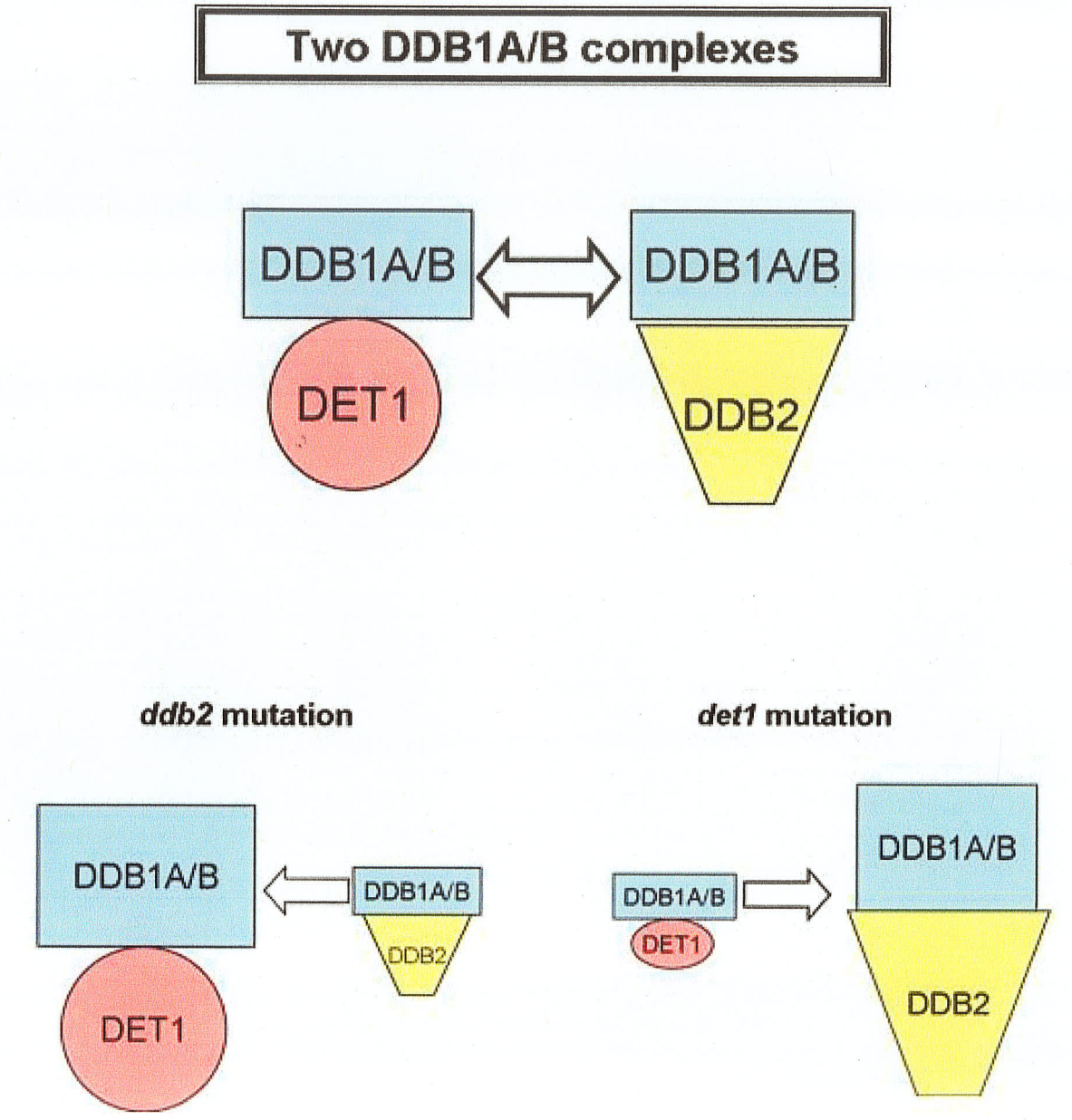
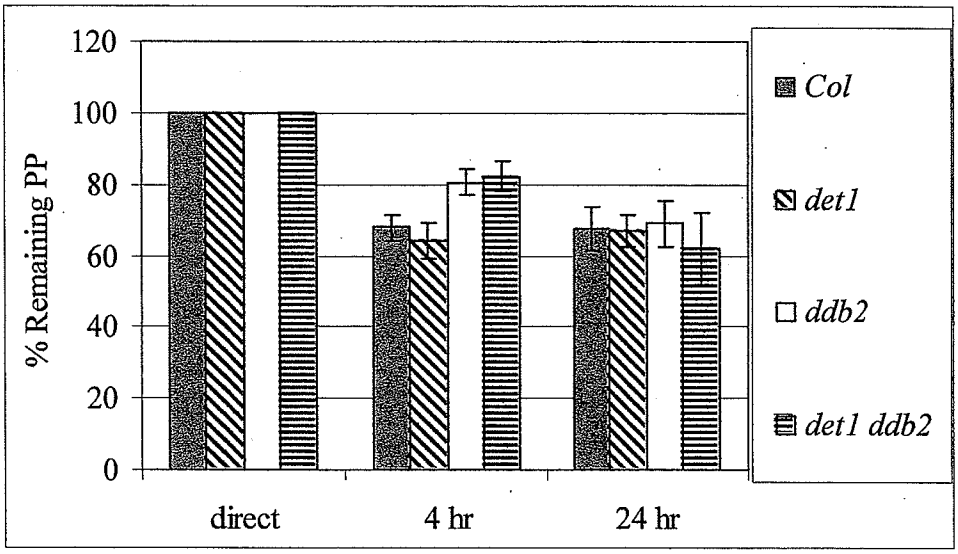


Figure 6.1 A model for the interaction between DDB1A/B complexes

A



B

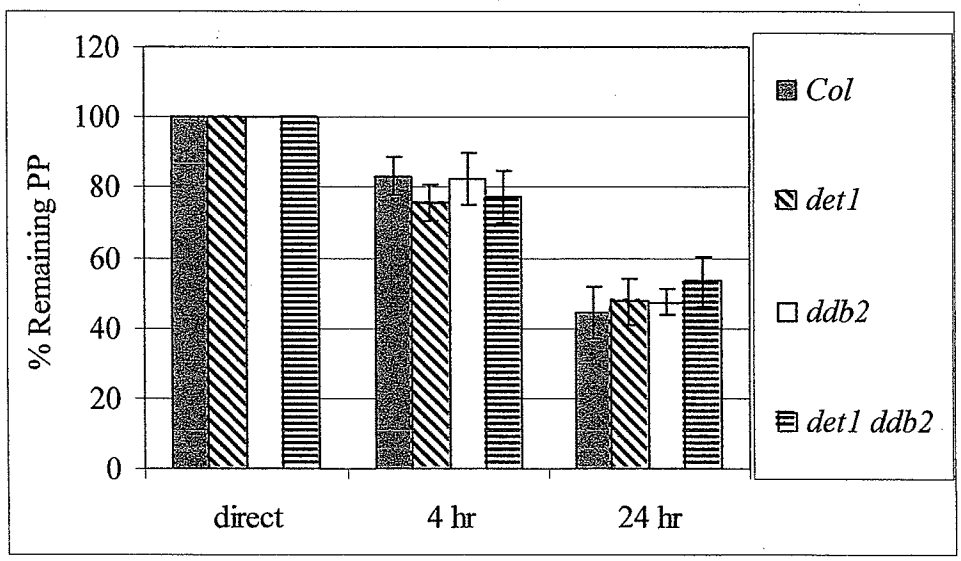


Figure 6.2 DNA photoproduct repair in wildtype, *det1*, *ddb2* and the *det1 ddb2* double mutant after UV-C irradiation.
A: Pyrimidine (6-4) pyrimidinone dimers (6-4 PPs) and **B:** Cyclobutane pyrimidine dimers (CPDs) levels directly, 4 and 24 hours after irradiation. Data are shown as the means ± SE (n=4).

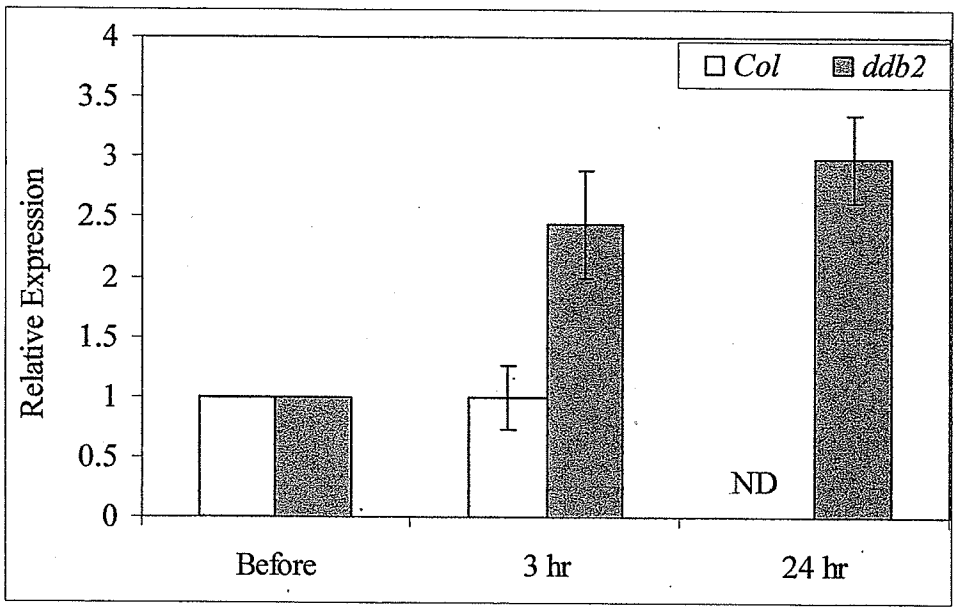


Figure 6.3 Effect of UV irradiation on mRNA level of *DET1* in wildtype and *ddb2*. Relative *DET1* mRNA levels before, 3 and 24 hours after UV-C exposure in 7-day-old wildtype and *ddb2* seedlings. Error bars indicate ± SE (n=3). mRNA level in non-irradiated samples (before) was designated 1. *UBQ10* was used to normalize mRNA level. ND: not determined.

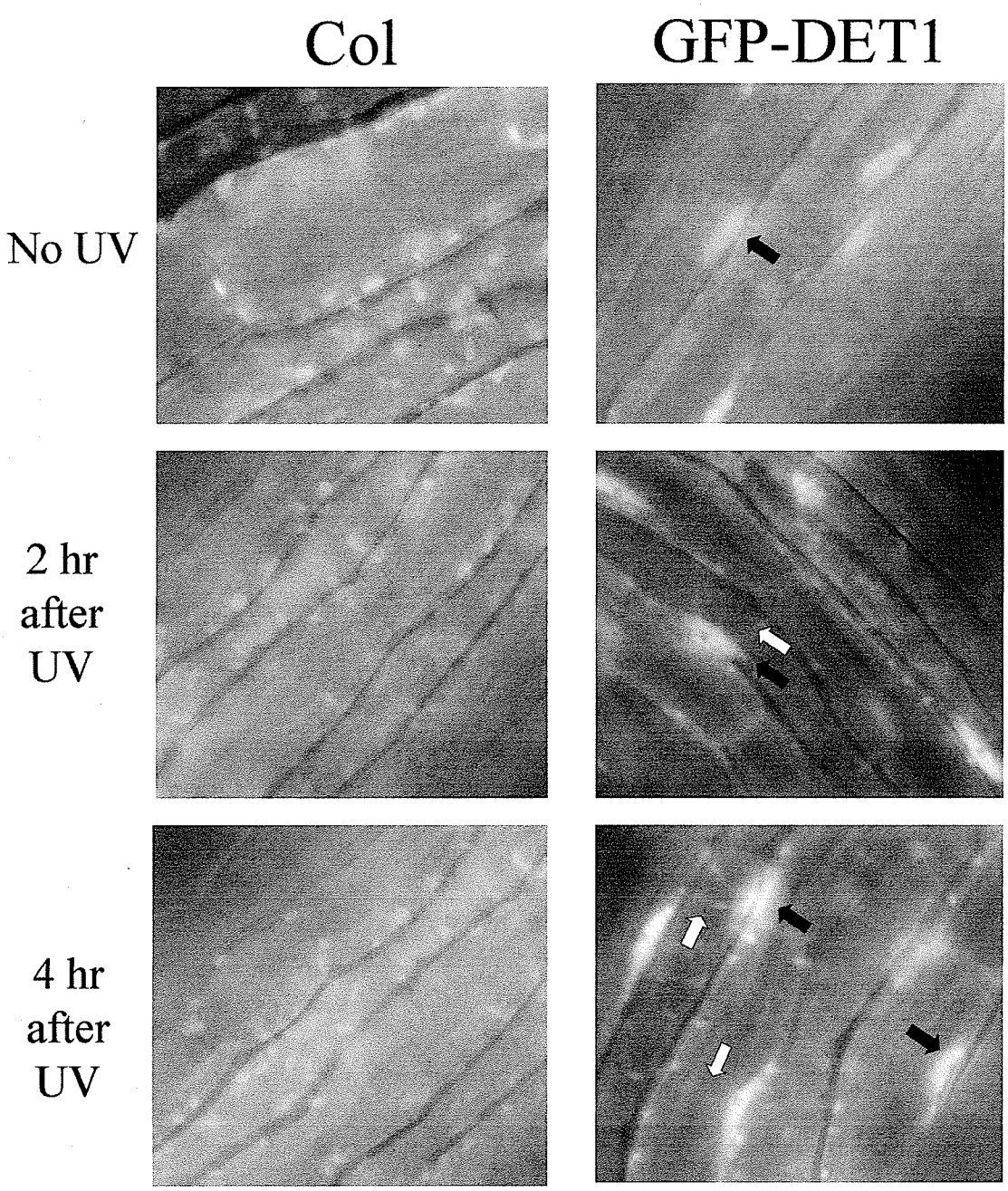
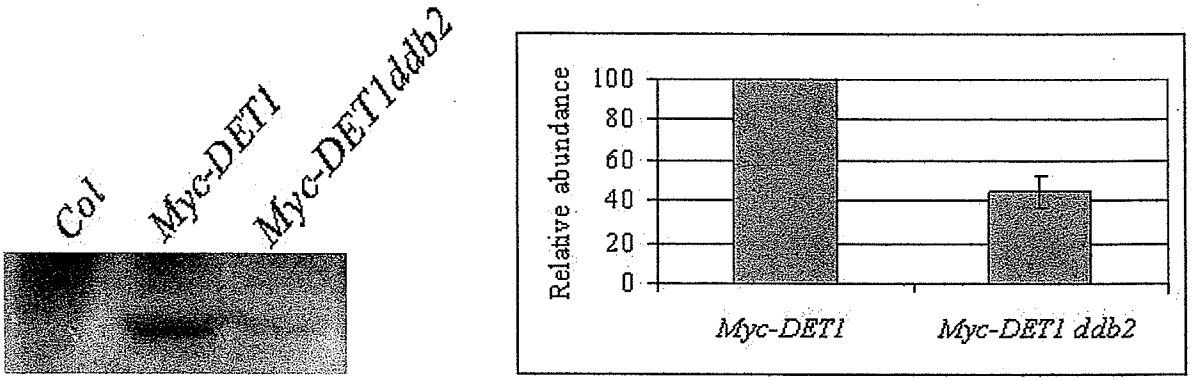
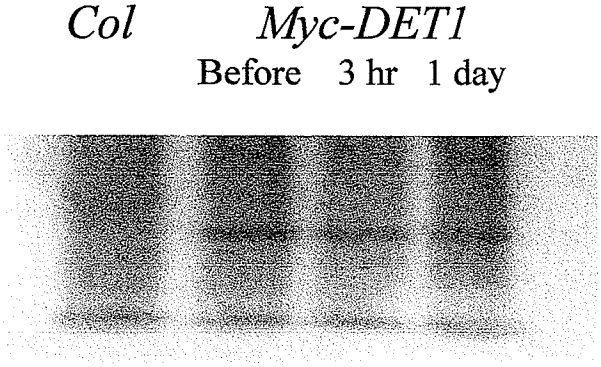


Figure 6.4 Effect of UV on GFP-DET1 localization. White arrow indicates potential cytoplasmic strand fluorescence. Black arrow indicates nuclear fluorescence. Pictures were taken at 40x

A



B



C

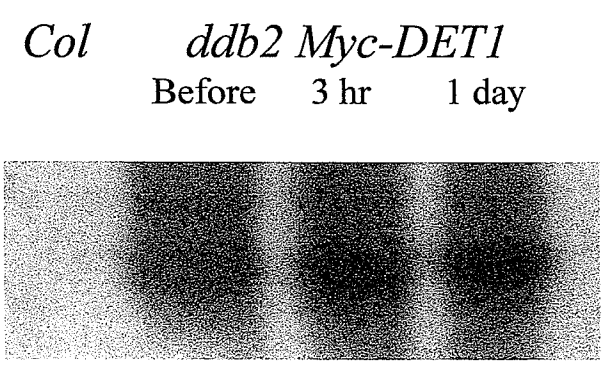


Figure 6.5 Abundance of Myc-DET1 Protein
A: Effect of *ddb2* mutation on Myc-DET1 abundance. **B:** Abundance of Myc-DET1 protein level before, 3 and 24 hours after UV exposure in *Myc-DET1*. **C:** Abundance of Myc-DET1 protein level before, 3 and 24 hours after UV exposure in *ddb2 Myc-DET1*.

D

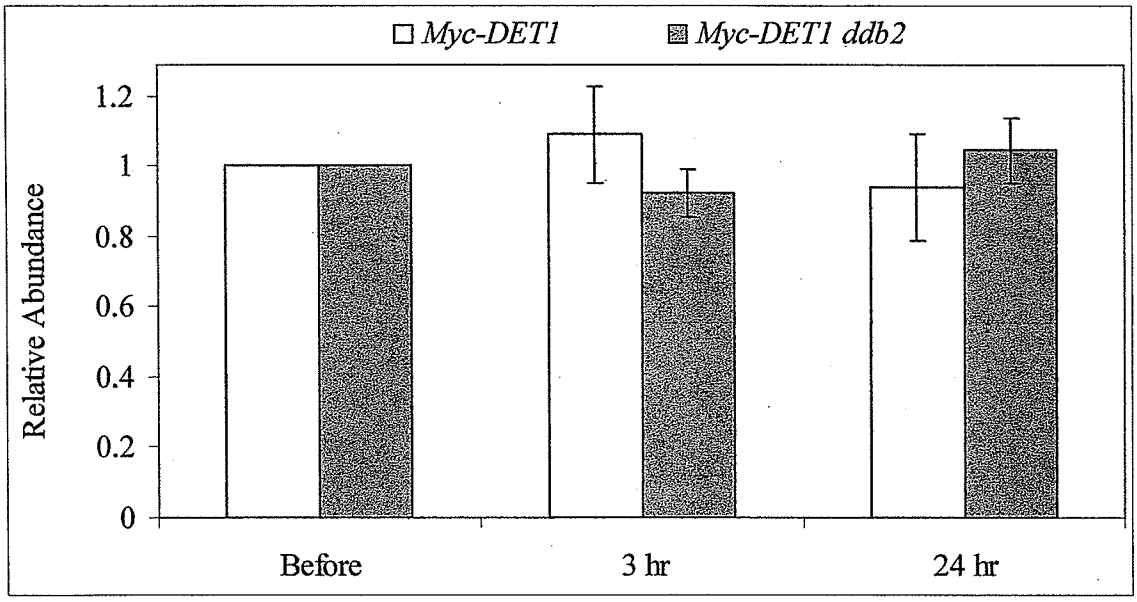


Figure 6.5 Abundance of Myc-DET1 Protein (cont.). D: Relative Myc-DET1 protein level before, 3 and 24 hours after UV exposure in *Myc-DET1* and the *ddb2 Myc-DET1* double mutant. Error bars indicate ± SE (n=2). Myc-DET1 protein level in non-irradiated samples (before) was designated 1.

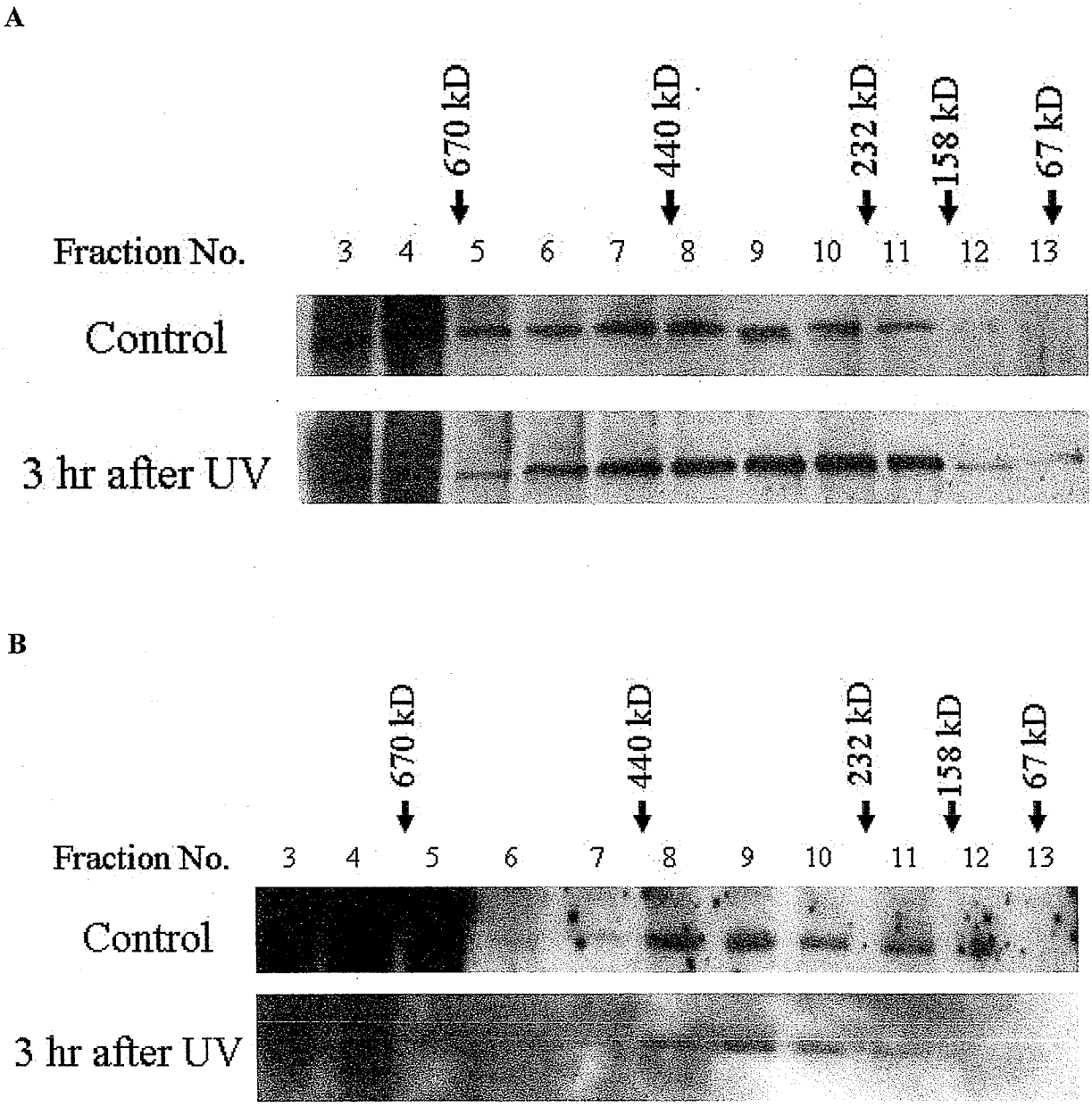


Figure 6.6 Effect of UV-C on Myc-DET1 complex size.
Total protein of 7-day light grown *Myc-DET1* (A) and the *ddb2 Myc-DET1* double mutant (B) seedlings (dark control and 3 hours after UV exposure) was extracted, separated by gel filtration and analyzed via anti-Myc western blot.

CHAPTER 7: SUMMARY AND CONCLUSIONS

7. SUMMARY AND CONCLUSIONS

The immobile nature of plants renders them susceptible to variations in the surrounding environment. They often experience wide variations both in the abiotic and biotic components of their environment. For growth and development to proceed harmoniously, plants have to adjust to these changes. The effect of environmental changes such as light results in many physiological, chemical and developmental responses as an adaptation reaction (Crawley, 1986).

The main goal of this study was to examine the genetic interaction between *DET1*, *DDB1A* and *DDB2* in response to visible and UV light in *Arabidopsis*. Thus, this thesis was divided to two main streams. The first stream investigated the role of *DET1*, *DDB1A* and *DDB2* in *Arabidopsis* growth and development under visible light. The second stream examined the role of *DET1*, *DDB1A* and *DDB2* in *Arabidopsis* DNA damage repair. The results of these studies are summarized in Table 7.1.

7.1 Loss of function analysis reveals an interaction between DET1- and DDB2-type-DDB1A/B complexes under visible light

In the first stream of this thesis, we showed that the *ddb2* partial loss of function plants exhibit wildtype phenotypes for all of the studied growth parameters mentioned in chapter 2. It had been shown previously that mutation of *ddb1a* results in a wildtype phenotype (Schroeder *et al.,* 2002). Here, further characterization of *ddb1a* loss of

function was performed. In full agreement with Schroeder *et al.* (2002), all the additional growth parameters showed that *ddb1a* mutation results in a wildtype phenotype.

det1-1 is a partial loss of function mutation. *det1* seedlings exhibit short hypocotyls, open cotyledons and high anthocyanin content under dark conditions (Chory *et al.,* 1989) and small rosette, reduced apical dominance, and day length insensitive flowering when grown under light conditions (Pepper and Chory, 1997). Schroeder *et al.* (2002) found that mutation of *ddb1a* in the *det1* background enhanced the short *det1-1* hypocotyl, the high anthocyanin content, the short plant and reduced apical dominance phenotypes. Here I was interested in examining the effect of *ddb2* mutation in the *ddb1a*, *det1*, and *det1 ddb1a* backgrounds. Two main phenotype categories were detected: *DDB1A*-dependent and *DDB1A*-independent.

For the *DDB1A*-dependent phenotypes, no significant differences were observed between *det1 ddb1a* and *det1 ddb1a ddb2*. Examples for this category are rosette diameter and hypocotyl length (dark grown seedlings). The genetic model that explains this category states that there is a competition between DDB2 and DET1 containing complexes for the available DDB1A. For the *DDB1A*-independent phenotypes, the *det1 ddb1a* mutant showed significantly different phenotypes than *det1 ddb1a ddb2*. Examples of this category are chlorophyll content and flowering time. The genetic model that explains this category states that DDB1B was able to fulfill the role of DDB1A.

Subsequently, the role of *DDB1A* overexpression in *Arabidopsis* growth and development was studied using 3 independent *DDB1A* overexpression lines. No significant differences were observed between any of the *DDB1A* overexpression lines and wildtype. As mentioned earlier, *ddb1a* is also similar to wildtype. These two findings

together suggest that there is a certain threshold for *DDB1A/DDB1B* in the cell that in the case of *ddb1a* mutation, the level of *DDB1B* is sufficient. In the case of *DDB1A* overexpression, this will not change the scenario because the cell has sufficient *DDB1A* levels to accomplish the developmental processes.

Reverse genetics have been successfully used in studying and understanding *Arabidopsis* development. Loss of function mutants are available to the public via the *Arabidopsis* stock centers. Each line of these mutants is a result of random T-DNA insertion that affects gene activity. One major obstacle for this approach is genetic redundancy. Analysis of the *Arabidopsis* genome sequence revealed that more than 80% of the genome corresponds to duplicated areas. These duplication events could limit the understanding of gene function due to the lack of obvious mutant phenotype (Briggs *et al.*, 2006). Here we used double and triple mutant analysis to distinguish the roles of DDB1A and DDB1B.

7.2 *DDB1A* overexpression lines exhibit UV but not visible light phenotypes

The second stream in this thesis aimed to investigate the role of *DET1*, *DDB1A* and *DDB2* in *Arabidopsis* DNA damage repair. In chapter 4, the *ddb1a* T-DNA mutant was used. Using RT-PCR analysis we showed that this mutation is a null mutation. In addition, the same *DDB1A* overexpression lines that had been used in chapter 3 were used here. Using these valuable genetics tools in which no *DDB1A* is available in one case (*ddb1a*) or more *DDB1A* is available in a second case (the overexpression lines), in addition to the wildtype plants, which represents the normal level of *DDB1A*, we were

able to examine the genetic role of *DDB1A* in DNA damage repair. Using shoot, root and photoproduct repair assays, I found that *DDB1A* overexpression enhanced UV tolerance in *Arabidopsis.* 6-4PP repair analysis showed for the first time that loss of function of *ddb1a* significantly reduced the repair rate of 6-4PP 4 hours after UV exposure. In addition, I found that UV exposure increases *DDB1A* mRNA level. The maximum level was 3 hours after UV exposure. Similarly, DDB1A-HA protein levels increased after UV exposure. As explained previously, DDB1 forms many complexes in the cell. Using gel filtration, a comparison between control and UV irradiated seedlings showed that UV exposure results in a decrease in fractions corresponding to molecular weights between 160 and 370 kD. In conclusion, these results indicate that *DDB1A* has an important role in *Arabidopsis* DNA repair.

Comparing the lack of *DDB1A* overexpression phenotype under visible light conditions to its strong effect under UV light and DNA damage repair could be explained due to the role of DDB1A in each pathway. The endogenous amount of DDB1A could be sufficient for normal growth and development under visible light conditions. In contrast, under UV irradiation, more DDB1A may be required to repair DNA damage. The increase in *DDB1A* mRNA level after UV exposure supports this hypothesis. In addition, the level of DDB1B in the cell could be sufficient to fulfill the absence of *ddb1a* under visible light but not under UV light. This also suggests that the required DDB1A level in the cell is higher under UV irradiation than visible light.

7.3 Loss of function analysis reveals roles of DDB complex in damage recognition

Next I examined the interaction between *DDB1A* and *DDB2* in *Arabidopsis* DNA repair. *ddb2* single mutants exhibit wildtype phenotypes in shoot and root assays. In contrast, 6-4PP repair analysis showed that *ddb2* mutants exhibit reduced repair 4 hours after exposure. No significant difference was observed in shoot and root assays between the single *ddb2* mutant and the double mutant *ddb1a ddb2*.

Our photoproduct analysis allowed us to examine the relative role of global genomic repair (GGR) and transcription coupled repair (TCR) in *Arabidopsis* DNA damage repair. GGR repairs damage in the whole genome, while TCR is specific to the transcribed regions of genes. The difference between these two pathways is in the damage recognition step. GGR requires DDB1, DDB2 and XPC-HR23B for damage recognition. TCR on the other hand initiates with RNA polymerase stalling at the site of DNA damage. CSA and CSB are also required in the damage recognition step in TCR (Fleck and Nielsen, 2004). In addition, it has been shown that DDB1 forms a complex with CSA that is important in TCR damage recognition (Groisman *et al.*, 2003). This means that DDB1 is an important component of both NER pathways (Figure 7.1). Photoproduct analysis data from chapter 4 and 5 is summarized in Table 7.2.

Arabidopsis has two copies of DDB1: DDB1A and DDB1B that are 91% identical. The question is, is it DDB1A or DDB1B or both that interacts with DDB2 or CSA during damage recognition. This is still unclear. Also, as described earlier, DDB1-DDB2 and XPC-HR23B are proposed to act as damage recognition factors. But are these two complexes involved or is one of them enough? Are they dependent on each other? Is their a limiting factor in each complex, and if yes which one is it? All these questions are still not

understood. Using *ddb1a* and *ddb2* single mutants and the double mutant *ddb1a ddb2* helped us to answer some of these questions.

Table 7.1: Summary the genetic interaction between *DET1*, *DDB1A* and *DDB2* in response to visible and UV light.

	Visible light phenotypes	UV phenotypes
det1	Many (Chory *et al.*, 1989)	None (Chapter 6)
ddb1a	None (Schroeder *et al*, 2002)	PP repair defect (Chapter 4)
DDB1A overexpression	None (Chapter 3)	Increased UV tolerance (Chapter 4)
ddb2	None (Chapter 2)	PP repair defect (Chapter 5)
ddb1a ddb2	None (Chapter 2)	PP repair defect (Chapter 5)
det1 ddb2	Suppression of some *det1* phenotypes (Chapter 2)	None (Chapter 6)
det1 ddb1a	Enhancement of some *det1* phenotypes (Schroeder *et al*, 2002)	ND
det1 ddb1a ddb2	Enhancement of some *det1 ddb1a* phenotypes (Chapter 2)	ND

PP: photoproduct, ND: not determined

Table 7.2: Summary of the remaining (%) 6-4PP and CPD in Col, *ddb1a*, *ddb2*, *ddb1a ddb2* and *uvh1* 4 and 24 hrs after UV exposure. Col, *ddb1a*, *ddb2*, *ddb1a ddb2* from Figure 5.2. *uvh1* from Figure 4.3.

	Time	*Col*	*ddb2*	*ddb1a*	*ddb1a ddb2*	*uvh1*
6-4PP	4 hrs	70	80	90	90	90
	24 hrs	70	70	80	80	90
CPD	4 hrs	80	90	90	90	100
	24 hrs	40	45	55	70	80

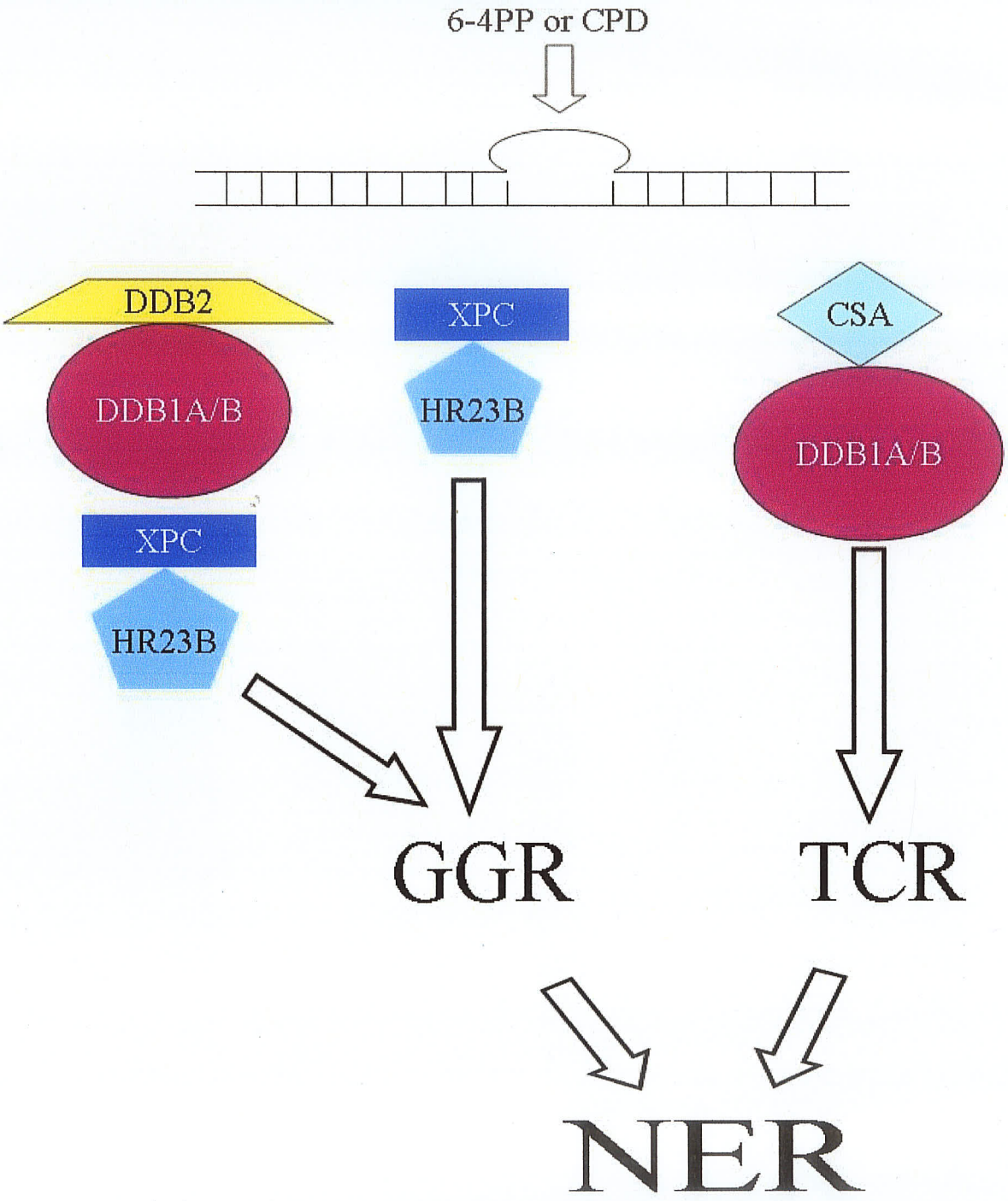


Figure 7.1 Suggested models for DNA damage recognition in *Arabidopsis* NER

The results of this study show that 6-4PP repair 4 hours after UV exposure in the *ddb1a* loss of function mutant exhibits similar repair rate as the *uvh1* mutant (Figure 4.3). This suggests that DDB1A is essential for 6-4PP repair at this point. Similarly, the *ddb2* partial loss of function mutant also exhibits a decrease in 6-4PP repair rate at 4 hours after exposure (Figure 5.2). In addition, the 6-4PP repair rate in the *ddb1a ddb2* double mutant is similar to *ddb1a* mutant which indicates a *DDB1A*-dependent phenotype (Figure 5.2). That is, DDB1B complexes are not active at this stage. These results indicate that 6-4PP recognition in *Arabidopsis* GGR 4 hours after UV exposure is achieved via the DDB1A-DDB2 complex. This is in agreement with the increase in *DDB1A* mRNA level 3 hours after UV exposure.

A different scenario occurs for 6-4PP repair 24 hours after UV exposure. *ddb1a* loss of function mutants show repair rates intermediate between wildtype and *uvh1* (Figure 4.3). This suggests that DDB1B, CSA or XPC-HR23B complexes recognize the damage at this point. In addition, *ddb2* mutants show similar repair rate to wildtype (Figure 5.2). Similarly, mutation of *ddb2* in the *ddb1a* background does not modify its repair rate which indicates that the DDB1B-DDB2 complex is not active at this point. Therefore, we expect that XPC-HR23B or DDB1B-CSA complexes to recognize 6-4PP 24 hours after UV exposure.

For CPD repair, 4 hours after UV exposure, *ddb1a*, *ddb2* and *ddb1a ddb2* mutants exhibit levels of CPD repair rates close to wildtype (Figure 5.2). This suggests that DDB1B-CSA or XPC-HR23B complexes are important in CPD damage recognition 4 hours after exposure. In contrast, 24 hours after UV exposure, the *ddb1a ddb2* double mutant showed slower CPD repair rate than the single mutants or the wildtype. This suggests that the DDB1B-DDB2 complex is now active in damage recognition.

Also, the double mutant *ddb1a ddb2* show different remaining CPD levels (70%) than the *uvh1* mutant (80%) 24 hours after exposure. This suggests that DDB1B-CSA or XPC-HR23B complexes are also involved in damage recognition at this stage.

In response to UV exposure, the *DDB2* mRNA level increased in wildtype plants 24 hours after irradiation (Figure 4.4). In contrast, *ddb1a* mutants exhibit a dramatic decrease in *DDB2* mRNA starting 3 hours after exposure. At 24 hours after exposure, *ddb1a* plants showed only 30% *DDB2* mRNA level compared to non-irradiated seedlings. Regarding *DDB1A* mRNA response, *DDB1A* mRNA increased 3 hours after UV exposure in wildtype plants. However, in the *ddb2* mutant, no signify cant change was observed 3 and 24 hours after exposure (Figure 5.3). These results together indicate that there is reciprocal regulation of *DDB1A* and *DDB2* mRNA abundance in the cell in response to DNA damage. This model supports the hypothesis that DDB1 and DDB2 are both required in DNA damage recognition.

7.4 DET1 has a role in *Arabidopsis* development under visible light but not in DNA damage repair

The results of chapter 2 showed that the double mutant *det1 ddb2* is significantly different than the *det1* and *ddb2* single mutants. This means that *DET1* genetically interacts with *DDB2* in *Arabidopsis.* This encouraged me to examine if *det1 ddb2* double mutant exhibit a different phenotype than *ddb2* in response to UV irradiation, and if mutation of *ddb2* and/or UV treatment affects DET1 behavior.

det1 seedlings showed similar photoproduct repair rates to wildtype. The partial loss of function *ddb2* exhibits less 6-4PP repair than wildtype. On the other hand, *det1 ddb2* exhibits similar 6-4PP and CPD repair rates to the *ddb2* mutant. In addition, Myc-DET1 abundance showed similar responses to UV exposure in wildtype or *ddb2* background. These results suggest that there is no genetic interaction between *DET1* and *DDB2* during *Arabidopsis* DNA damage repair.

7.5 Suggested future research

Numerous models for the role of DDB in DNA repair have been proposed in the literature. One suggests that DDB is important in chromatin remodeling and accessibility of other repair factors to the damage site (Li *et al.,* 2006). A second hypothesis suggests that the interaction of DDB with the damaged DNA could enhance the recruitment of NER factors (Sagasawa *et al.,* 2005). Others suggest that DDB could work as a molecular chaperone that facilitates the interaction between the damaged DNA and NER factors (Tang and Chu, 2002). On the other hand, it has been shown that the DDB complex is not the only damaged DNA recognition factor (Naegeli, 1999).

The results of this thesis show that *DET1*, *DDB1A* and *DDB2* interact genetically and regulate *Arabidopsis* development under visible light. In contrast, only *DDB1A-DDB2* interaction was observed under UV irradiation and DNA damage repair. Further studies that could illuminate the significance of these genes include:

- We found that overexpression of *DDB1A* enhances UV tolerance. It would be interesting to examine if this enhancement is *DDB2*-dependent or not. This could be

examined by generating a line with *DDB1A* overexpression and *ddb2* loss of function, then examining the response to UV exposure.

- By generating *DDB2* or *DDB1B* overexpression lines, it will be possible to examine the effect of having more *DDB2* or *DDB1B* on *Arabidopsis* growth and development under both visible and UV light.

- Since only *Arabidopsis* has *DDB1B* and its function is still unclear, further work is needed to generate either a *ddb1b* mutant and/or mutant of the DDB1 family (*DDB1A* and *DDB1B*). This could be achieved by RNAi. Also, it will be interesting to examine the effect of loss of function of the whole DDB complex (*ddb1a ddb1b ddb2*) in *Arabidopsis* development and DNA repair.

- Further work is required to examine the interaction between the DDB complex and other NER factors at the damage recognition step. Generating *Arabidopsis* double mutants of *ddb1a* or *ddb2* with *xpc* or *centrin2* could be one possible way to study that interaction.

Arabidopsis has been broadly used as a model organism for studies not only in the plant kingdom but beyond. The results of this study, in addition to other findings in the field, could also have implications in human health research.

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9. Appendix

9. Appendix: Biological repeats of the effect of UV on mRNA level.

Col

		Rep 1	Rep 2	Rep 3
DDB1A	Before	1	1	1
	3 hr	3.49	4.00	2.36
	6 hr	2.08	2.39	1.23
	24 hr	0.91	1.22	1.14
DDB1B	Before	1	1	
	3 hr	1.44	2.28	
	6 hr	1.19	1.78	
	24 hr	0.83	1.33	
DDB2	Before	1	1	
	3 hr	0.95	0.94	
	6 hr	0.87	0.90	
	24 hr	1.16	1.17	

ddb1a

		Rep 1	Rep 2	Rep 3
DDB1B	Before	1	1	
	3 hr	0.82	0.99	
	6 hr	0.82	1.10	
	24 hr	0.76	0.96	
DDB2	Before	1	1	1
	3 hr	0.72	0.62	0.31
	6 hr	0.32	0.45	0.21
	24 hr	0.16	0.31	0.29

5-1

		Rep 1	Rep 2	Rep 3
DDB1A	before	1	1	1
	3 hr	3.70	2.70	2.66
	6 hr	1.35	1.49	1.29
	24 hr	1.51	1.45	0.94
DDB1B	before	1	1	
	3 hr	1.26	1.25	
	6 hr	1.23	1.18	
	24 hr	1.13	1.33	
DDB2	before	1	1	1
	3 hr	0.92	0.81	0.97
	6 hr	1.28	1.14	0.95
	24 hr	1.27	1.37	1.13

ddb2

		Rep 1	Rep 2
DET1	before	1	1
	3 hr	3.13389	2.86
	24 hr	3.91964	3.18