The Role of Arabidopsis UV-Resistance Locus 8 Protein in Regulating Photosynthetic Competence

Novita Indah Susanti

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Division of Molecular and Cellular Biology
Faculty of Biomedical and Life Sciences
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To all the people I love who believe in dream and hope

“God does not ask us to be successful but faithful. Faithful means that we will do our best in everything“

(Mother Theresa)

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Abstract

Arabidopsis thaliana UV-Resistance Locus 8 (UVR8) is a UV-B-specific protein that regulates genes concerned with protection against ultraviolet-B (UV-B) radiation. Some of these genes encode chloroplast proteins including the RNA polymerase sigma factor, sigma 5 (*SIG5*) and one of the Early Light Induced Proteins in *Arabidopsis thaliana* (*ELIP1)*. According to this discovery, UVR8 had been proposed to be involved in regulating chloroplast related genes that encode Photosystem II Reaction Center core proteins, the D1 and D2 proteins. This hypothesis was examined in this study. Several physiological approaches and measurements of transcripts and protein were done using *uvr8-1*, *sig5.1*, *sig5-2* and *elip1/2* mutants. This study showed that the *uvr8-1* mutant is very sensitive to UV-B compared to wild type and other mutants and *uvr8-1* had a reduction of its photosynthetic efficiency (measured as Fv/Fm values). Assessments of *SIG5* and *ELIP1* transcripts and measurements of photosynthetic efficiency showed that these genes are not essential in UV-B protection. Further, transcript measurements of *psbA* and *psbD*-BLRP, which encode the D1 and D2 protein respectively, showed that UVR8 is involved in accumulation of *psbD*-BLRP transcripts but little affected *psbA* transcripts. Moreover, UV-B caused reduction of D1 protein consistent with the reduction of the Fv/Fm values when wild type and *uvr8-1* plants were exposed to UV-B, but the role of UVR8 in this mechanism needs to be investigated further. However, the effect of UV-B on D2 protein still remains unclear.

List of Figures and Tables

Tables :

Abbreviation

APS	Ammonium Persulafate
BSA	Bovine Serum Albumine
Chl(s)	Chlorophyll (s)
CHS	Chalcone Synthase
CBR	Carotene Biosynthetsis Related
Col-0	*Arabidopsis thaliana* Columbia-0 ecotype
Cry	Cryptochrome
ELIP	Early Light-Inducible Protein
Et-Br	Ethidium Bromide
HY5	HYPOCOTYL ELONGATION5
HYH	HYPOCOTYL ELONGATION HOMOLOG
L*er*	*Arabidopsis thaliana* Landsberg *erecta* ecotype
NEP	Nuclear-encoded RNA polymerase
PAR	Photosynthetically Active Radiation
PEP	Plastid-encoded RNA polymerase
Phy	Phytochrome
psbD-BLRP	*psbD*-Blue Light Responsive Promoter
PSII RC	Photosystem II Reaction Center
PTF1	Plastid Transcription Factor1
Q_A	$Quinone_A$
Q_B	$Quinone_B$
R/FR	Red/Far Red
rbcL (Rubisco)	Ribulose 1,5-biphosphate carboxylase/oxygenase large subunit
RCC1	Regulator of Chromatin Condensation 1
ROS	Reactive Oxygen Species
SDS/PAGE	Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis

SIG(1-6)	Plastid factor Sigma (1-6)
sqRT-PCR	Semi quantitative Reverse Transcriptase Polymerase Chain Reaction
UVR8	UV-Resistance Locus 8 protein
UV-B	Ultraviolet B

Table of Contents

Chapter 1. Introduction

1.1. Introduction

1.1.1. General knowledge of Photosynthesis and UV-B radiation

Plants require sunlight for photosynthesis - a process that converts solar energy into chemical energy - in order to sustain life. There is no doubt that photosynthesis is a very important process. Photosynthetic organisms such as plants generate O_2 for the environment and sugar to provide energy for itself and others. Photosynthesis takes place in subcellular organelles called chloroplasts **(Figure 1.1.)**. The chloroplast has inner, outer and thylakoid membranes. Among these three membranes, the thylakoid membrane is the most important in photosynthesis. The photosynthetic machines such as light harvesting-proteins and reaction centres are attached to the thylakoid membrane.

Figure1.1. A chloroplast
(Source:http://student.ccbcmd.edu/courses/bio141/lecguide/unit3/eustruct/u4fg41.html)

Green plants capture light using sensitizers. The well known sensitizer which is involved in photosynthesis is *Chlorophyll* (*Chl*). *Chls* are tetrapyrrole molecules that strongly absorb bands in the visible region of spectrum. Chlorophylls and others pigments attach to proteins to build a photochemical system machine called Photosystem. In higher plant, there are two photosystems, i.e. Photosystem II (PSII) and Photosystem I (PS I). The initial process of photosynthesis takes place in PSII. In PSII, energy from these photons is used to oxidize water gradient across the membrane to generate oxygen. As a photochemical machine, it is possible for PSII or other photosynthetic apparatus to be impaired somehow. Naturally, the plant has an ability to repair damage; however under extreme stress the impairment of its components is unavoidable.

One of the possible causes of photodamage of photosynthetic apparatus is UV radiation. Since UV radiation is a constituent of the solar spectrum, it is impossible for plants to avoid UV light exposure. There are three types of UV radiation, i.e. UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (less than 280 nm) **(Figure 1.2)**.

Figure 1.2.

Among these types, UV-A and UV-B have the most biological importance because the stratospheric ozone layer very effectively absorbs UV radiation that has wavelengths below 290 nm (Ulm and Nagy, 2005). The amount of UV-B reaching the earth surface varies and is influenced by many factors such as cloud, latitude, altitude, season, solar angle, aerosol and Ozone (O_3) layer (reviewed by Allen *et al.*, 1998; Hollósy, 2002). For decades, the effects of UV-B radiation have attracted many groups of researchers. Perhaps it relates to the environment changing in the past few decades (Hollósy, 2002). The amount of UV-B radiation increased recently as a result of mankind activities such as the usage of chloro-fluoro-carbon (CFC). Ozone depletion as a consequence of CFC reaction may increase the amount of UV-B reaching the earth's surface. The most suspected targets for this environmental change are plants since they cannot move or hide themselves from external threat. In other point of view, plants are very important for human life. Thus, any environment threats to plants may impact either directly or non-directly to human life.

Plant perceive light-signalling by photoreceptors. Phytochrome (Phy) is photoreceptor for Red/Far-Red (R/FR) light, whilst cryptochrome (Cry) and phototropins strongly absorb blue/UV-A light. In contrast to other light-signalling regulation, UV-B photoreceptors are still unknown. The complexity of UV-B signalling, variation of responses and the unknown photoreceptor is thus interesting to be investigated.

UV-B acts both as a non-damaging and damaging agent. A-H-Mackerness (2000) recorded UV-B induced changes in plant growth and development. UV-B causes changes in pigment composition, loss of photosynthetic activity, alteration in the timing of flowering, and inhibits reproduction (A-H-Mackerness, 2000). Ulm and Nagy (2005) provided evidence that at molecular level the growth and photomorphogenic response to UV-B is distinct at short (280-300 nm) and long wavelength (300-320 nm). In parallel to this report, different fluence rate of UV-B also has been reported to stimulate different responses as stated by Brown *et al* (2005). These responses to different fluence rates are mediated by distinct regulation. At present little is known of UV-B signalling

regulation in higher plants and so much research needs to be done in order to understand the complexity of UV-B signalling.

As mentioned before, UV-B stimulates different responses as a damaging and non-damaging agent. Experiments in higher plants revealed the responses to UV-B depend on fluence rates as noted in Brown *et al* (2005). At low fluence rates, UV-B stimulates some genes that are involved in a wide range of processes in UV protection (Brown *et al.*, 2005), including genes that are responsible for flavonoids and phenolics production. Flavonoids accumulated in the epidermis provide a shield to protect plant from UV-B radiation (Reviewed by Teramura and Sullivan, 1994), as its component strongly absorbs UV-B (Hollósy, 2002). In higher plant accumulation of flavonoid is distinct in two main taxonomic groups. In most dicotyledon plants flavonoid accumulated in epidermis, whilst in monocotyledon plants flavonoid is distributed in epidermis and mesophyll (Hollósy, 2002).

How plants protect themselves from UV-B by producing flavonoids and other secondary metabolites is well documented. Li and co-workers (1993) used *Arabidopsis* mutants the *transparent testa -4*, *5* and *6* mutants (*tt4*, *ttt5* and *tt6*), which have reduced flavonoid and phenolic compounds. As noted in their report, the *tt4* mutant is the chalcone synthase mutant and *tt5* is chalcone isomerase mutant (Li *et al*., 1993). The experiments showed these mutants were more sensitive than wild type to UV-B. Another mutant identified by Lois and Buchanan (1994), the *uvs* mutant, also showed the alteration in flavonoid compounds caused sensitivity to UV-B. The Arabidopsis ferulic acid hydroxylase mutant (*fah1*) suffers more growth–inhibition and UV-B-injury than wild type (Landry *et al.*, 1995). Study in *Brassica napus* revealed the enhancement of flavonoid content when leaves were exposed to UV-B (Olsson *et al.*, 1998). This study implied that flavonoids are involved in UV-B protection responses. Using the *tt4*, *tt5* and *fah* mutants, Booij-James and co-workers (2000) found that alteration in phenolic compounds affect PSII heterodimer in *Arabidopsis* under mixture of photosynthetically active radiation (PAR) and UV-B. Recent study showed UV-B failed to induce chalcone synthase (*CHS*) and other UV protection genes in *Arabidopsis uvr8* mutant causing hypersensitivity to UV-B (Brown *et al.*, 2005). CHS is a key enzyme in

flavonoid biosynthesis. Expression of *CHS* is light dependent and regulated by distinct UV-B, UV-A and blue light transduction pathways (Fuglevand *et al.,* 1996). Related to defense mechanism in plants, UV-B stimulates expression of some pathogenesis related proteins such as PR-1,-2 and -5 (A-H-Mackerness, 2000).

Low fluence rates of UV-B also have been reported to mediate photomorphogenic responses. In 1998, Kim and co-workers provided evidence that phyA and phyB are required to UV-B-induce photomorphogenesis in *Arabidopsis thaliana*. Moreover, the authors also provide evidence that this response is not mediated by DNA damage signalling (Kim *et al*., 1998). Further, Boccalandro and co-workers (2001) observed cotyledon opening in Arabidopsis was mediated by phytochrome but the enhancement of this phenomenon under UV-B radiation was regulated by unidentified UV-B photoreceptor. On the other hand, Bertram and Lercari (2000) found evidence that UV-B-induced photomorphogenic response did not require phytochrome B in tomato plants (*Solanum lycopersicum*). The authors suggested distinct mechanism and photoreceptors involved in UV-B mediated photomorphogenic responses. Stratmann (2003) noted that photomorphogenic responses to low fluence rate of UV-B were not regulated by phytochrome, cryptochrome and phototropin photoreceptor. This implied that unknown UV-B photoreceptor may be involved in photomorphogenic regulation in UV-B signalling pathways.

At high fluence rates UV-B acts as a damaging agent. It causes damage to biomolecules. In extremes, UV-B can cause cell necrosis. At high fluence rates UV-B generates ROS (Reactive Oxygen Species), which can cause cell death. Several experiments showed that ROS can cause oxidation of lipid and protein and damage DNA (Kliebenstein *et al.,* 2002). UV-B radiation caused enhancement in lipid peroxidation (Hollósy, 2002). In order to lessen the impact of ROS, plant produces antioxidants such as ascorbic acid (Kliebenstein *et al.,* 2002). Study in *Arabidopsis thaliana* showed that ROS was generated by multiple sources under UV-B exposure (A-H-Mackerness *et al.,* 2001). However, ROS-mediated UV-B activities are not only detected in higher plants. ROS activity also was detected in cyanobacterium *Anabaena* sp, which were illuminated by UV-B (He and Häder, 2002a; 2002b).

As mentioned above, UV-B causes changes in pigment composition (A-H-Mackerness, 2000). In agreement with this statement, Hollósy (2002) in his review paper reported that UV-B causes reduction on *Chl b* content. In contrast to this report, Rao and co-workers (1995) reported that UV-B increased total amount of chlorophyll and carotenoid in both Arabidopsis wild type (Lansberg *erecta*) plant and *tt5* mutant.

Investigation of the effect of UV-B on photosynthetic activity in algae *Dictyota dichotoma* was reported by Ghetti and co-workers (1999). Other experiments in *Dictyota dichotoma* showed UV-B involved both in repair mechanism and turnover of photosynthesis (Flores-Moya *et al.*, 1999). Rajagopal and co-workers (2000) observed UV-B radiation (1.9 mW m^{-2}) on intact cell of cyanobacterium *Spirulina plantesis* caused reduction in photosystem II activity. Sunlight containing UV-B has been reported to cause reduction in photosystem II activity in phytoplankton (Marwood *et al.*, 2000).

In higher plants, UV-B causes turnover of the D1 protein of PSII Reaction Centre and reduction of mRNA transcripts of Ribulose 1,5-biphosphate carboxylase oxygenase (Rubisco) (Teramura and Sullivan, 1994). In 1995, Wilson and co-workers presented that UV-B-induced photomodification of Rubisco Large subunits on *Brassica napus*, tomato (*Lycopersicon esculentum*), pea (*Pisum sativum* L.) and tobacco (*Nicotiana tabacum*). The 66-kD protein was detected in plants exposed to 65 μmol $m^{-2}s^{-1}$ PAR plus UV-A (1.7 μmol $m^{-2}s^{-1}$) and supplementary UV-B (1.5 μmol $m^{-2}s^{-1}$) for 4 hours (Wilson *et al*., 1995). This protein was considered as a photomodification product of Rubisco Large subunits (Wilson *et al*., 1995). Allen *et al* (1998) in their critical review presented that UV-B declined the activity of large sub-unit of Rubisco in mature leaf of oilseed rape. This reduction was due to a reduction in amount of Rubisco presented in the leaf. The authors also quoted some researches that reported effect of UV-B radiation on reduction of Rubisco activity and content in higher plants (Allen *et al*., 1998). In agreement with previous reports Hollósy (2002) presented reduction of UV-B-induce Rubisco activity. However, a study in *Arabidopsis thaliana* showed that UV-B did not affect the amount of Rubisco protein both in wild type (Lansberg *erecta*) and *tt5* mutant exposed to 15 kJ $m^{-2}day^{-1}$ of UV-B for 5 days (Rao *et al*., 1995). Further,

UV-B decreased Rubisco protein in *tt5* mutant only when plants were exposed in prolongation time of exposure to 7 days (Rao *et al*., 1995). Moreover, UV-B declined initial and total activities of Rubisco only in *tt5* plants (Rao *et al*., 1995). However, the decrease in the activity of Rubisco was not accompanied by a decrease in the amount of protein (Rao et al., 1995). A-H Mackerness and co-workers (1997) investigated the effects of supplementary UV-B on mRNA transcripts and chloroplast protein i.e. Lhcb, D1 and RUBISCO in *Pisum sativum* L. Plants were grown in 150 μmol $m^{-2}s^{-1}$ of PAR then exposed to PAR with supplementary UV-B (estimate dose was 182 mW m^{-2}). The results showed that UV-B did not affected *psbA* transcripts during 4 days treatment. In contrast, the level of D1 protein declined after 2 days (A-H Mackerness *et al*., 1997). Furthermore, the *rbcL* mRNA level was not affected for the first two days of experiment, despite the reduction in Large SubUnit (LSU) of the protein in two days experiment (A-H Mackerness *et al*., 1997).

The effects of UV-B on PSII are well documented as mentioned briefly. In contrast, UV-B has less impact on the PSI relative to PSII. Thus, many researchers concluded PSII is the main possible target in UV-B destruction effect (Hollósy, 2002). Allen *et al* (1998) in their critical review presented evidence that UV-B causes reduction in stomatal conductance (*G*) leading to stomatal closure.

1.1.2. Photosystem II Reaction Centre (PSII RC)

As mentioned previously, the heart of photosynthesis can be addressed to PSII-RC since the initial energy conversion takes place in PSII reaction centre. Oxidation of H_2O to O_2 takes place in PSII-RC. The complexity of PSII, both in structure and function had been observed in photosynthetic bacteria, cyanobacteria and higher plants (Seibert, 1993). There were speculations about PSII complex structure and function. Perhaps significant contribution was provided by Nanba and Satoh (1987) when they successfully isolated PSII-RC. This invention elucidated location of D1 and D2 proteins in PSII-RC. It revealed that the isolated PSII-RC contain D1, D2 and Cyt b_{559} proteins

(Seibert, 1993), moreover, heterodimer D1 and D2 proteins are the primary separation sites in PSII (Nanba and Satoh, 1987; Seibert, 1993). To date, the structure of PSII in higher plants has been established. It is composed of two major polypeptides, the D1 (*psbA* product) and D2 (*psbD* product) proteins, the α- (*psbE* product) and β- (*psbF* product) sub-units of Cytochrome b_{559} and the PsbI protein (Seibert, 1993) as illustrated in Figure 1.3.

Figure 1.3.

The D1 and D2 heterodimer are encoded by plastid *psbA* and *psbD* genes, respectively. The study in amino acid sequence provided information that the D1 and D2 sub-units are homologous with L and M sub-units of PSII of purple bacteria and might possibly have similar function with L and M sub-units (Nanba and Satoh, 1987). Although there are some similarities between PSII Reaction Centre and purple bacterial reaction centre, they differ in structure and activity (Telfer and Barber, 1994). As mentioned above, the success in PSII-RC isolation by Nanba and Satoh (1987) contributed to insight knowledge in PSII-RC and led to advance many researches in photosynthetic mechanisms, including studies in PSII-RC-related proteins, i.e. D1 and D2 protein. To date, the function of D1 and D2 proteins in PSII Reaction Centre was

elucidated. Jansen and co-workers (1996a) noted D1 protein provides binding environment for several chemical herbicides.

Since PSII is the site of energy separation, its apparatus is easy to damage. As mentioned briefly, plants have an ability to repair damage. This means that the damaged component can be replaced by a new one to achieve a balance. In extreme cases, if the rate of photodamage is higher than the ability of PSII to recover, it will cause photoinhibition. The term photoinhibition, as described by Kok in 1956 (Osmond, 1994), is a light-dependent reduction in photosynthetic efficiency. This term apparently is a simple way to describe the complexity of the process of photodamage of photosynthetic apparatus. When photoinhibition occurs dramatically, photosynthesis will end and plants will no longer survive.

1.1.2.1. Photodamage of PSII : D1 and D2 protein degradation

Since the inhibition of electron transport can be initiated at different sides in PSII, photoinhibition was divided into two mechanisms i.e. donor and acceptor side mechanism (Telfer and Barber, 1994). The donor side mechanism occurred when the acceptor side could not maintain electron donation from water, thus extending the life time of excited donor molecule ($P680^+$). The acceptor side mechanism occurred at the level of quinones and reduction of plastoquinone pool. The idea of donor and acceptor side mechanisms was proposed by Barber and Anderson in 1992 (Telfer and Barber, 1994). The primary electron donor is chlorophyll molecule (P680). The electron acceptor is pheophytin and two types of Quinones, i.e. Q_A (bounds to D2) and Q_B (bounds to D1) (Styring and Jegerschold, 1994). These donor and acceptor molecules are bound to D1 and D2 proteins. Tyr_Z and Tyr_D, the Tyr161 of D1 and D2 respectively, are the immediate and accessory electron donors for P680 (Styring and Jegerschold, 1994).

The mechanism of electron transfer in PSII Reaction Centre is described as follows. Light excites the P680 molecule which transfers its electron to pheophytin. The

pheophytin molecules transfer the electron to the first acceptor quinone (Q_A) which continues it to secondary quinone acceptor (Q_B). Then Q_B leaves its site in D1 protein and exchange with an oxidized quinone molecule from plastoquinone pool (Styring and Jegerschold, 1994) **(See Figure 1.3)**.

Barbato and co-workers (1995) observed the degradation of D1 protein *in vivo* and *in vitro* of PSII in Spinach (*Spinacia oLeracea* L.) under UV-B radiation. They found 20 kDa of protein fragment which corresponded to a degradation product of D1 protein. This study also provided evidence that degradation of D1 under UV-B depends on the presence of manganese. The manganese cluster known to be bound to D1 and D2 proteins is in donor side of PSII reaction centre. The authors suggested that D1 degradation under UV-B depends on manganese on the donor side of PSII. Shipton and Barber (1991) provided evidence that degradation of D1 and D2 *in vitro* study of peas was caused by an autoproteolytic process and occurred in oxidizing side of photosystem II. In 1999, Babu and co-workers revealed a mixture of PAR and UV-B radiation rapidly degrade D1 and D2 proteins and this phenomenon is dependent on the redox (reduction-oxidation) status of PSII.

Inactivation of PSII Reaction Centre or in extreme photoinhibition also can be enhanced by low temperature (Krause, 1994). In his review paper, Krause (1994) provided evidences that photoinhibition has been observed *in vitro* under chilling temperature. Moreover, he proposed several factors may contribute in the enhancement of photoinhibition in low temperature. First, low temperature can decrease carbon metabolism. As a consequence, reduction of primary acceptor electron (Q_A) is increased. Second, D1 synthesis is inhibited in low temperature. Third, low temperature inhibited formation of zeaxanthin (Krause, 1994).

An enzymatic process which involves some proteases has been speculated to cause D1 degradation. A recent study carried out by Huesgen and colleagues (2006) showed D1 protein degraded in Arabidopsis mutant lacking the Deg2 protease had similar rate with wild type when plants were subjected to 1500μmol $m^{-2}s^{-1}$ fluorescent light source. This result differed from *in vitro* experiments. The authors proposed that D1 degradation *in vivo* is controlled by several mechanisms. Another protease proposed

to be involved in D1 degradation is FtsH protease (Nixon *et al*., 2005; Yu *et al*., 2005). FtsH is an ATP-dependent metalloprotease. This enzyme is found in bacteria, mitochondria, and plastid (Yu *et al*., 2005). At present, 12 FtsH proteins have been identified in *Arabidopsis thaliana*. Three of them are found in mitochondrion and the rest in chloroplast (Yu *et al*., 2005).

Other studies in relation to D1/D2 degradation with Early Light-Inducible Proteins (ELIPs) were quoted by Adamska and Kloppstech in their review (1994). They concluded that degradation of D1 protein or photodamage to PSII is related to accumulation of ELIPs under various stress conditions (Adamska and Kloppstech, 1994).

Studies in D1 and D2 degradation under several light stress experiments also have been reported by numerous groups of researchers. D1 degraded rapidly in an extreme level of photosynthetically active radiation (PAR, 400-700 nm). In 1996, Jansen and co-workers provided evidence that D2 as well as D1 protein was degraded in *Spirodella oligorizha* under UV-B radiation (Jansen *et al.,* 1996a). The same group of researchers also proposed degradation of D1 and D2 protein under UV-B radiation was coupled, which D2 degradation was influenced by D1 (Jansen *et al*., 1996b). Barbato and co-workers (2000) found UV-B radiation promoted rapid turnover of D1 and D2 protein in detached barley leaves and affected the structure and functional organization of PSII. Olsson and co-workers (2000) demonstrated D1 protein in *Brassica napus* (oilseed rape) turnover rapidly after irradiation with high intensities of PAR alone or added with UV-B. Other approaches to obtain insight knowledge in D1 and D2 degradation mechanism were carried out by Booij-james and co-workers (2000). Their studies of Arabidopsis mutants deficient in phenolic metabolism showed that either UV-B alone or mix with PAR cause rapid degradation of D1 and D2 proteins (Booij-james *et al.,* 2000). Taken together, these findings showed that the D1 and D2 proteins degradation could be mediated by different wavelength of spectrum. In UV-B particularly, degradation is regulated by distinct photoreceptor from other light-signalling photoreceptor.

In vitro experiments carried out by Friso *et al* (1994) showed degradation of D2 protein after illumination with UV-B. In their experiments, isolated PSII reaction centre from pea was subjected to UV-B at wavelength 312 nm. The result detected fragments of D2 degradation products only when the isolated PSII added with external quinone. The authors conclude damage in D2 after UV-B illumination was dependent on binding quinones.

Despite numerous studies of D1 and D2 degradation under light stress experiments the mechanism of these processes is still unclear, particularly in UV-B radiation. In attempt to gain insight into UV-B signalling pathways, a recent study carried out by Brown *et al* (2005) characterized Arabidopsis UV Resistance locus 8 (UVR8) that is specific to UV-B. The *uvr8* mutants failed to induce expression of genes concerned with UV protection. Some of these nuclear genes encode chloroplast proteins. The authors speculated that UVR8 might play an important role in photosynthesis activity. To date there is no evidence for a correlation between UVR8 and D1/D2 regulation. To gain insight into this possibility, several approaches were done in this study. All the basic theory related to this will be explained in next sections.

1.1.2.2. An approach to investigate photosynthetic activity: Chlorophyll fluorescence

As stated above, plants depend on light to drive the photochemical reaction in PSII-RC. Light is perceived abundantly by chlorophylls. However, not all the energy is used for photosynthesis. To maintain energy efficiency, excess energy can be dissipated as heat or re-emitted as light/chlorophyll fluorescence. The increase in one process will reduce the other two (Maxwell and Johnson, 2000). For example, if most of the amount of photon energy is used for photochemical reaction (photosynthesis activity) the yield in heat dissipation and chlorophyll fluorescence are reduced.

The chlorophyll fluorescence has been used as a physiological parameter to observe photochemical efficiency of PSII. In the study of chlorophyll fluorescence,

some consensus terms are offered. When plant or any samples are shifted from dark to light, the open state of reaction happened. The photon is absorbed by chlorophyll (P680) which becomes excited ($P680^+$). Following this process, the electron is transferred from $P680^+$ to primary acceptor molecule (Q_A) in D2 protein. The Q_A is oxidized. In this case, the level of chlorophyll fluorescence is low. This condition is known as F_0. All the energy is trapped and used in photochemistry reaction (Blankenships, 2002). Electron from Q_A then is transferred through processes to Q_B. During this processes, the PSII Reaction Centre is closed. The fluorescence rises to maximum (F_m) and goes through until steady state is reached (Blankenship, 2002). The possible fluorescence is calculated as a result of (F_m- F_0), called F_v. Then maximum quantum yield of PSII is calculated as a relative unit of F_v/ F_m.

$$F_v/F_m = (F_m - F_0)/F_m$$

1.1.3. SIG5 and ELIPs respond to light-stress environment

In regard to damaging effects of UV-B, this section will describe genes related to light-stress responses i.e. *SIG5* and *ELIP1/ELIP2*. These genes are nuclear genes that encode chloroplast proteins. This section will describe any possible correlation between these genes and the photosynthetic-related proteins, D1 and D2 protein of PSII Reaction Centre.

1.1.3.1. SIG5

Transcription in higher plant plastids is directed by two distinct RNA polymerases, i.e. nuclear-encoded RNA polymerase (NEP) and plastid-encoded RNA polymerase (PEP) (Fujiwara *et al.*, 2000; Nagashima *et al.*, 2004). NEP is a T7

bacteriophage-type RNA polymerase, involved in transcription of housekeeping genes. PEP is a eubacteria-type RNA polymerase, responsible for the transcription of photosynthesis genes in the chloroplast. PEP is composed of the plastid-encoded core sub-units, 2α, β, β', β'' (encoded by *rpoA, rpoB, rpoC1* and *rpoC2)* and one of nuclear-encoded sigma (σ) factors (Nagashima *et al.,* 2004). The sigma sub-units mediate promoter recognition (Yao *et al.,* 2003). A phylogenetic analysis for σ factors identified so far in plants shows that plant σ factors are members of bacterial σ^{70} family and these factors are encoded in nuclear genome, expressed in cytosol and transported into plastid (Reviewed by Toyoshima *et al.,* 2005). Fujiwara *et al* (2000) noted PEP transcribes most photosynthesis genes. Phylogenetic analysis divided σ factors into four distinct clusters (Toyoshima *et al*., 2005). Cluster I is composed of Sig1 and Sig4 groups. The Sig2 and Sig3 are members of cluster II. Cluster III is a group of Sig6 and cluster IV is a group of Sig5 (Toyoshima *et al*., 2005).

Arabidopsis thaliana has six σ factors, SIG1-SIG6 encoded by nuclear *SIG1-SIG6* genes respectively. The last three genes *SIG4-SIG6* (designed as *sigD, sigE* and *sigF* in original study) were identified by Fujiwara and co-workers (2000). Among these six σ factors, SIG5 is unique. As mentioned above, this sigma factor does not share a cluster with other SIG factors in phylogenetic tree (Fujiwara 2000; Toyoshima 2005). The initial studies in SIG5 were conducted in relation to light perception. Recent studies carried out by Nagashima and co-workers (2004) showed SIG5 is also induced by low temperature, high salt and high osmotic stress. The authors conclude that this sigma factor is induced by multiple stress conditions (Nagashima *et al.,* 2004). Experiments conducted under white, blue and red light showed that *SIG5* is induced by blue light, not by red light (Tsunoyama *et al.,* 2002). In their experiments, all the *SIG* transcripts were accumulated in rosette leaves of 4 weeks-old *Arabidopsis thaliana* under growth conditions of 10-20 μmol $m^{-2}s^{-1}$ white light. Increasing light intensity to 100 μmol $m^{-2}s^{-1}$ enhanced accumulation of *SIG5* transcripts. *SIG5* transcripts also accumulated under blue-light but never in red-light. Moreover, this group showed evidence of a correlation between *SIG5* and *psbD*-BLRP transcripts accumulation under

blue-light illumination. However, *SIG5* and *psbD*-BLRP differ in intensity requirement (Tsunoyama *et al*., 2002). Other experiments carried by Onda *et al* showed *SIG2*, *SIG3*, *SIG4* and *SIG6* transcripts increased slowly and were lower than *SIG5* in blue-light illumination (Onda *et al*., 2008). Moreover, this group of researchers provide evidence that *SIG5* transcript was not expressed strongly in red-light illumination compared to *SIG1*. Experiment in blue-light showed *SIG5*-induction in *Arabidopsis thaliana* is mediated by cryptochrome rather than phototropin (Onda *et al*., 2008).

As stated above, Tsunoyama and co-workers (2002) revealed that there is correlation between *SIG5* and *psbD*-BLRP. The evidence of the activation of *psbD*-BLRP also was provided by Nagashima *et al* (2004), who reported that *psbD*-BLRP tanscripts were lost in a mutant deficient in *SIG5*. These findings showed that *SIG5* is required to activate *psbD*-BLRP. The *psbD*-BLRP is one of the *psbD* promoters that regulates D2 protein accumulation in PSII Reaction Centre. Interestingly, *SIG5* also recognized *psbA* gene that encodes D1 protein of PSII RC.

There are four conserved regions in eubacterial σ^{70} family. Among those four regions, region 2 and 4 are highly conserved (Toyoshima *et al*., 2005). Region 2 is divided into five subdomains, 2.1 to 2.5 whilst region 4 is divided into subdomain 4.1 and 4.2. Onda and co-workers (2008) provided evidence that Asn484 in the conserved region 4.2 in *Arabidopsis thaliana* was required to activate *psbD*-BLRP, whilst Arginine 493 is involved in *psbA* recognition. Although there is evidence of correlation between *SIG5, psbA* and *psbD*-BLRP, to date no report has been done to investigate the role of SIG5 in transcript level of *psbA* and *psbD*-BLRP and protein level of D1 and D2 under UV-B illumination.

In order to investigate SIG5, several mutants have been employed by researchers. The *sig5-1* (ecotype WS) and *sig5-2* (ecotype Columbia) mutants are the first isolated AtSig5 mutants (Yao *et al.,* 2003) as shown in Figure 1.4 (A). The *sig5-1* mutant has a T-DNA insertion at exon 5 which would generate SIG5 lacking conserved regions 4 and 3. The *sig5-2* (ecotype Columbia) mutant has a T-DNA insertion at exon 2 that would generate SIG5 missing all conserved regions needed to activate bacteria sigma factors (Yao *et al*., 2003) **(Figure 1.4 (A))**. The authors reported that the

disruption in SIG5 caused embryonic lethally. The failure to recover homozygous mutants after SIG5 disruption led the authors to speculate that *SIG5* acts in plant reproduction (Yao *et al*., 2003; Nagashima *et al*., 2004; Tsunoyama *et al*., 2004). In contrast to Yao *et al* (2003), Nagashima and co-workers succeeded in isolating the *sig5-2* homozygous mutant **(Figure 1.4 (B))**. The authors confronted Yao and co-workers' work and stated that Yao's result may come from unknown elements during experiments.

The *sig5.1* mutant (ecotype Columbia) is a knock out Arabidopsis *SIG5* mutant with a T-DNA insertion in the last exon of *SIG5* (Tsunoyama *et al*., 2004). The phenotype of *sig5.1* mutant is identical to wild type under normal growth condition. Nucleotide sequencing revealed that insertion of T-DNA is located 1,931 bp downstream from initiation site. This mutant failed to show *psbD*-BLRP induction. Further, RNA analysis provided evidence that *psbA* and *rbcL* transcripts were decreased slightly. The authors concluded that SIG5 is specifically required to activate *psbD*-BLRP. Tsunoyama and co-workers also provide evidence that expression of *SIG5* correlated with development stage of chloroplast.

A.

B.

Figure 1.4.

As mentioned above, the initial studies of SIG5 were conducted in relation to blue-light-mediated responses. In addition to these studies, Nagashima and co-workers (2004) provide evidence that SIG5 is also induced by multiple stress condition such as salt, osmolality and low temperature. No report has been made that SIG5 also induced by UV-B until Brown *et al* (2005) showed evidence that *SIG5* transcripts were expressed after UV-B radiation. Moreover, Brown *et al* (2005) demonstrated that expression of *SIG5* transcripts was detected weakly in *uvr8* mutant compared to wild type. This finding suggested that *SIG5* is regulated under UVR8 pathways, which acts specifically in low fluence rate of UV-B.

To gain insight knowledge in UV-B perception and transduction, correlation between multiple-stress responsive SIG5 with UVR8 was examined in this study. The *uvr8-1*, *sig5.1* and *sig5-2* mutants were used. All data will be presented in chapter 3.

1.1.3.2. Early Light-Inducible Proteins (ELIPs)

The Early Light-Inducible Proteins (ELIPs), as described by Heddad *et al* (2006), are nuclear-encoded proteins that accumulate in thylakoid membranes and are related to light-harvesting chlorophyll *a*/*b*-binding proteins (LHC Cab). The ELIPs initially are synthesized as pre-protein in the cytoplasm, translocated into the chloroplast and inserted in thylakoid membranes (Adamska and Kloppstech, 1994; Casazza *et al.,* 2005; Rossini *et al.,* 2006). ELIPs have three transmembrane domains and their central helices have similar sequence to LHC Cab proteins (Grimm *et al.,* 1989; Adamska and Kloppstech, 1994; Hutin *et al.,* 2003; Casazza *et al.,* 2005). Both ELIPs and LHCs bind chlorophyll and carotenoid. Even though ELIPs and LHCs have similar sequence and bind to pigments in photosynthetic system, they differ in protein structure (Adamska and Kloppstech, 1994). Hutin and co-workers (2003) noted differences between ELIPs and LHCs is in the expression under high light condition. *ELIP* is expressed transiently under high light, whilst *LHC* is not.

Initial studies of ELIPs were carried out in pea (*Pisum sativum* L.) and barley plants. Studies in etiolated pea and barley plants revealed transcription of ELIPs is regulated by phytochrome (Adamska and Kloppstech, 1994). In mature pea and barley plants transcription and accumulation of ELIPs protein are regulated by the well known photoreceptor, cryptochrome which is activated strongly by blue and UV-A light (Adamska and Kloppstech, 1994). Further, study in pea revealed that both transcript and translation of ELIPs were not detectable in leaves exposed to red/far-red (Adamska *et al.,* 1992a) and UV-B (Adamska *et al.,* 1992b). Moreover, Adamska and co-workers (Adamska *et al.,* 1992b) provided information that transcription of ELIP under UV-B was only detected in the presence of white light. UV-B alone failed to induce ELIP. The authors also revealed addition of herbicide that blocked carotenoid synthesis enhanced ELIP accumulation but did not affect *ELIP* transcription (Adamska *et al.,* 1992b). According to these findings Adamska and Kloppstech (1994) noted UV-B does not induce ELIP transcription but prevents its degradation. The authors conclude that UV-B acts at more than one point of regulation of ELIP (Adamska and Kloppstech, 1994).

ELIPs are distributed in various plants e.g. pea, barley, wheat, tomato, tobacco and beans (Adamska and Kloppstech, 1994). Studies of ELIP-like proteins showed that these proteins also have been found in algae and ferns (Adamska and Kloppstech, 1994). Chen *et al* (2008) recently succeed in cloning a putative carotene biosynthesis related (*cbr*) gene from algae *Dunaliella salina*. CBR is homologous to ELIP-like protein in higher plants. In *Arabidopsis thaliana* there are two types of ELIP genes i.e. *ELIP1* and *ELIP2,* which encode two ELIP proteins ELIP1 and ELIP2 respectively (Casazza *et al.,* 2005).

Localization studies of ELIPs in thylakoid membranes of pea showed ELIPs were localized in stroma thylakoids and the intermediate fraction (Adamska and Kloppstech, 1994). Heddad and co-workers (2006) showed both ELIP1 and ELIP2 in *Arabidopsis thaliana* were found in isolated mLhcb and tLhcb but in different LHCII sub population.

ELIPs are only detectable when mature plants are exposed to a number of environmental conditions (high light, UV radiation, cold, salt stress, nutrient

deprivation, senescence) that inhibit photosynthetic activity (Casazza *et al.,* 2005). ELIPs are accumulated transiently when plants are exposed to high light condition. As mentioned briefly in the previous section there is evidence that ELIPs interact with D1 protein in higher plants. Adamska and Kloppstech (1991) reported D1 protein is one of ELIP crosslinking products. Study in *Arabidopsis thaliana* showed that the amount of ELIP1 accumulated linearly with increasing light intensities and photoinhibition (Fv/Fm measurements) whilst ELIP2 started to accumulate massively when photoinhibition reached 40% level (Heddad *et al.,* 2006). The authors concluded that under high light-stress condition, ELIP1 and ELIP2 protein in *Arabidopsis* respond differentially and these responses are regulated at the transcript level. Moreover, the responses are also related to photodamage of PSII (Heddad *et al.,* 2006).

The physiological function of ELIPs is still not clear yet. Since ELIPs were found induced and stable under light stress conditions, ELIPs were proposed as photoprotective proteins (Adamska and Kloppstech, 1994). Numerous studies in *Arabidopsis* (Hutin *et al.,* 2003), pea, barley, and tomato (Reviewed by Adamska and Kloppstech, 1994) revealed that ELIPs may function in photoprotection against light stress. Particularly in *Arabidopsis thaliana*, Hutin *et al* (2003) provided evidence that ELIP acts as a photoprotective protein. They succeeded in generating an Arabidopsis mutant called *chaos*. This mutant was lacking cpSRP43, a sub-unit of the cpSRP (signal recognition particle) complex (Hutin *et al*., 2003). Even though the *chaos* mutation was specific to LHCs, this group of researchers used this mutant in ELIPs study. They referred to previous study that cpSRP pathway was used to insert ELIPs into thylakoid membranes (Hutin *et al*., 2003).

In contrast to previous researchers and Hutin *et al* (2003) particularly, Rossini and co-workers (2006) observed that light induction of ELIP1 and ELIP2 in *Arabidopsis* did not affect either photoinhibition or photooxidative stress. This finding led to novel possibilities that ELIPs may not serve as a photoprotective protein. The authors suggested that further research was needed to assess their hypothesis.

Bruno and Wetzel (2004) reported that *ELIP* mRNA accumulates during the earliest transition process from chloroplast to chromoplast in tomato fruit (*Lycopersicon*

esculentum Mill. cv. Rutgers). The authors concluded ELIP may play a role in chloroplast-to-chromoplast transition process. Bruno and Wetzel (2004) also noted there were some reports about the role of ELIP in drought-stress tolerance.

As mentioned previously, Adamska and co-workers (1992b) provided evidence that UV-B did not induce ELIPs in pea plants. Studies in *Arabidopsis thaliana,* so far, were conducted under high white light conditions. To date, no reports have been proposed in regard to UV-B radiation of ELIPs in *Arabidopsis thaliana*. Recent experiment carried out by Brown and co-workers (2005) showed that transcript level of *ELIP1* was detected in wild type plants of *Arabidopsis thaliana* subjected to UV-B. Interestingly, *ELIP1* was not expressed in Arabidopsis mutant that fails to induce *CHS* gene and other UV protection genes. The mutant, called *uvr8*, is deficient in UVR8 protein. The finding indicates that *ELIP1* is regulated under UVR8 pathways. Profound experiment in UVR8 pathway studies proposed that expression of *ELIP1* is influenced by HY5 or HYH transcription factors (Brown and Jenkins, 2008).

Although there was evidence that ELIPs interact with D1 protein in PSII Reaction Centre (Adamska and Kloppstech, 1991; Heddad *et al.,* 2006) and are involved in protection against photooxidative stress (Hutin *et al.,* 2003) and other suggested functions (Bruno and Wetzel., 2004) the physiological role of ELIPs is still unclear. To date, little is known about UV-B effects on ELIPs in *Arabidopsis thaliana*. Moreover, no report has been made for investigate the correlation between UVR8 protein, ELIPs and their roles in photosynthetic activity under UV-B radiation.

To gain insight of the potential role of ELIPs in *Arabidopsis thaliana*, several mutant deficient in ELIPs were identified. The Arabidopsis *elip1/2* mutant characterized by Rossini and co-workers (2006) was obtained by crossing *elip1* and *elip2* single mutants **(Figure 1.5)**. As described by Casazza and co-workers (2005), the *elip1* line consisted of two lines 691E05 and 369A04 carrying T-DNA insertion in *ELIP1* gene. The lines 252D03 and 292H03 were carrying T-DNA insertion in *ELIP2* gene (Casazza et al., 2005).

Figure 1.5.

The *elip1/2* double mutant had been assessed in several light stress condition but not in UV-B (Rossini *et al*., 2006). Here we employed this mutant to assess whether the lack of ELIPs proteins affects photosynthetic activity under UV-B illumination. Several approaches were conducted e.g. UV-B sensitivity assay, transcript and F_v/F_m measurements.

1.1.4. The genes encoding PSII RC core proteins: *psbA* and *psbD*

1.1.4.1. *psbA*

The *psbA* gene encodes the D1 protein in PSII Reaction Centre. This gene is transcribed by PEP, a member of a eubacterial-type enzyme. In higher plants, the *psbA* promoter contains conserved region -35 and -10 element and a TATA motif element (Nickelsen and Rochaix, 1994).

In cyanobacteria *Gloeobacter violaceus* PCC 7421 there is a family of five *psbA* genes. These five genes encode three isoform variants of D1 protein (Sicora *et al*., 2008). When *Gloeobacter violaceus* PCC 7421 cells were exposed to supplemental UV-B or high light irradiance, PSII activity was inhibited. Parallel to this result, the amount of PsbA protein (D1) was reduced to 50%. In contrast, there was no evidence that the amount of PsbA protein declined under high light irradiation (Sicora *et al*., 2008). In transcripts level, the amount of *psbA* transcripts decreased in UV-B radiation compared to standard growth condition, except for *psbAIV*. However, the response in transcripts level varied for each member of *psbA*. The authors suggested that the *psbA* gene family in *Gloeobacter violaceus* PCC 7421 responds differentially to UV-B and high light (Sicora *et al*., 2008).

The *psbA* gene study in pea leaves was reported by Kettunen and co-workers in 1997. When a leaf was shifted to photoinhibitory light (2000 μmol $m^{-2}s^{-1}$, 20°C) from growth light condition, Fv/Fm values were reduced but there was no indication of loss of the amount of D1 protein. Related to this measurement, the D1 synthesis measurement showed rapid synthesis of D1 protein during the photoinhibitory period. The authors concluded that during the photoinhibitory period, D1 was synthesized rapidly to replace the damaged ones. However, the rate of repair mechanism was lower than inhibition and thus Fv/Fm showed a decrease. The idea of rapid turnover was supported by mRNA assays. The *psbA* transcripts increased in the photoinhibitory period. Further, the authors examined thylakoid-associated *psbA* mRNA. The result showed that the increase in *psbA* transcript was accompanied by the increase in

translation initiation and docking of *psbA* mRNA ribosome to thylakoid membrane (Kettunen *et al*., 1997).

In their review, Nickelsen and Rochaix (1994) wrote that transcription of the *psbA* gene needs involvement of bacterial-type RNA polymerase recognized by its sigma factors. A recent study of *psbA* gene regulation in *Arabidopsis thaliana* provided information that *psbA* promoter is recognized by plastid sigma factors. Onda *et al* (2008) wrote that plastid sigma factors SIG1, SIG2, SIG5 and SIG6 recognized the *psbA* promoters. So far, how these plastid sigma factors regulate *psbA* transcription is not clear. Moreover, no report has been made of *psbA* transcript regulation under UV-B radiation. Assuming that SIG5 recognizes both *psbD*-BLRP and *psbA* promoters and their correlation to D2 and D1 protein activity in PSII of *Arabidopsis thaliana*, transcript level of *psbA* will be assessed in this study in relation to UV-B signalling pathways.

1.1.4.2 *psbD*-Blue Light Responsive Promoter (*psbD*-BLRP)

As mentioned briefly in the previous section, the D2 protein in PSII is encoded by the *psbD* gene. Together with *psbC* gene that encodes CP43 in PSII Reaction Centre, the *psbD* forms a *psbD/psbC* operon. Nickelsen and Rochaix (1994) wrote in their review paper that at least three different promoters transcribe this operon. One of these promoters is strongly regulated by blue light and has an unusual and complex structure. This unique promoter is called *psbD*-Blue Light Responsive Promoter (*psbD*-BLRP). Hoffer and Christopher (1997) reported that activation of *psbD* mRNA in *Arabidopsis thaliana* was initiated from three different positions, i.e -550, -190 and -950 bp upstream from translational start codon. The -950 bp position has conserved nucleotide sequence of Blue-light responsive promoter as found in barley (*Hordeum fulgare*). This finding agreed to previous studies that one of the *psbD* promoters is strongly regulated by blue-light. Further study in wheat revealed that there are four different promoters of transcript initiation sites of *psbD* (Nakahira *et al*., 1998).

Generally the plastid-encoded RNA polymerase (PEP) recognizes -10 and -35 conserved elements of plastid eubacterial-σ^{70} promoters. Unlike other chloroplast promoters recognized by PEP, *psbD*-BLRP lacks a functional -35 element (Tsunoyama *et al.*, 2004). The well known blue light photoreceptors, Chryptochrome1 (Cry1) and Cryptochrome2 (Cry2), are required to co-activate *psbD*-BLRP (Thum *et al.*, 2001). Further, Thum and co-workers showed there was no evidence of the involvement of a transcription factor HY5 in this process. In fact, this promoter is not only stimulated by blue light, but also by UV-A light (Christopher and Mullet, 1994). A mixture of red and blue light also has been reported to enhance activation of *psbD*-BLRP transcript (Tsunoyama *et al.*, 2002). In addition to these studies, Mochizuki and co-workers (2004) revealed two independent light signals cooperate in activation of *psbD* BLRP. Blue light was perceived by cryptochrome to regulate *SIG5* which then activated the *psbD* BLRP.

Recent studies carried out by Nagashima and co-workers (2004) showed evidence that *psbD*-BLRP also responds to several stress condition such as high salinity, osmolality and low temperature. These responses are parallel to the level of *SIG5*. Thus, it was sensible to conclude that activation of *psbD*-BLRP under multiple stress experiment requires *SIG5*. Studies in *sig5* mutants showed reduction in activation of *psbD* BLRP due to the loss of *SIG5*. As reported by Tsunoyama (2004), *psbD* BLRP transcripts in Arabidopsis *sig5.1* mutant were lower than wild type when plants were exposed to high light condition.

In 1998, Nakahira and co-workers reported endogenous oscillators (circadian clocks) mechanism controlled the level of mRNA of *psbD* BLRP. This phenomenon was found in wheat (*Triticum aestivum*). Since *psbD* BLRP transcribed D2 protein in PSII Reaction Centre, the authors speculated that the circadian oscillation may control D2 protein synthesis (Nakahira *et al.*, 1998).

Other study in relation to *psbD*-BLRP and *psbA* was done by Baba and co-workers (2001). They found a novel protein called plastid transcription factor1 (PTF1). This protein is a chloroplast DNA binding protein (Baba *et al*., 2001). The Arabidopsis mutant deficient in PTF1 protein lost activity of *psbD*-BLRP (stated as psbD LRP in

original paper) but not in *psbA*. The author suggested that this protein is involved in transcription of *psbD* promoter.

Despite much research on *psbD*-BLRP, little is known about regulation of *psbD*-BLRP transcript in UV-B illumination. Recently, Brown and co-workers (2005) identified UV-B specific signalling component, called *Arabidopsis thaliana* UV-Resistant locus 8 (UVR8). Interestingly, the microarray study showed that UVR8 also regulates *SIG5* gene. The *uvr8* mutants showed less expression of *SIG5*. The authors suggested that UVR8 is involved in regulating photosynthetic genes. So far, no research has been reported on the pathway of *SIG5*, *psbD*-BLRP and D2 protein under UV-B radiation.

1.1.5. UVR8 and its role in UV-B signalling

Extensive research in light-signalling and perception have established photoreceptors which mediate different responses to different wavelengths. Phytochrome perceives Red/Far Red (R/FR) light, whilst cryptochrome and phototropin strongly absorb blue/UV-A light (Ulm and Nagy, 2005). So far no specific UV-B photoreceptor has been identified. In attempts to identify UV-B photoreceptors, numerous groups were working with different mutants and suggested different possible pathways but the UV-B photoreceptor remains unknown. Perhaps this is caused by the complexity of UV-B perception and signalling systems.

At present, many mutants had been generated and showed hypersensitivity to UV-B. Most of these mutants are altered in phenolic or flavonoid compounds as described in the previous section (Li *et al.*, 1993; Lois and Buchanan, 1994; Landry *et al.*, 1995; Liu *et al.*, 1995; Landry *et al.*, 1997; Booij-James *et al.*, 2000). Some of these mutants had alteration in *CHS* gene. This gene has been studied widely in defence mechanism against UV radiation. As mentioned before, CHS is a key enzyme in biosynthesis of flavonoids, which have an ability to protect plants from UV-B damage since they strongly absorb UV radiation.

The *Arabidopsis UV resistance locus 8-1* (*uvr8-1*) mutant, firstly characterized by Kliebenstein *et al* (2002) showed hypersensitivity to UV-B. The homozygous *uvr8-1* mutant was obtained after four rounds of outcrossing to the wild type (Lansberg *erecta* ecotype) *TT5* (Kliebenstein *et al.,* 2002). The parental *tt5* line is deficient in chalcone isomerase (Li *et al*., 1993; Kliebenstein *et al*., 2002). Furthermore, the *uvr8-1* mutant contains a single recessive mutation at the bottom of chromosome 5. The *uvr8-1* allele contains a 15-nucleotide deletion in a gene similar to the human guanine nucleotide exchange factor Regulator of Chromatin Condensation 1 (RCC1) (Kliebenstein *et al.*, 2002). The predicted UVR8 protein shares 50% similarity to the RCC1 family proteins (Kliebenstein *et al.,* 2002). However, RCC1 and UVR8 differ in function (Brown *et al*., 2005; Cloix and Jenkins, 2008). Mutation in *uvr8-1* alters phenylpropanoid metabolism and blocks induction of CHS protein. The *uvr8-1* mutant also reveals that it is not deficient in antioxidant defence (Kliebenstein *et al.,* 2002). The discovery of this mutant led to research to gain insight into UV-B signalling.

Figure 1.7.

Brown *et al* (2005) found that *CHS* induction was impaired in all mutants lacking in UVR8 protein. The impairment detected was specific to UV-B illumination and not mediated by cryptochrome 1 and phytochrome A photoreceptors (Brown *et al.*, 2005). This result suggested that *Arabidopsis* UV Resistance Locus 8 (UVR8) protein is a specific UV-B signalling component. Moreover, UVR8 also regulates transcription of

HY5 (Brown *et al*., 2005). At least 50% of genes regulated by UVR8 are also regulated by HY5 (HYPOCOTYL ELONGATION5), a bZIP transcription factor. The *hy5* mutant is sensitive to UV-B similar to *uvr8* mutant. This finding implied that HY5 also is needed in UV-B protection (Brown *et al*., 2005). Related to this finding, Ulm and Nagy (2005) reported that HY5 is regulated under UV-B by unknown signalling pathways in the long region of wave length (300-320 nm).

To obtain insight knowledge in UVR8, several approaches have been done. Initial study in UVR8 (Brown *et al.*, 2005) demonstrated that this protein is located in nucleus and associates with chromatin via histones. Further studies of UVR8 localization revealed that this protein is distributed abundantly in whole part of *Arabidopsis thaliana* such as leaves, root, rosette, stem and silique (Kaiserli and Jenkins, 2007). The abundance of this protein also had been assessed in different wavelength and fluence rate of light and the result established that accumulation of UVR8 protein is not dependent on fluence rate and wavelength (Kaiserli and Jenkins, 2007). This study demonstrated that UV-B stimulated relocation of UVR8 from cytoplasm to nucleus. Further, activation of UVR8 in nucleus still requires UV-B (Kaiserli and Jenkins, 2007). The authors concluded that UV-B promotes activation of UVR8 both in cytoplasm and nucleus (Kaiserli and Jenkins, 2007).

In addition to previous study (Brown *et al*., 2005), recent study of the interaction between UVR8 and chromatin showed that native UVR8 associated with chromatin in vivo (Cloix and Jenkins, 2008). This association does not require UV-B. The experiment also showed that UVR8 interacts with chromatin principally via histone H2B. Chromatin immunoprecipitation (ChIP) assays showed UVR8 associated with chromatin in *HY5* promoter region (Brown *et al*., 2005). In addition to this result, Cloix and Jenkins (2008) revealed that other regions of *HY5* gene are associated with UVR8 and binding is not restricted to the *HY5* promoter. Further, UVR8 interacted with chromatin of several regions of some UVR8-regulated genes (Cloix and Jenkins, 2008).

In a recent study of UVR8 and UV-B signalling pathways, Brown and Jenkins (2008) proposed several distinct pathways in which UV-B can stimulate gene expression. The UVR8-dependent pathway is regulated in low level of UV-B and

regulates UV-B protection. Genes regulated by UVR8 are also regulated under control of HY5 as reported in previous study (Brown *et al*., 2005). The model proposed by Brown and Jenkins (2008) showed that *CHS, ELIP1* and *CRYD* genes are regulated by UVR8 and HY5 transcription factor. Other UVR8-dependent genes i.e. *GPX7, WAKL8* and *SIG5* need both HY5 and HYH. HYH is bZIP transcription factor which has similar sequence to HY5 (Brown and Jenkins, 2008). The experiments using *hy5*, *hyh* and *hy5 hyh* double mutants showed HY5 is more important than HYH (Brown and Jenkins, 2008). The authors also proposed overlapping role of HY5 and HYH in UV-B regulation pathways.

The other side of UV-B signalling pathway is the UVR8-independent pathway. The UVR8-independent pathway was found to be regulated under higher level of UV-B (Brown and Jenkins, 2008). The genes regulated under this pathway include *WRKY30*, *FAD oxidored* and *UDP gtfp*.

As mentioned above, a previous study (Brown *et al*., 2005) revealed that UVR8 mediated expression of genes concerned with UV-B defences. Initial microarray study established that at low level of UV-B, UVR8 protein regulates approximately 72 UV-B-induced genes (5% False Discovery Rate), some of them are flavonoid-related genes and some are genes that encode chloroplast proteins including ELIPs and SIG5. Further study by Brown and Jenkins (2008) also showed that the UVR8-dependent pathway regulates expression of *ELIP1* and *SIG5*. This result suggested that UVR8 may affect photosynthetic activity (Brown *et al*., 2005) related to *ELIP1* and *SIG5*.

Despite many studies in UV-B and photosynthesis, little is known about UV-B signalling in photosynthetic pathways. Most research only showed a correlation between flavonoids level and sensitivity to UV-B. Interestingly, no research has been conducted in order to understand how UVR8 regulates the chloroplast genes and the photosynthesis apparatus, particularly D1 and D2 proteins. These questions will be addressed in this study.

1.2. The objectives of the study

The importance of D1/D2 proteins in responses to light-stress conditions, both in higher plants and cyanobacterium, is known (Shipton and Barber, 1991; Barbato *et al.*, 2000; Booij-James *et al.*, 2000; Ferjani *et al.*, 2001; Sicora *et al.*, 2008). The damage effects of UV-B are also well reported. The fact that no UV-B photoreceptor has been discovered has established a wide range of research in UV-B responses. Furthermore, the discovery of UVR8 in UV-B responses, may give insight in UV-B signalling studies. As mentioned before, experiments (Brown *et al.*, 2005) showed that this specific protein also controls genes for chloroplast protein e.g. *SIG5* and *ELIP*s. The *uvr8* mutant fails to induce *CHS* gene and is very sensitive to UV-B. According to Brown *et al* (2005) UVR8 may play an important role in photosynthetic activity. This hypothesis so far has not been investigated. Here, we hypothesize that UVR8 plays an important role in the regulation of photosynthetic activity, in particular D1 and D2 proteins. In relation to *ELIP1* and *SIG5*, transcript level of these genes also was measured.

In order to asses the hypothesis, several approaches were used using *uvr8-1* mutant (Lansberg *erecta* ecotype), *sig5.1, sig5-2* and *elip1/2* double mutant (Columbia ecotype). First, UV-B sensitivity assays were conducted with mutants under UV-B illumination. Molecular investigation was also done. In this approach, transcript and protein measurements were used. In transcript measurement, gene expression was analysed using Semi-quantitative Reverse-Transcriptase Polymerase Chain Reaction (sqRT-PCR) with specific primers for each gene of interest. Expression of *ACTIN2*, *SIG5*, and *ELIP1* were measured in plants subjected to different level of UV-B. In order to focus on photosynthetic apparatus, transcript and protein levels of D1 and D2 proteins were observed. In transcript level, Semi-quantitative RT-PCR on *psbA* and *psbD*-BLRP genes was conducted. The western blotting method was applied to analyze protein level of D1 and D2 proteins, using specific antibody against D1 and D2 proteins (Agrisera). Another approach to investigate UVR8 regulation in photosynthetic activity is by measuring PSII activity i.e. Fv/Fm, NPQ and Phi PSII. The measurements focused

on Fv/Fm values. Measurements of PSII activity were done in collaboration with Dr. Matthew Davy (University of Sheffield, UK).

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Plant materials

Seeds for wt L*er*, wt Col-0, and *uvr8-1* were provided by Prof. Gareth I Jenkins' group (University of Glasgow, UK). The *sig5.1* seeds were obtained from Takashi Shiina, Ph.D (Laboratory of Applied Biology, Kyoto Perfectural University, Shimogamo, Sakyo-ku, Kyoto, Japan) and *sig5-2* seed were derived from Kan Tanaka (Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan). The *elip1/2* seeds were obtained from Prof. Carlo Soave (Dipartimento di Biologia, Università degli Studi di Milano, Italy).

2.1.2. Chemicals

All the chemicals used in this study are commercial chemicals, purchased from SIGMA-ALDRICH Sci., FISHER SCIENTIFIC, BIORAD or stated.

2.1.3. Light measurement

White light was measured using *LI-COR* LI-250 light meter and for UV-B using Spectro Sense (Skye Instrument Ltd, Wales,UK).

2.2. Methods

2.2.1. Plant Growth and Treatment conditions

For transcript measurement and protein analysis, wild type ecotype Landsberg *erecta* (wt L*er*) and Columbia-0 (wt Col-0) were used as control. The *uvr8-1* (ecotype L*er*), *sig5.1*, *sig5-2*, *elip1/2* (ecotype Columbia) mutants were used in all treatment.

Plants were grown on compost for 14 days in continuous white light (120 ± 25 μmol $m^{-2}s^{-1}$) at 20°C. For wt L*er* and *uvr8-1* mutant, plants were illuminated to distinct level of UV-B, i.e. 1 ± 0.2 μmol $m^{-2}s^{-1}$, 3 ± 0.5 μmol $m^{-2}s^{-1}$ and 5 ± 0.5 μmol $m^{-2}s^{-1}$ UV-B for 2 hours, 4 hours and 6 hours. For transcript analysis of *psbA* and *psbD*-BLRP, plants were exposed to 3 ± 0.5 μmol $m^{-2}s^{-1}$ of UV-B for 4 hours, 7 hours and 14 hours. For D1 and D2 protein assays, total protein was extracted from 14 hours-illuminated-leaves. The remaining genotypes (wt Col-0 and its mutants) were exposed to 3 ± 0.5 μmol $m^{-2}s^{-1}$ UV-B for 14 hours.

2.2.2 UV-B Sensitivity Assays

The UV-B sensitivity assay method was undertaken according to Dr. Bobby Brown's method (Brown *et al*., 2005) with prolongation time of exposure (personal discussion with Dr Bobby Brown, University of Glasgow, Scotland, UK). Plants were grown on compost for 12 days under continuous white light (120 ± 25 μmol $m^{-2}s^{-1}$) then exposed to white light (kept constant) with supplementary UV-B (5 ± 0.5 μmol $m^{-2}s^{-1}$) for 60 hours and 72 hours. Cellulose acetate filter was used to prevent UV-C radiation. Filter was changed every 24 hours. After each time point, plants were returned to 120 ± 25 μmol $m^{-2}s^{-1}$ continuous white light for five days to recover. Photographs were taken before treatment and after 5 days of recovery period.

2.2.3. The efficiency of photosynthesis: Fv/Fm measurements

Experiments were done in collaboration with Dr. Matthew Davy from University of Sheffield, UK. All data reported were a combination from two experiments. Measurements and statistical data analysis were under taken in collaboration with Dr. Matthew Davy.

Plants were grown on compost, one plant in each insert, for 14 days under continuous white light (120 ± 25 μmol $m^{-2}s^{-1}$). The surface of the compost was covered with black plastic beads before treatments to prevent algal growth, which would interfere with chlorophyll fluorescence imaging. 14-days-old plants were transferred to 1 ± 0.2 μmol $m^{-2}s^{-1}$ and 5 ± 0.5 μmol $m^{-2}s^{-1}$UV-B (for wt L*er* and *uvr8-1* only) and 3 ± 0.5 μmol $m^{-2}s^{-1}$ (for all plants) for 2, 4, 6, 7, 11, 14, 15, and 20 hours (duration time vary to each genotypes). Plants were adapted in the dark for 30 minutes before measurements. Actinic level was either 120 or 500 μmol $m^{-2}s^{-1}$. The saturating light white pulse was 3000 μmol $m^{-2}s^{-1}$ for 200 ms.

2.2.4. Transcript Measurement

2.2.4.1. RNA Isolation

RNA was extracted using Qiagen RNase Mini Kit. Mature leaves were harvested and ground in liquid nitrogen and decanted into 450 μl of RLT buffer. The sample was transferred to QIAshredder and centrifuged for 2 minutes at 13200 rpm. Then the supernatant was transferred to a fresh tube containing 225 μl of ethanol without disturbing the pellet. The sample was transferred to an Rneasy spin column and centrifuged at 11000 rpm for 15 seconds. The supernatant was discarded and 700 μl of RW1 buffer added to the column. The column was centrifuged at 11000rpm for 15 seconds. 500 μl of RPE buffer was added afterwards. Then the column was centrifuged

for 15 second at 1100rpm and the supernatant was discarded. 500 µl was added for the second time and the column was centrifuged at 11000 rpm for 2 minutes to wash. To dry the column membrane, the column was placed in fresh 2 ml collection tube and centrifuged at 13000 rpm for 2 minutes. Then it was placed in a 1.5 ml fresh tube and 30 µl DEPC-treated H_2O was added directly to the membrane and centrifuged at 11000 rpm for 1 minute to elute RNA.

RNA concentration was measured by spectrophotometry (SmartSpec ^{TM} 3000, BIORAD). RNA was diluted in DEPC-treated H_2O (dilution factor = 100) and absorbance was measured at λ 260 nm, 280 nm and 320 nm to know the appropriate volume to make 1 µg of cDNA. The total concentration of RNA was calculated as in the formula below (See Table 2.1 for calculation example)

$$\text{Concentration } \mu g/\mu l \text{ RNA} = (40 \times OD_{260} \times df) / 1000$$

OD_{260} = Optical density at λ 260 nm

df = dilution factor

Table 2.1. Example for RNA calculation

Sample	A260	A280	A320	A260/A280	Concentation (µg/µl)	Volume for 1µg (µl)
Wt L*er*	0.042	0.030	0.006	1.400	0.168	5.95

An appropriate volume of RNA was aliquoted to be used in DNase treatment. RNA stock was stored in -80°C.

2.2.4.2. DNase Treatment

Following RNA isolation, DNase treatment was used to eliminate contamination of genomic DNA in RNA samples. 3.5 µl 10× DNase I buffer and 1 µl DNase I (3 units, Ambion) were added to the RNA with an appropriate amount of DEPC-treated H_2O to make total volume 35 µl. The sample was incubated for 1 hour in 37°C. 5 µl of slurry (Ambion) was added and incubated for 2 minutes in room temperature. The tube was flicked 2-3 times during incubation and centrifuged at 13200 rpm for 2 minutes. DNased-RNA was stored in ice ready for further procedures.

To check whether the DNase treatment worked properly, 2.5 µl of DNased-RNA was amplified with *ACTIN2* primers using Semi quantitative RT-PCR (see section 2.4.4. for PCR method). Amplification was conducted for 35 cycles. The PCR product was run on EtBr-stained 1% agarose gel and documented in Gel-Doc imaging software (BIORAD). If DNased-RNA still has DNA contamination, the whole procedure should be repeated. For Double DNase treatment, an amount of DNased-RNA was taken and added to 0.15 × vol of 10× DNase I buffer (for example, if 30 µl of DNased-RNA was used, add 4.5 µl of 10× DNase I buffer) and 1 µl DNase I (3 units). Then procedure was repeated until DNased-RNA is free from genomic DNA.

2.2.4.3. Reverse Transcriptase Reaction (cDNA synthesis)

For cytosolic mRNA, which includes trancripts of *SIG5* and *ELIP1* genes, 0.6 µl of oligodT was added into 10 µl of Dnased-RNA sample. To ensure synthesis of cDNA from plastid mRNA (including *psbA* and *psbD*-BLRP transcripts) which does not have any poly-A tail, 2 µl of random primer (Invitrogen) was used instead. The sample was incubated at 70°C for 10 minutes and immediately cooled in ice for 1 minute. To the sample, 5 µl AMV Reverse transcriptase 5× reaction buffer (Promega), 2.5 µl of 10 mM dNTPs (1 mM final, Promega), 0.6 µl of 40 u/µl RNAse inhibitor (1 u/µl final,

Promega), 1 µl 25 mM DTT (1mM final), 1 µl of 10 u/µl AMV (0.4 u/µl final) AMV Reverse Transcriptase were added to appropriate DEPC-treated H_2O to make total volume 25 µl. The sample was incubated at 48°C for 45 minutes, then at 95°C for 5 minutes and centrifuged briefly. cDNA samples were stored in -20°C to be used in further procedures.

2.2.4.4. Semi-quantitative RT-PCR (sqRT-PCR) Reaction

2.5 µl of cDNA was used as template and added to 22.5 µl of basal mix solution consisting of 5 µl of 5 × Green GoTaq Flexi Buffer (Promega), 1.5 µl of 25 mM $MgCl_2$ (Promega), 0.5 µl of 10 mM dNTPs, 0.625 µl each of 20 µl sense and antisense Primers, 0.125 µl of 5 u/µl Taq Polymerase (Promega) and DEPC-treated H_2O to make total volume 25 µl. For negative control, 2.5 µl DEPC- treated H_2O was used instead cDNA. For positive control, 2.5 µl genomic DNA was used. Primers used in this study are:

Table 2.2. Primers were used in the study

Gene	Primers	References
ACTIN2	s : 5'-CTT ACA ATT TCC CGC TCT GC-3' a : 5'-GTT GGG ATG AAC CAG AAG GA-3'	Brown and Jenkins (2008)
ELIP1	s : 5'-GTA GCT TCC CTA ACC TCA AG-3' a : 5'- GAA TCC AAC CAT CGC TAA AC-3'	Brown and Jenkins (2008)
SIG5	s : 5'-TCCTTC GTG TTC GTT AGG AG-3' a : 5'- CAG TCC AAG CTC ACT ATA TC-3'	Brown and Jenkins (2008)
psbD-BLRP	s : 5'-GGA AAT CCG TCG ATA TCT CT-3' a : 5'- CTC TCT TTC TCT AGG CAG GAA C-3'	Mochizuki *et al* (2004)
psbA	s : 5' TTA CCC AAT CTG GGA AGC TG-3' a : 5'GAA AAT CAA TCG GCC AAA AT-3'	Wormuth *et al* (2001)

Transcripts of genes of interest were always normalized to *ACTIN2* bands. PCR reactions are amplified in number of cycles as follows:

Table 2.3. Number of cycles was used in RT-PCR reaction

Gene	Cycles	Sources
ACTIN2	24	Dr. Bobby Brown
ELIP1	24	Dr. Bobby Brown
SIG5	26	Dr. Bobby Brown
psbD-BLRP	18	Sami Khan (MRes Report, 2007)
psbA	16	See Appendix 1

PCR reactions were run under the following conditions (according to Dr Bobby Brown protocols, with modification from Chiara Tonelli's protocols, University of Milan, Italy):

Step 1: 2 minutes 30 seconds at 94°C, 1 minute at 55°C and then 2 minutes at 72°C

Step 2: 45 seconds at 94°C, 1 minute at 55°C and then 2 minutes at 72°C in appropriate number of cycles

Step 3: 5 minutes at 72°C

Step 4: forever at 10°C

2.2.4.5. Running PCR products on agarose gel

PCR products were run in EtBr-stained agarose gel. 1% agarose-TAE gel was used for *ELIP1*, *SIG5* and *psbA* transcripts as they produce bands around 400-500 bp. For *psbD*-BLRP, 2% agarose was used as this product appears around 80 bp. For example, to make 50 ml 1% agarose-TAE gel, 0.5 grams agarose powder (SIGMA) was added to 50 ml 1× TAE Buffer and solubilized by heating. 2 µl EtBr was added for every 50 ml agarose solution.

The gel was poured into a tank and left until solid. Each PCR product was loaded into a well and run with a buffer consisting of 1× TAE Buffer at 100 V. 1 kb plus marker (Invitrogen) was used as a ladder. The gel was documented using imaging Gel-Doc *Quantity One* software (BIORAD) in saturation pixel mode.

2.2.5. Protein Analysis

2.2.5.1. Protein Extraction

Total protein was extracted according to Agrisera's protocol, modified by Jane Findlay (University of Glasgow, UK). Mature leaves were harvested and ground in liquid Nitrogen to make fine powder. The powder was transferred to a 1.5 ml eppendorf tube containing 200 µl of extraction buffer (kept in ice) and frozen immediately in liquid Nitrogen (LN_2); buffer contained 140 mM Tris Base, 105 mM Tris-HCl, 0.5 mM ethylendiaminetetraacetic acid (EDTA), 2% Sodium Dodecyl Sulfate (SDS), 10% glycerol. Buffer stock was stored in a cold room (4°C) and stirred carefully before use to mix in glycerol. A half tablet of protein inhibitor (Complete mini plus, Roche) was added to 1 ml buffer. All the extraction processes were undertaken in cold room (4°C) to avoid protein degradation.

The frozen tissues were sonicated at 30 % power until just thawed using Soniprep 50 (SANYO). During sonication, the tube was placed in ice to avoid heating inside the sample. The sonicated sample was then put immediately in Liquid N_2. Before centrifugation, the tube was transferred from Liquid N_2 into ice to defrost the sample briefly (never put sample too long in ice before centrifugation). Then the sample was centrifuged in 4°C for 3 minutes at 10000 rpm. The pale colour of the pellet is an indicator of whether the cell lysis has worked properly or not. After centrifugation, the supernatant was transferred carefully to a fresh tube containing 1 M DTT to make 50 mM DTT final concentration (for example, 15 µl 1 M DTT was added to 285 µl supernatant to make 50 mM 300 µl total volume). Following the extraction processes,

the total protein extract was measured using Bradford assay against Bovine Serum Albumin (BSA) standard curve. Bradford assay was always done on the same day protein extraction was conducted.

2.2.5.2. Bradford Assay

For Bradford analysis, Bradford Reagent (BIORAD) was diluted 5-fold in demineralized-water (for example, to make 25 ml Bradford Reagent, 20 ml demineralized-water was added to 5 ml Bradford Reagent). 2, 4, 6 and 8 µl of standard BSA were pipetted into 1.5 ml eppendorf tubes. 1 ml 5-fold diluted Bradford solution was added to each tube. The solution was mixed carefully using a Gilson pipette. The solution was then transferred to a plastic cuvette and put in a spectrophotometer (WPAbiowave CO8000 Cell Density Meter) to read the absorbance. A standard curve was plotted in linear graph as in formula below.

$$Y = mX + c$$

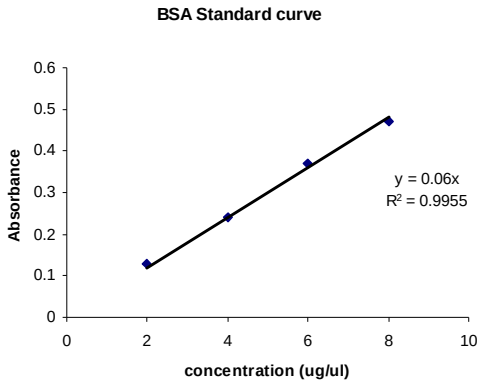
Axis Y = absorbance

Axis X = concentration

A good standard curve was achieved if the slope was linear (R^2) ≥ 0.97. For sample measurement, 2 µl protein was added to 1 ml Bradford using the same method as used in standard curve measurement. Total protein was calculated as µg/µl (Total concentration was divided by 2). For D1 analysis, 10 µg/µl of total protein was used whilst 20 µg/µl was used for D2 **(Table 2.4)**. Once total protein had been measured, some amounts of total protein were aliquoted in several tubes and stored at -80°C.

Table 2.4. Example of Bradford Assay

BSA (µg/µl)	OD
2	0.13
4	0.24
6	0.37
8	0.47



Sample	OD	Concentration (µg/2 µl)	Concentration (µg/µl)	Volume for 10 µg (µl)	Volume for 20 µg (µl)
1	**0.23**	**3.83**	**1.91**	**5.22**	**10.45**

2.2.5.3. Western Blotting and Immunodetection

An equal amount of 10 µg of total protein (for D1) or 20 µg (for D2) was added to 5 µl protein dye (**do not** heat sample) and an appropriate amount of extraction buffer to make same total volume for each sample. The samples were loaded into SDS/PAGE containing 40% Acrylamide, 1.5 M Tris Buffer, demineralized-H_2O, 10% SDS, 0.1% SDS, 10% APS (Amonium Persulfate), TEMED and 0.5 M Tris Buffer (See Appendix for complete recipe). 20 µl protein ladder (New England Biolabs) was used. The gel was run in SDS Running Buffer at 200 Volt in PAGE tank. D1 and D2 proteins appear around 28-30 kDa (Agrisera's protocols), took approximately 40 minutes to run in gel.

Following SDS/PAGE process, transfer process was performed. The gel was transferred to PVDF (Amersham Bioscience) membrane for 60 minutes at 100 Volt. The membrane was wet briefly in methanol before use. After the transfer process, the membrane was stained with Ponceau until bands appeared and then washed briefly in

demineralized water. The washed membrane was put in two pieces of plastic cover and scanned. After taking picture, membrane was block in 8% milk in TBST overnight in a cold room (4°C).

On the next day, primary antibody (D1 or D2) was diluted in 8% milk-TBST to make total concentration 1/100.000. The blocking solution was discarded and antibody solution was poured onto the membrane. The membrane was incubated in primary antibody for 1 hour at room temperature on a shaker. Then the primary antibody was removed (primary antibody can be used 2-3 times, stored at -20°C) and membrane was washed 3 times in TBSTT (5 minutes each) and once in TBST.

The washing solution was discarded and secondary antibody (anti-rabbit HRP, Promega) was added; 1/200.000 dilution in TBST 8% milk was used. The membrane was incubated for 1 hour at room temperature on a shaker. After incubation, the secondary antibody was discarded and the membrane was washed 5 times in TBSTT (5 minutes each) and once in TBS. Then the membrane was covered with ECL+ solution for 5 minutes and developed in X-Omat machine to reveal bands.

2.2.6. Data Analysis

Data from the experiments were analyzed in two different approaches. First, data were analyzed descriptively according to photographs recorded from gels (transcripts level) and western blot scan photographs (protein analysis). Ponceau and western blotting result scanning pictures were saved in TIFF files.

Second, data was analyzed quantitatively. To convert bands from photographs to quantitative value, each bands shown in Gel-Doc, Ponceau and Western Blotting photographs were quantified using *Quantity One* ® software. For transcripts level, band of gene of interest was normalized to *ACTIN2*. For protein level, band of protein was normalized to rbcL bands.

Chapter 3. Results

3.1. The *uvr8-1* mutant is very sensitive to UV-B

To observe sensitivity of plants under UV-B exposure, several types of Arabidopsis mutants i.e. *uvr8-1* (ecotype Landsberg *erecta*), *sig5.1*, *sig5-2* and *elip1/2* (ecotype Columbia) were examined according to Brown *et al* (2005) and personal discussion with Dr Bobby Brown (University of Glasgow, Scotland, UK). Plants were grown in continuous white light (120 ± 25 μmol $m^{-2}s^{-1}$) for 12 days and transferred to white light with supplementary UV-B (5 ± 0.5 μmol $m^{-2}s^{-1}$) for 60 and 72 hours then returned to continuous white light to recover for 5 days **(Figure 3.1)**. The aim of this experiment was to confirm that *uvr8-1* mutant is hypersensitive to UV-B and to compare the sensitivity of the other mutants relative to *uvr8-1* and wild type.

As shown in Figure 3.1 (A), *uvr8-1* mutant is very sensitive to UV-B. *uvr8-1* plants failed to survive after 5 days recovery period. A similar result has been demonstrated previously by Brown *et al* (2005). The sensitivity of *uvr8-1* is caused by failure to induce genes concerned with UV protection.

Figures 3.1(B) and (C) showed that *elip1/2* and *sig5* mutants are apparently tolerant to UV-B, no difference was seen in survival compared to wild type. With regard to *elip1/2* mutant, this observation is consistent with Rossini *et al* (2006) in which *elip1/2* null mutant was apparently tolerant to high light irradiance (less than 400 μmol $m^{-2}s^{-1}$). Thus our result may be added to *elip1/2* studies, that this mutant is also tolerant to UV-B.

3.2. Mutant deficient in UVR8 suffers damage to photosynthetic apparatus on UV-B exposure (measurement of the photosynthesis efficiency)

Following the UV-B sensitivity assays, the photosynthesis efficiency was measured on plants. The aim of these measurements was to investigate UV-B effects on photosynthetic apparatus, PSII particularly, and to see whether UVR8 plants were different from wild type in their sensitivity to UV-B.

The idea of Fv/Fm measurement is to provide information about PSII efficiency. When dark-adapted plants are exposed to light, PSII pigments absorb the light energy and use it to drive photochemical reactions. The basic theory of this measurement is explained in Chapter 1. Fv/Fm values of 0.7 – 0.8 indicate that plants are healthy. The decrease in Fv/Fm values indicates reduction in PSII efficiency. Statistical analysis of Fv/Fm measurements is shown in Figure 3.2. All data are from experiments undertaken with Dr. Matthew Davy (University of Sheffield, UK). Experiments were undertaken with plants grown in either 120 ± 25 μmol $m^{-2}s^{-1}$ or 140 ± 25 μmol $m^{-2}s^{-1}$ but since they gave similar results only those for 120 ± 25 μmol $m^{-2}s^{-1}$ are presented.

As shown in Figure 3.2, wt L*er* and *uvr8-1* mutant were exposed to three distinct UV-B levels i.e. 1 ± 0.2 μmol $m^{-2}s^{-1}$, 3 ± 0.5 μmol $m^{-2}s^{-1}$ and 5 ± 0.5 μmol $m^{-2}s^{-1}$ at duration stated. At low level UV-B (1 ± 0.2 μmol $m^{-2}s^{-1}$), there was no indication of photoinhibition or other disruption in photochemical activities **(Figure 3.2 (A))**. Fv/Fm values were maintained at a healthy level (0.7-0.8) both in wild type and mutant.

When plants were exposed to ambient level of UV-B, both wt L*er* and *uvr8-1* had decreased values of Fv/Fm **(Figure 3.2 (B))**. The values in *uvr8-1* mutant differ from wild type after 7 hours exposure. Significant deference between the two genotypes was shown when they were exposed to 11 and 14 hours at 3 ± 0.5 μmol $m^{-2}s^{-1}$ UV-B. This indicates photoinhibition or other damages to PSII activity occurred in *uvr8-1* mutant after 14 hours exposure whilst in wt L*er*, the damages did not occur massively. This difference in Fv/Fm can be seen in colour images of leaf fluorescence shown in Figure 3.3.

To test whether higher intensity of UV-B causes severe damage to PSII in both genotypes, plants were exposed to high level of UV-B (5 ± 0.5 μmol $m^{-2}s^{-1}$). As shown in Figure 3.2(C), severe damages were detected in plants after 14 hours exposure in both wt L*er* and *uvr8-1* mutant; Fv/Fm had decreased rapidly to 0.5 and 0.3 respectively.

These findings led to conclusion that significant damages occurred at 14 hours of UV-B at 3 ± 0.5 μmol $m^{-2}s^{-1}$, particularly in *uvr8-1* mutant. This condition then was used in further experiments to analyze photoinhibition at the molecular level. Both transcript and protein levels were studied as demonstrated in the next sections.

3.3. Transcription of genes encoding chloroplast proteins controlled by UVR8

According to Brown *et al* (2005), UVR8 significantly regulated genes that encode chloroplast proteins, e.g. *ELIP1* and *SIG5*. Further, *SIG5* is known to recognize the promoter of *psbA* and the BLRP of *psbD*, genes that encode PSII core proteins, D1 and D2 respectively. In order to obtain knowledge of UVR8 regulation pathways in transcription level of PSII core proteins, transcript measurements of *SIG5*, *ELIP1*, *psbA* and *psbD*-BLRP were assessed using Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction (sq RT-PCR) as described in Materials and Methods. This following section will be focused on analysis of genes encoded by cytosolic mRNA, i.e. *SIG5* and *ELIP1*. Transcription of *psbA* and *psbD*-BLRP will be shown in further section in correlation with protein assays of D1 and D2 proteins.

3.3.1. Transcript level of *SIG5* and *ELIP1* in wt L*er* and *uvr8-1*

Transcript levels of *SIG5* and *ELIP1* were measured in three different levels of UV-B, i.e. 1 ± 0.2 μmol $m^{-2}s^{-1}$, 3 ± 0.5 μmol $m^{-2}s^{-1}$ and 5 ± 0.5 μmol $m^{-2}s^{-1}$. Plants were grown in continuous white light (120 ± 0.5 μmol $m^{-2}s^{-1}$) and illuminated with UV-B for

duration noted **(Figure 3.4 (A))**. In all cases, transcript levels were normalized to transcripts of the *ACTIN2* gene, which was used as a loading control.

As shown in Figure 3.4 in both wt L*er* and *uvr8-1*, *ELIP1* transcripts do not appear in untreated plants. Casazza *et al* (2005) stated that ELIPs are only expressed when plants are exposed to stress environments that inhibit photosynthetic activity. Adamska and co-workers (1992b) found both transcript and translation level of ELIP were not detected in pea subjected to UV-B. However, Figure 3.4 revealed that transcripts of *ELIP1* are detectable in Arabidopsis plants subjected to UV-B. Compared to wt L*er*, expression of *ELIP1* in *uvr8-1* mutant is not detected in any condition. These results indicate that *ELIP1* transcript is controlled by UVR8. Accumulation of *ELIP1* appears in wt L*er* plants exposed to UV-B. For *ELIP1*, 1 ± 0.2 μmol $m^{-2}s^{-1}$ UV-B is sufficient to induce *ELIP1* expression and transcripts are detected 2 hours after exposure to 3 or 5 ± 0.5 μmol $m^{-2}s^{-1}$ UV-B. *ELIP1* expression is shown when wt L*er* plants were exposed to 3 ± 0.5 μmol $m^{-2}s^{-1}$ for 4, 7 and 14 hours. Accumulation of *ELIP1* transcripts in 4 and 14 hours exposure was less than 7 hour, indicating that the peak was reached when plants were exposed to UV-B for 7 hours **(Figure 3.4 (B))**.

Expression of *SIG5* gene still appears in *uvr8-1* mutant but is much less compared to wt L*er*. These findings confirm previous experiments (Brown *et al*. 2005) that UVR8 regulates *SIG5*. *SIG5* transcripts increased in 1 to 5 μmol $m^{-2}s^{-1}$ UV-B and after 2 to 14 hours illumination.

3.4. How does UVR8 regulate PSII core proteins in transcript and protein level?

As described in chapter 1, the intention of this study is to investigate UVR8 function in regulating expression of PSII core proteins under UV-B, both in transcription and translation level. In order to obtain the goals, *psbA* and *psbD*-BLRP transcript levels were examined in wt L*er* versus *uvr8-1* mutant. Transcripts of the gene of interest were always adjusted to *ACTIN2*. This section first demonstrates results in

transcript measurements on *psbA* and *psbD*-BLRP genes. Data of D1 and D2 proteins will be shown in section 3.4.2.

3.4.1. *psbD*-BLRP and *psbA* transcripts in response to UV-B

3.4.1.1. *psbD*-BLRP transcripts accumulated in response to UV-B in wild-type but less in *uvr8-1* mutant

The *psbD*-BLRP transcript was examined both in wt L*er* and *uvr8-1* mutant at different levels of UV-B for 2, 4 or 6 hours **(Figure 3.5)**. In mature leaf wt L*er* plant, *psbD*-BLRP transcripts accumulated linearly with increasing intensity and time of exposure to UV-B. These transcripts in wild type appeared stronger than in *uvr8-1* mutant relative to *ACTIN2*. In the absence of UVR8 protein, there was little change in the transcript level in response to UV-B. Since time was limited in this study, measurement at different fluence rates was not repeated.

The second type of experiment was done in triplicate. 14 days-old-plants were illuminated with UV-B at 3 ± 0.5 μmol $m^{-2}s^{-1}$ for 4, 7 and 14 hours (See Materials and Methods). Semi-quantitative RT-PCR and simple statistical analysis of quantified *psbD*-BLRP are shown in Figure 3.6 (A and C) respectively. In three different experiments, two of them were done in duplicate. In one of these experiments, one sample failed to be expressed in semi-quantitative RT-PCR. To assure there was nothing wrong in this result, the Semi-quantitative RT-PCR process was repeated. Since not enough cDNA was left, new cDNA was made and all the samples once again adjusted to obtain similar expression in *ACTIN2*. For each set of new cDNA Semi quantitative RT-PCR assessment for *ACTIN2*, *psbA* and *psbD* was taken to be calculated in statistical analysis.

The images of semi-quantitative RT-PCR showed that the absence of UVR8 impairs the UV-B induction *psbD*-BLRP transcript **(Figure 3.6 (A))**. Visual analysis of three experiments in each case shows increases of the amount of *psbD*-BLRP transcripts

in wt L*er* in response to UV-B but less effect in *uvr8-1* mutant. The *psbD*-BLRP transcripts were less in *uvr8-1* mutant compared to wt L*er*, consistent with previous result **(Figure 3.5)**.

To assess this result, imaging expression of each band was quantified using Gel Doc Quantifying method (*Quantity-One* Software, BIORAD, calculated in local background) according to Dr Helena Wade and Lauren Headland's method and personal discussion with Dr Bobby Brown (University of Glasgow, Scotland, UK). Data are presented in histogram **(Figure 3.6 (C))**. However, the quantification method has a weakness. Since bands should be subtracted to background, different background would give different normalization factor. Therefore, error bars could be quite large. However, trend of means is *psbD*-BLRP transcripts in UV-B induced wild type are higher than untreated plants. Further, the means bars showed that transcripts of *psbD*-BLRP in *uvr8-1* mutant are less compared to wild type, consistent with the imaging expressions (**Figure 3.6 (A)**). Observation focus in the error bars showed statistical differences for 7 hours treatment.

3.4.1.2. Is *psbA* regulated by UVR8 in ambient level of UV-B?

To investigate whether UV-B affects *psbA* transcripts in the presence and absence of UVR8 protein, *psbA* transcripts were measured. Since this had not been done previously in Prof. Gareth I. Jenkins' laboratory, the condition for amplification needed to be optimised. Semi Quantitative RT-PCR was undertaken using several cycles of PCR and 16 cycles chosen for all experiments **(Appendix 1)**. The measurements were done in triplicate along with *psbD*-BLRP measurements. From Figure 3.6 (A) apparently *psbA* transcripts are not affected by UV-B in both genotypes. The bands apparent had similar intensities in all lanes. To assess this result, the same approach as *psbD*-BLRP was used. The bands were quantified using *Quantity One* Quantifying method. Data are exhibited in histogram **(Figure 3.6 (B))**. Despite the weakness in the quantification method, the observation of Standard Error (S.E) at 14 hours UV-B

treatment appears to be significant difference between wild type L*er* and *uvr8-1* mutant. This difference is not seen in imaging picture of sqRT-PCR **(Figure 3.6 (A))**. However, the cause of greater accumulation of *psbA* transcripts in *uvr8-1* mutant compared to wild type at 14 hours UV-B treatment is unclear.

3.4.2. D1 and D2 Protein assays

3.4.2.1. UV-B radiation causes reduction in D1 protein

Among chloroplast proteins, D1 protein is known easily to degrade when plants are exposed to photoinhibitory light conditions. D1 also may be a target of UV-B. Fv/Fm values as shown in section 3.2 indicate reduction of PSII activity under 3 ± 0.5 μmol $m^{-2}s^{-1}$ UVB for more than 6 hours. To attain knowledge at the molecular level, western blot analysis was conducted using an anti-D1 antibody (Agrisera). Total proteins were extracted from 14-days-old-plants exposed to UV-B at 3 ± 0.5 μmol $m^{-2}s^{-1}$ for 14 hours.

Pictures shown in Figure 3.7(A) are taken from three independent experiments. As expected, bands migrated to apparent 28-30 kDa in SDS/PAGE gel. These bands corresponded to D1 protein (According to Agrisera's leaflet from whom the antibodies were purchased). In some journals, D1 protein is corresponded to 32 kDa product. Immunoblot assay showed this protein consistently declined after 14 hours illumination with UV-B both in wild type and *uvr8-1* mutant but there was much change in wild type. Two of these experiments showed that D1 protein in UV-B induced *uvr8-1* mutant have less amount compared to wild type. This indicates that in the absence of UVR8 protein, UV-B may promote increased degradation of D1 protein.

As same as in transcripts level, scan of ponceau staining and western blotting proteins were quantified and presented in histogram **(Figure 3.7(B)).** Statistical differences of Standard Error observation showed there is significant difference between UV-B and non UV-B treatment. This finding suggested that UV-B may

increase degradation of D1 protein, consistent with visual analysis **(Figure 3.7 (A))**. However, there is no significant difference between the two genotypes.

3.4.2.2. Regulation of D2 protein level is not clear yet

In an attempt to investigate D2 protein under UV-B exposure, four independent experiments were done. In each experiment, several replications were conducted. The total protein from 14-days-old *Arabidopsis thaliana* was extracted as described in Materials and Methods section. 20 μg of total protein were loaded to SDS/PAGE gel. Western blot analysis was conducted using an anti-D2 antibody (Agrisera). The cross-reacting protein had an apparent molecular mass of 28-30 kDa, as stated in the Agrisera leaflet. Unfortunately, western blotting assay of D2 protein failed to determine whether UV-B affects the level of this protein in the absence of UVR8 protein. There were variations in every experiment as shown in Figure 3.8 and more in Appendix 2.

To look for a trend in D2 regulation under UV-B, each experiment was analyzed independently. All bands of protein were quantified using *Quantity-One* Software and normalized to rbcL bands. Statistical analysis of all the experiments is presented in Figure 3.9. The histograms showed variation in each experiment which made it difficult to establish a trend.

3.5. Response to UV-B in mutant deficient either in SIG5 or ELIP1/ELIP2 proteins

As mentioned above, there is evidence that UVR8 regulates *ELIP1* and *SIG5*. To compare to UVR8 deficient mutant, experiments using mutants deficient in SIG5 and ELIP1 were conducted. Transcript levels of *ACTIN2*, *SIG5*, *ELIP1* and *psbD*-BLRP were measured for plants exposed to 3 ± 0.5 μmol $m^{-2}s^{-1}$ UV-B for 14 hours. All genes of interest were adjusted to *ACTIN2* **(Figure 3.10)**. Fv/Fm values for these mutants

exposed to either 3 ± 0.5 μmol $m^{-2}s^{-1}$ UV-B alone or plus supplementary high white light (150 ± 25 μmol $m^{-2}s^{-1}$) also were measured **(Figure 3.11)**. All data for Fv/Fm measurements were obtained in experiments with Dr. Matthew Davy (University of Sheffield, UK).

In all untreated genotypes, *ELIP1* was not expressed, as shown in Figure 3.10 (A). This behaviour is similar to that seen in wt L*er* and *uvr8-1* untreated plants **(Figure 3.4)**. When plants were transferred to 3 ± 0.5 μmol $m^{-2}s^{-1}$ UV-B for 14 hours, *ELIP1* failed to be expressed in the *elip1/2* double mutant as expected. Strongest expression was shown in *sig5.1* mutant. Whether there is relation between *SIG5* mutation in *sig5.1* mutant and the amount of *ELIP1* transcript and why *sig5-2* had different expression is still unknown. This possibility was not tested in this study.

SIG5 transcript appeared weak in untreated wild type and *elip1/2* double mutant and was induced by UV-B in both genotypes. Very weak expression of *SIG5* was detected in *sig5.1* mutant exposed to UV-B. This was an unexpected result as this mutant is a knock out *SIG5* mutant (Tsunoyama *et al*., 2004). Whether the seeds were contaminated during sowing plants or other possibilities were not assessed since there was not enough time to repeat and assess the unexpected results in this study.

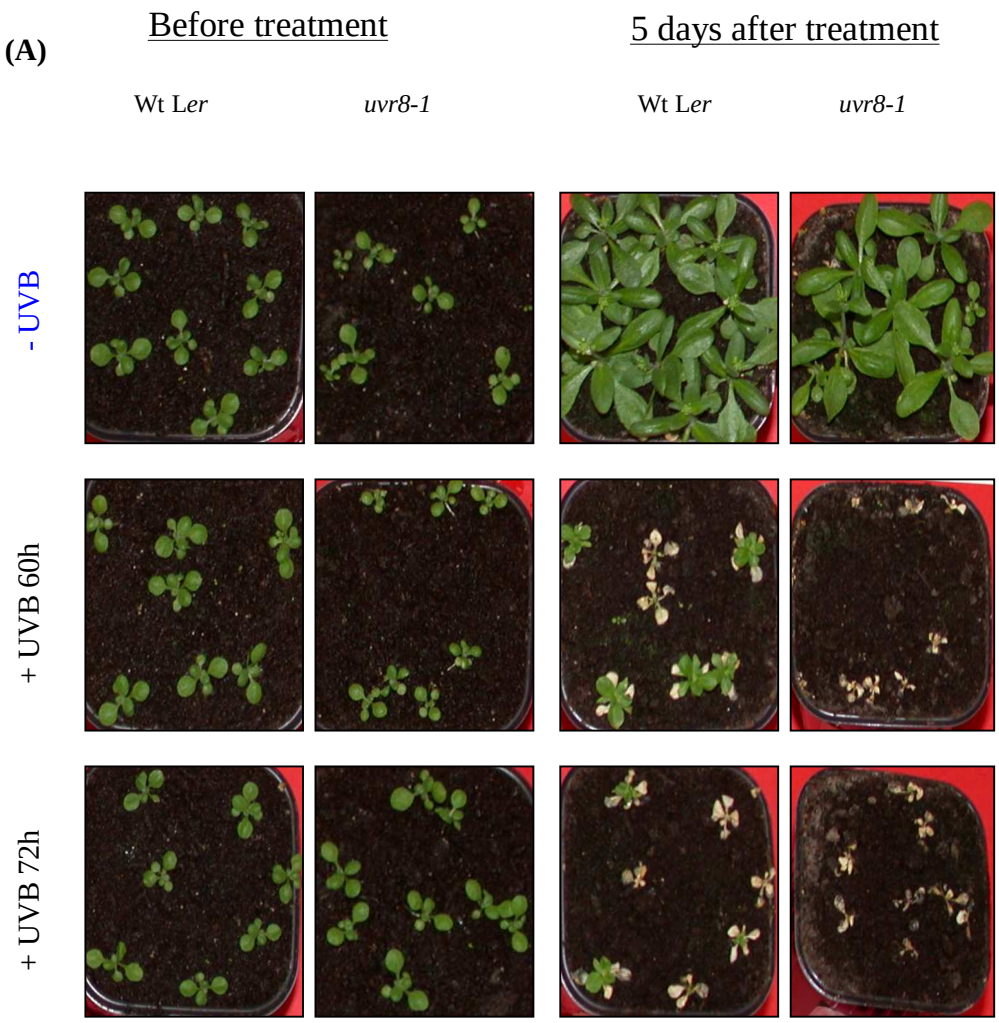
That *SIG5* mediates activation of *psbD*-BLRP gene is well documented (Mochizuki *et al*., 2004; Nagashima *et al*., 2004; Tsunoyama *et al*., 2004; Onda *et al*., 2008). To investigate whether the absence and presence of *SIG5* in different genotypes affected *psbD*-BLRP transcript in UV-B exposure, semi quantitative RT-PCR of *psbD*-BLRP was assessed in four different genotypes **(Figure 3.10 (B))**. It is clearly shown that plants which are not deficient in *SIG5* strongly induce *psbD*-BLRP gene following UV-B exposure. The *sig5* mutant plants fail to show *psbD*-BLRP induction. This indicates that *SIG5* is important in the activation of *psbD*-BLRP by UV-B.

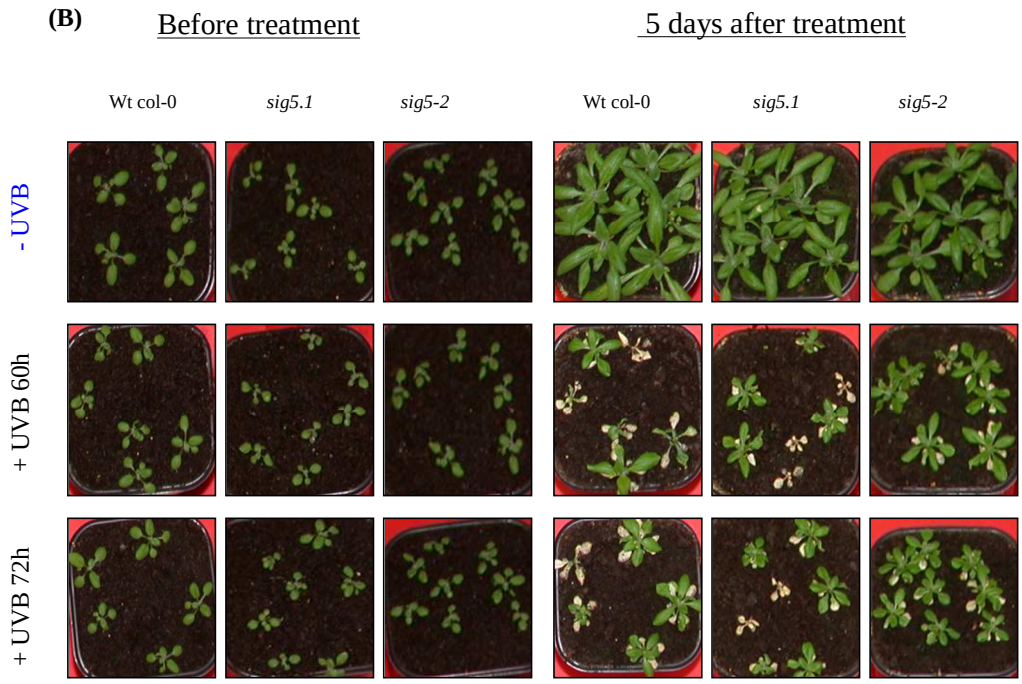
3.6. Fv/Fm measurement on Arabidopsis mutants deficient in either SIG5 or ELIP1/ELIP2 proteins

Along with transcript analysis, Fv/Fm values were measured on *sig5.1*, *sig5-2* and *elip1/2* mutants (ecotype Columbia). Statistical analyses on Fv/Fm values in mutants compared to wild type are shown in Figure 3.11. Consistent with UV-B sensitivity assay, the mutants had Fv/Fm values higher than wild type (wt Col-0) after exposure to UV-B. Apparently in these mutants photosynthetic activity was maintained properly.

In addition, to assess whether high white light also contributes to PSII activity, measurement was also done on wt col-0 and *sig5* mutants exposed to a mixture of UV-B (3 ± 0.5 μmol $m^{-2}s^{-1}$) and high white light (150 ± 25 μmol $m^{-2}s^{-1}$). The *sig5* mutants were chosen based on knowledge that *SIG5* has close relation to *psbA* and *psbD*-BLRP transcript regulation. Data are shown in Figure 3.11 (C). Compared to Fv/Fm values in Figure 3.11 (A), plants exposed to a mixture of UV-B and white light had higher Fv/Fm values. This result implied that UV-B in the presence of high white light did not impact on photosynthetic activity in *sig5* mutants in this experiment.

Figure 3.1. UV-B Sensitivity assay on **(A)** wt L*er* vs *uvr8-1* **(B)** wt Col-0 vs *sig5.1* and *sig5-2* and **(C)** wt Col-0 vs *elip1/2*. Plants were grown under continuous white light (120 ± 25 µmol $m^{-2}s^{-1}$) for 12 days and transferred to UV-B (5 ± 0.5 µmol $m^{-2}s^{-1}$) for 60 and 72 hours. After treatment plants were returned to white light to recover. Photographs were taken before treatment and after 5 days of recovery period.



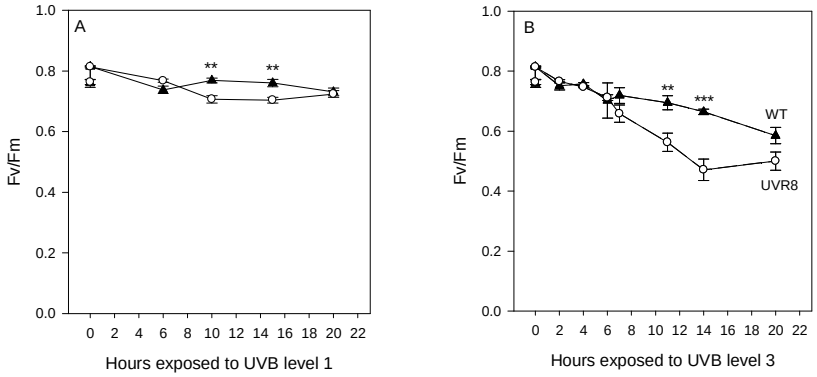

(B)
Before treatment
5 days after treatment
Wt col-0
sig5.1
sig5-2
Wt col-0
sig5.1
sig5-2
- UVB
+ UVB 60h
+ UVB 72h

(C)


Before treatment
5 days after treatment
Wt Col-0
elip1/2
Wt Col-0
elip1/2
- UVB
+ UVB 60h
+ UVB 72h

Figure 3.2. Fv/Fm values of wt L*er* (closed triangle) and *uvr8-1* (open circle) in **(A)** 1 ± 0.2 µmol $m^{-2}s^{-1}$ **(B)** 3 ± 0.5 µmol $m^{-2}s^{-1}$ **(C)** 5 ± 0.5 µmol $m^{-2}s^{-1}$ UV-B. Plants were grown in continuous white light and transferred to UV-B for duration shown. n=6 ± S.E. Statistically significant differences are indicated by * = P < 0.05; ** = P < 0.01; *** = P < 0.001. Data shown are combination from two experiments in collaboration with Dr. Matthew Davy (University of Sheffield, UK).

(A) **(B)**



(C)

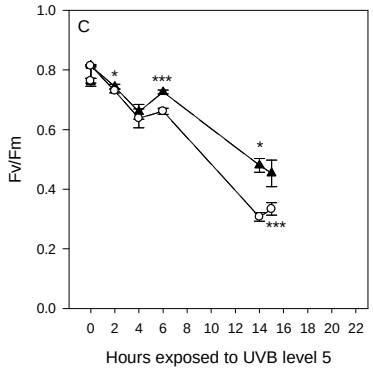


Figure 3.3. Color images of Fv/Fm measurements on **(A)** wt L*er* and **(B)** *uvr8-1* either untreated or exposed to 3 ± 0.5 µmol $m^{-2}s^{-1}$ UV-B for 14 hours. Fv/Fm values 0.7-0.8 indicate plants are healthy. Plants were dark adapted 30 minutes before measurement. Photographs are courtesy of Dr. Matthew Davy (University of Sheffield, UK).

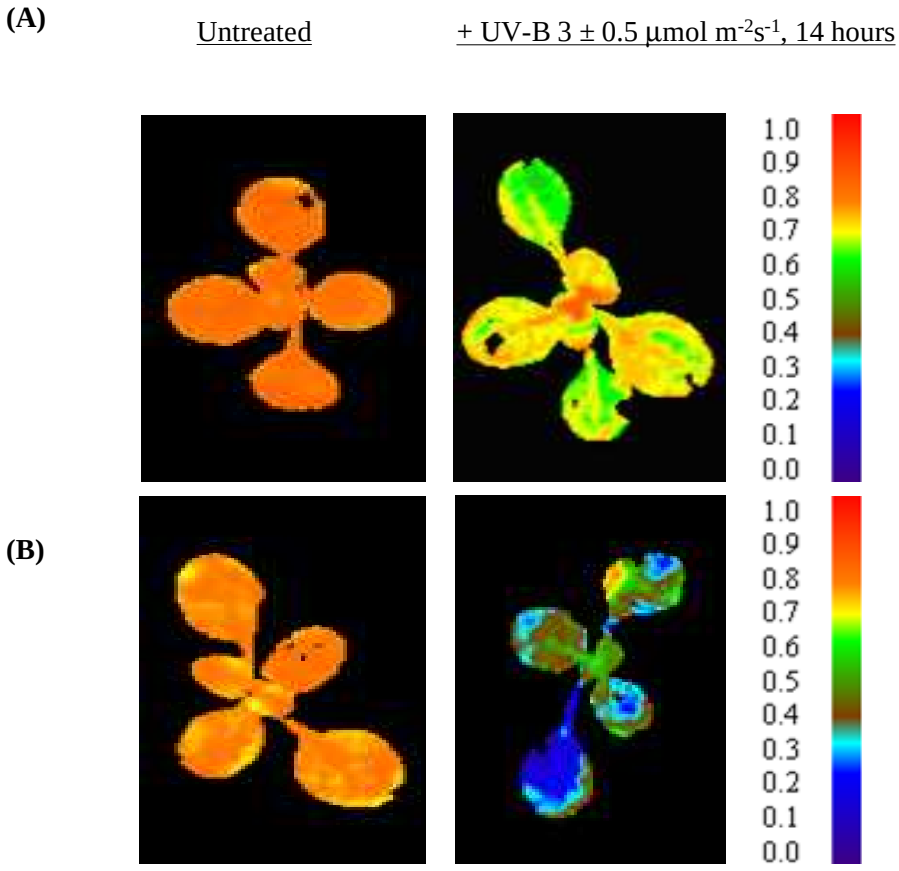


Figure 3.4. Semi quantitative RT-PCR of *ACTIN2*, *ELIP1* and *SIG5* gene expressions in wt L*er (wt)* and *uvr8-1 (u)* **(A)** at 1, 3 and 5 µmol m^{-2} s^{-1} for 2, 4 and 6 hours of UV-B **(B)** at 3 ± 0.5 µmol $m^{-2}s^{-1}$ UV-B for 4, 7 and 14 hours

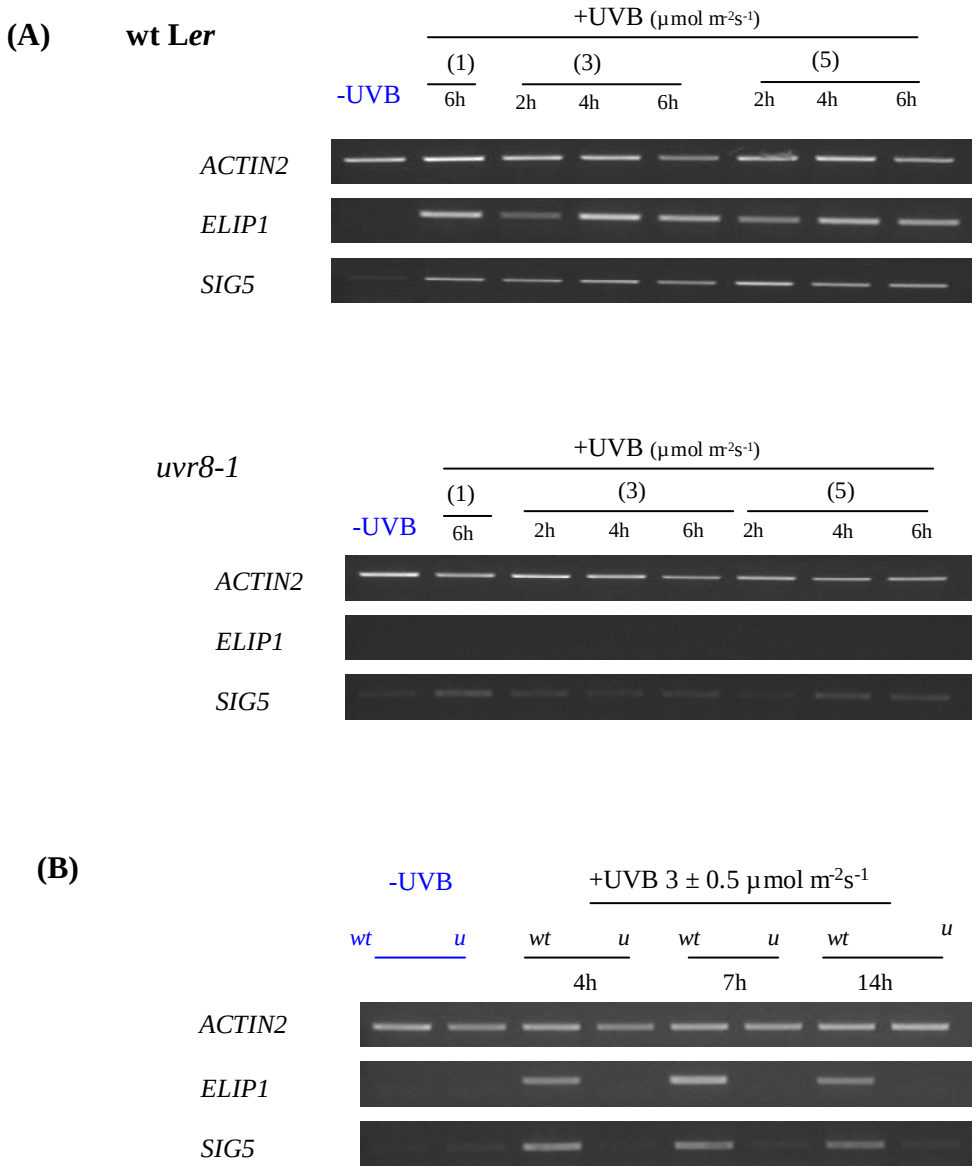


Figure 3.5. Semi quantitative RT-PCR analysis of *ACT2* and *psbD*-BLRP transcripts in **(A)** wt L*er* and **(B)** *uvr8-1*. Plants were grown in white light (120 ± 25 µmol $m^{-2}s^{-1}$) for 14 days then exposed to UV-B at 1, 3 and 5 µmol $m^{-2}s^{-1}$ for 2, 4 and 6 hours.

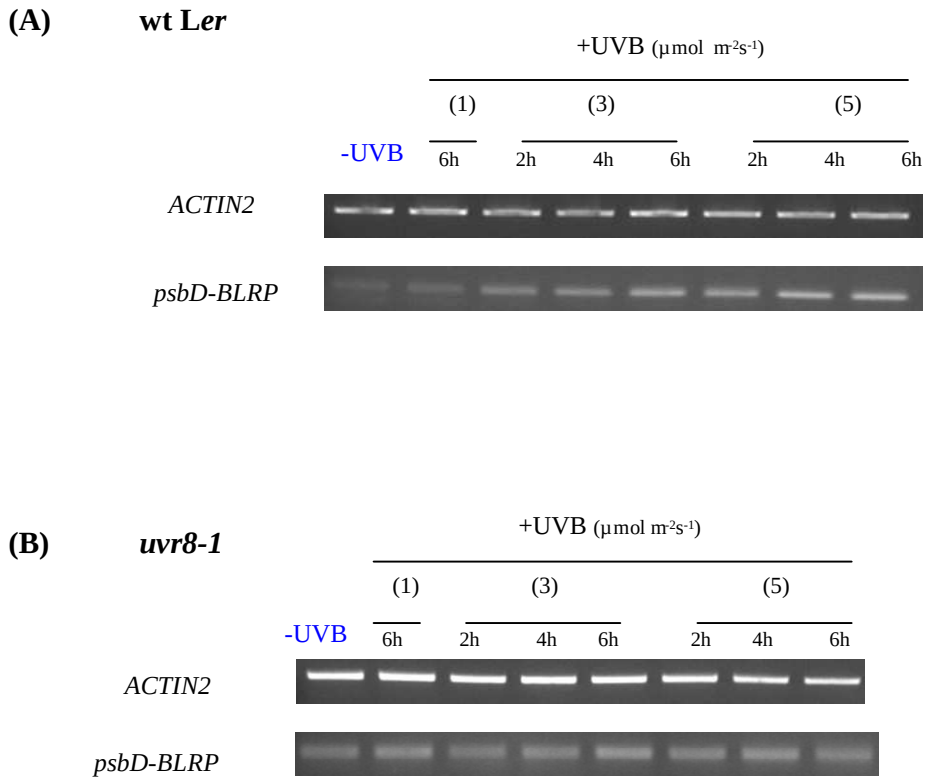


Figure 3.6. (A) Semi quantitative RT-PCR of *ACTIN2*, *psbA* and *psbD*-BLRP transcripts in 14-days-old wt L*er* (*wt*) and *uvr8-1*(*u*) either untreated or exposed to UV-B at 3 ± 0.5 µmol $m^{-2}s^{-1}$ for 4, 7 and 14 hours. Photographs are taken from 3 different set of experiments. Statistical analysis of **(B)** *psbA* and **(C)** *psbD*-BLRP transcripts. Data were normalized to *ACTIN2*. All data shown are mean ± S.E (n=3), analyzed using *Quantity One* software (BIORAD), local background subtraction.

(A)

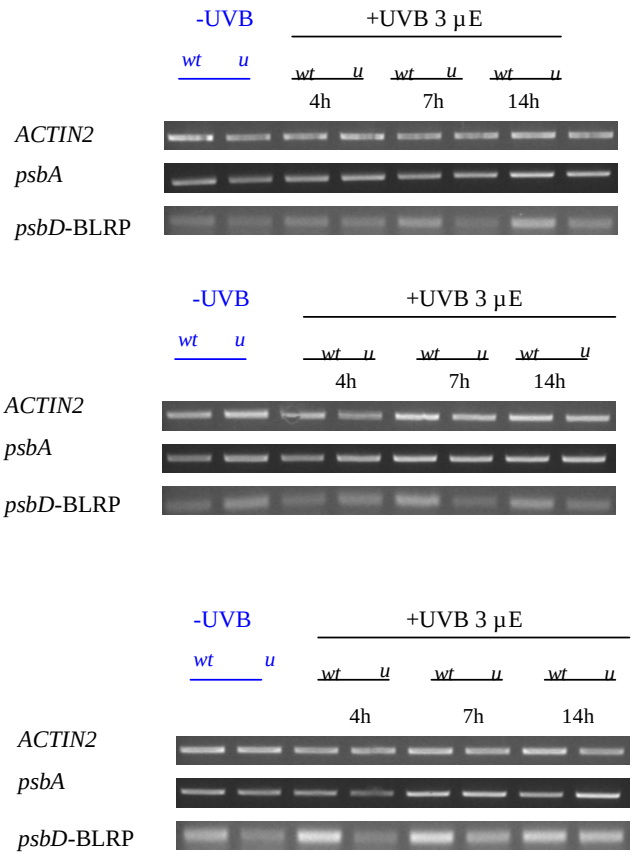
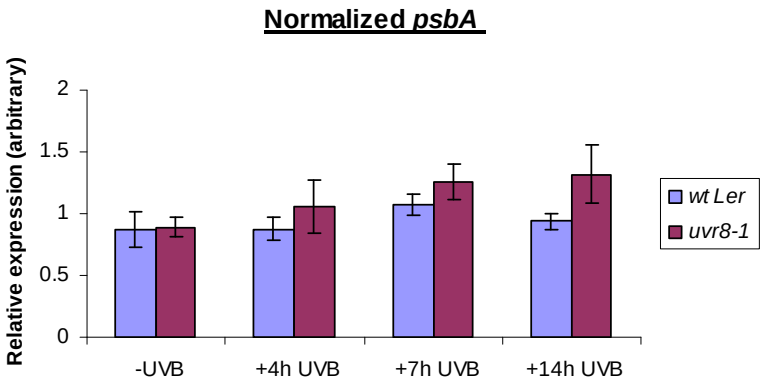


Figure 3.6. (Cont.) **(B)** *psbA* and **(C)** *psbD*-BLRP transcripts. Data were normalized to *ACTIN2*. All data shown are mean ± S.E (n=3), analyzed using Gel Doc software (*Quantity One*), local background subtraction

(B)



(C)

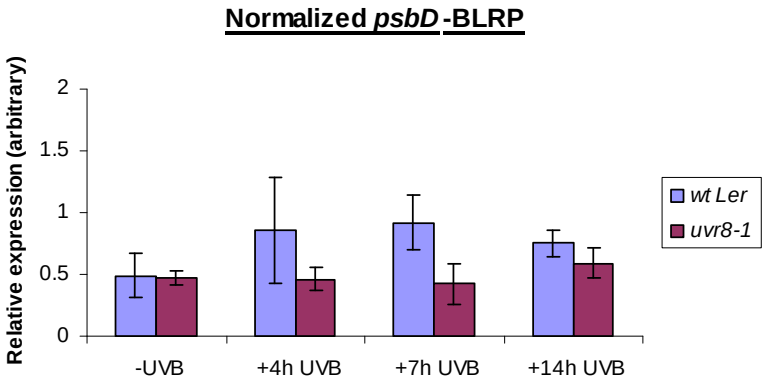


Figure 3.7. (A) Western blot photographs of D1 protein in wt L*er* (*wt*) and *uvr8-1* (*u*). Ponceau staining of ribulose-1,5-biphosphate carboxylase large subunit (rbcL, 47.5 kDa) was used as a loading control. Figure shown are taken from three indeendent experiments. 14 days-old-plants were exposed to 3 ± 0.5 µmol $m^{-2}s^{-1}$ UV-B for 14 hours. Untreated and treated tissues were extracted and fractionated in SDS/PAGE Gel, then probed by specific antibody against D1 protein (Agrisera). Equal amount of 10 µg of total protein was loaded to each lane. **(B)** Statistical analysis of quantified-D1 protein adjusted to rbcL bands. Data are mean ± S.E (n=3).

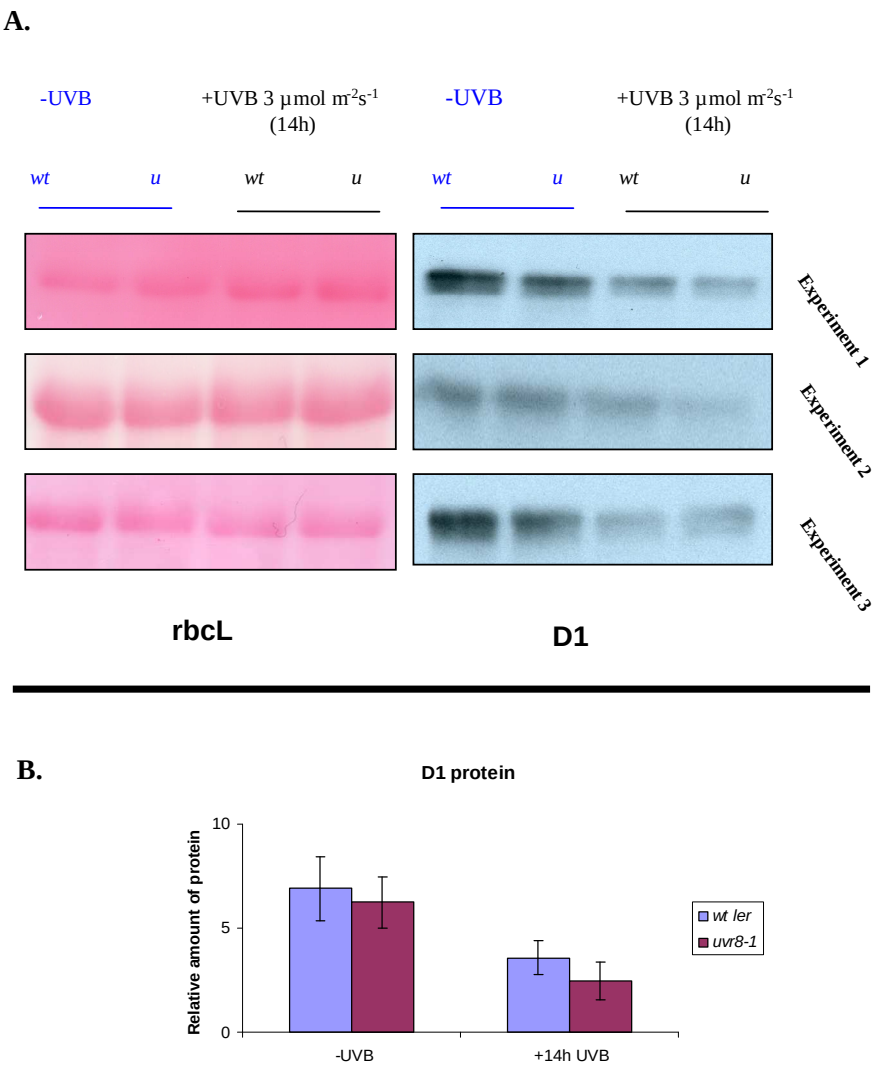


Figure 3.8. Western blot photographs of D2 protein in wt L*er* (*wt*) and *uvr8-1* (*u*). Ponceau staining of ribulose-1,5-biphosphate carboxylase Large subunit (rbcL) was used as a loading control. Figures shown are taken from four independents experiments. All the figures taken from experiments are provided in **Apendix 2**. 14 days-old-plants were exposed to 3 ± 0.5 µmol $m^{-2}s^{-1}$ UV-B for 14 hours. Untreated and treated tissues were extracted and fractionated in SDS/PAGE Gel, then probed by specific anti D2 antibody (Agrisera). Equal amount of 20 µg total protein was loaded in each lane.

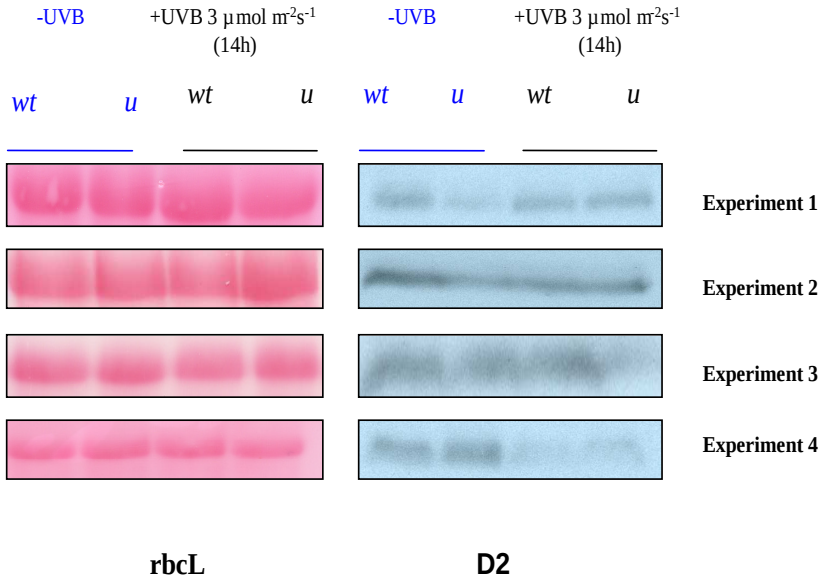


Figure 3.9. Statistical analysis of D2 protein in wt L*er* (*wt*) and *uvr8-1* (*u*) from four independent experiments, each replicated two to four times **(See Appendix 2)**. Data are mean ± SE (n=2-4).

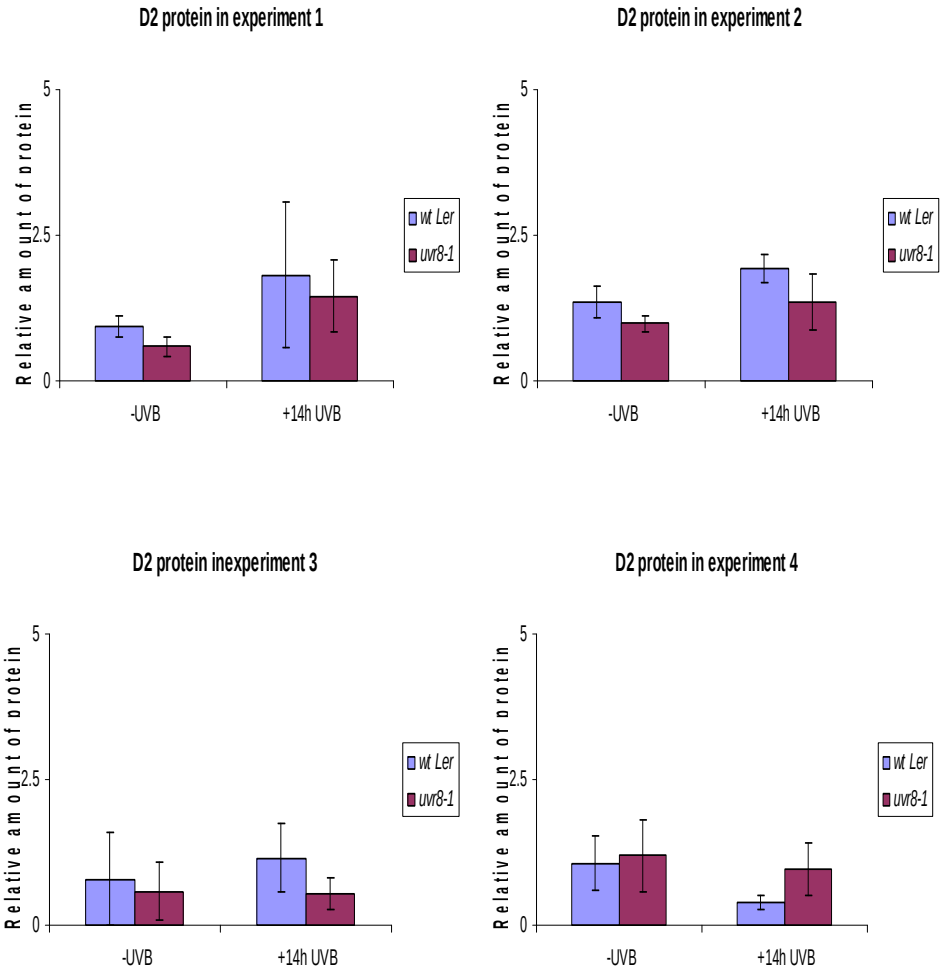


Figure 3.10. Semi quantitative RT-PCR analysis of **(A)** *ACT2*, *ELIP1* and *SIG5* and **(B)** *ACTIN2* and *psbD*-BLRP in wt Col-0, *sig5.1*, *sig5-2* and *elip1/2*. Plants were grown in continuous white light (120 ± 25 µmol $m^{-2}s^{-1}$) for 14 days then transferred to 3 ± 0.5 µmol $m^{-2}s^{-1}$ UVB for 14 hours.

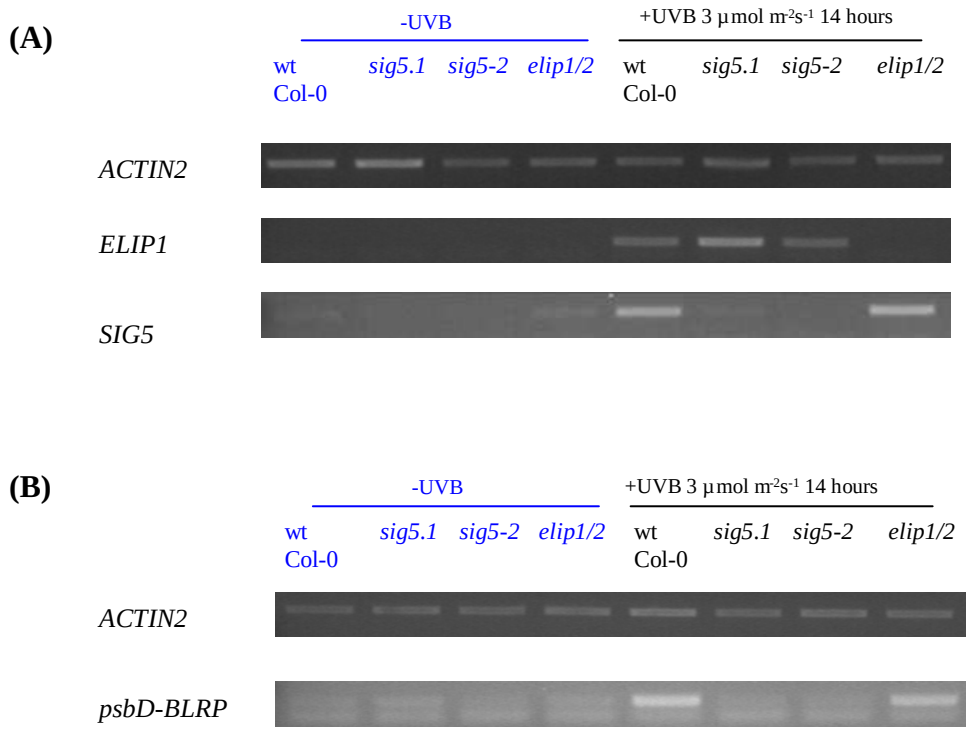
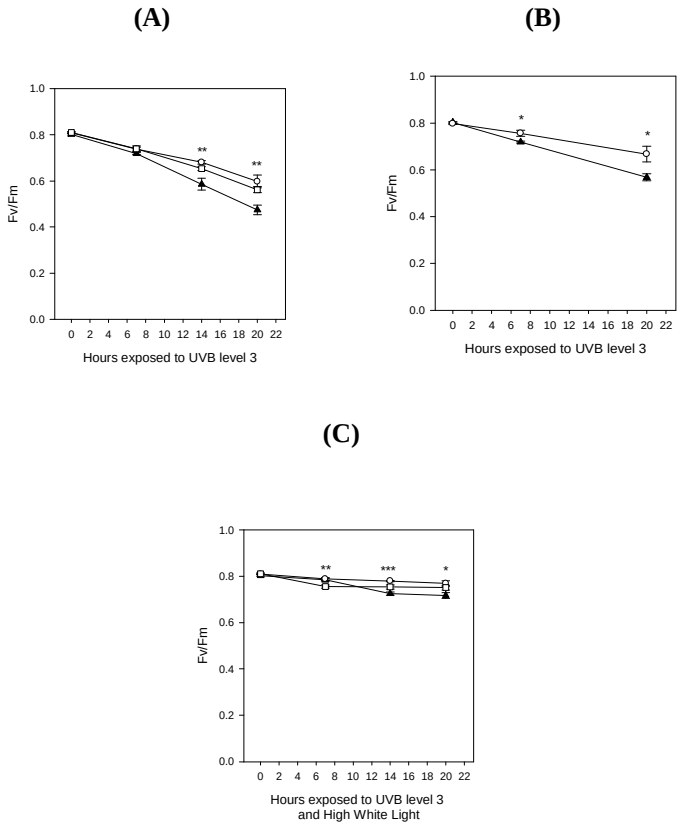


Figure 3.11. Fv/Fm values of **(A)** wt Col-0 (closed triangle), *sig5.1*(open circle), *sig5-2* (open square) and **(B)** wt Col-0 (closed triangle) and *elip1/2* (open circle). Plants were grown in continuous white light (120 ± 25 µmol $m^{-2}s^{-1}$) for 14 days then transferred to 3 ± 0.5 µmol $m^{-2}s^{-1}$ UVB for duration stated **(C)** Fv/Fm values of wt Col-0 (closed triangle), *sig5.1* (open circle), *sig5-2* (open square) at 3 ± 0.5 µmol $m^{-2}s^{-1}$ UVB plus supplementary high white light (150 ± 25 µmol $m^{-2}s^{-1}$) for duration stated. Values were obtained at actinic light level of 20 or 500 Photosynthetic Photon Flux Density (n=6 ± S.E). Statistically significant differences between wild type and mutants are indicated by * = P<0.05, ** = P<0.01 and *** = P<0.001. All data are obtained from experiment in collaboration with Dr. Matthew Davy (University of Sheffield, UK).



Chapter 4. General Discussion

4.1. *SIG5* and *ELIP1* are not substantial in UV-B protection mechanism

As mentioned in the previous chapter and shown in Figure 3.1 (A), the *uvr8-1* mutant is very sensitive to UV-B compared to wild type. After exposure to UV-B, *uvr8-1* plants suffer damage. Consistent with this result, Fv/Fm values of *uvr8-1* mutant decreased under UV-B **(Figure 3.2)**. A significant difference between wild type and *uvr8-1* mutant was shown at 3 ± 0.5 μmol $m^{-2}s^{-1}$ UV-B. This result indicated that a deficiency in UVR8 protein caused damage to the photosynthetic apparatus. According to Brown and co-workers (2005) the sensitivity of *uvr8-1* is caused by failure to induce genes concerned with UV protection, and some of these genes encode chloroplast proteins such as *SIG5* and *ELIP1*. Contrary to Adamska and co-workers (1992b), who reported that UV-B did not induce ELIPs in pea plants and in agreement with Brown *et al* (2005), the result shown in Figure 3.4 showed that accumulation of *SIG5* and *ELIP1* transcripts following UV-B exposure was impaired in *uvr8-1* mutant compared to wild type. However, whether the reduction in Fv/Fm values or the impairment of PSII activity relates to *SIG5* and *ELIP1* deficiency was not clear yet. To investigate whether *SIG5* and *ELIP1* deficiency contributed to photodamage of PSII in *uvr8-1* mutant, several approaches were used in this study.

As described in chapter 1, PSII RC core proteins D1 and D2 are encoded by *psbA* and *psbD* genes respectively. One of the *psbD* promoters is unique, called *psbD*-BLRP. This promoter is strongly regulated by blue light and its activation specifically requires SIG5. As shown in Chapter 3 (**Figure 3.5 and 3.6 (A)**), accumulation of *SIG5* transcripts was much lower in *uvr8-1* mutant compared to wild type following UV-B exposure. The same pattern can be seen in *psbD*-BLRP transcripts. The UV-B stimulation of *psbD*-BLRP transcripts appeared to be inhibited in *uvr8-1* mutant. Thus it was possible that the inactivation of *psbD*-BLRP was related to the lack of *SIG5*.

However, there was no evidence that *SIG5* and *psbD*-BLRP deficiency contributed to D2 protein regulation under UV-B exposure since there was much variation in western blotting assays of D2 protein (**Figure 3.8**).

On the other hand, UV-B caused a decrease in the amount of D1 protein as shown in Figure 3.7 (A). The decrease in the amount of D1 protein appeared to be greater in the *uvr8-1* mutant compared to wild type although the difference was not statistically significant. The decrease in D1 protein in wild type and *uvr8-1* was consistent with the UV-B sensitivity assay **(Figure 3.1 (A))** and Fv/Fm measurements **(Figure 3.2. and 3.3)**. D1 protein declined parallel to the decrease in photosystem efficiency in both wild type and *uvr8-1* mutant. This finding agreed with previous studies of the turnover and damage of D1 protein. UV-B promotes rapid turn over of D1 and D2 proteins in barley leaf (Jansen *et al*., 1996a; Babu *et al*., 1999; Barbato *et al*., 2000). However, the reduction in amount of D1 protein and Fv/Fm values was not determined by the transcript level. Semi quantitative RT-PCR of *psbA* transcripts indicated that they were little affected by UV-B **(Figure 3.6 (A))**. Although there was inhibition in *SIG5* transcripts in *uvr8-1* mutants, *psbA* transcript did not change during UV-B illumination. These findings imply that SIG5 is not crucial in UV-B protection. A possible reason is that *SIG5* may not regulate *psbA* at the trancription level or that another sigma factor can replace its function.

In an attempt to elucidate the role of *SIG5* and *ELIP1* in UV-B signalling pathways related to photosynthesis activity, *sig5.1*, *sig5-2* and *elip1/2* mutants were used in this study. As described in chapter 1, *sig5.1* contains a T-DNA insertion in the last exon of *SIG5* (Tsunoyama *et al*., 2004). This mutant failed to show *psbD*-BLRP induction under high light irradiation. As mentioned in the previous chapter, our finding showed very little *psbD*-BLRP transcript was expressed when the mutant was irradiated with 3 ± 0.5 μmol $m^{-2}s^{-1}$ of UV-B for 14 hours. In addition, *psbD*-BLRP was not expressed in *sig5-2* mutant. The *sig5-2* mutant has a T-DNA insertion at exon 2 of SIG5. However, both these *sig5* mutants are deficient in SIG5. Interestingly, UV-B treatment of *sig5* mutants did not drastically change Fv/Fm values as shown in Figure 3.11. In agreement with this measurement, UV-B sensitivity assay showed the *sig5*

mutants were tolerant of UV-B. In contrast, the *uvr8-1* mutant, which also lacks the expression of *SIG5* transcripts showed hypersensitivity to UV-B and reduction in Fv/Fm level. Taken together, our findings indicate that *SIG5* is not substantially involved in photosynthetic regulation under UV-B radiation.

Parallel to *SIG5* observation, *ELIP1* was also investigated in the presence and absence of UVR8 protein. As described in chapter 1, several evidences have been reported that ELIPs might act as a photoprotective protein. Adamska and Kloppstech (1991) provided evidence that ELIPs interact with D1 protein in PSII Reaction Center. Further, studies of mRNA and protein level showed that the ELIP mRNA level and protein increased parallel with the decrease in D1 protein (Adamska *et al*., 1992a). A recent study carried out by Heddad and co-workers (2006) revealed that accumulation of ELIP1 transcript and protein in green leaf of wild type *Arabidopsis thaliana* are correlated with the degree of photodamage of PSII Reaction Center. Contrary to previous research, studies of ELIP1 and ELIP2 deficient mutants (*elip1* and *elip2*, respectively) showed that there was no significant difference between wild type and mutants in photoinhibitory treatments (Casazza *et al*., 2005). Further, double null mutant *elip1/2* behaved as wild type in a high light experiment (Rossini *et al*., 2006). In agreement with Casazza *et al* (2005) and Rossini *et al* (2006), the findings as shown in Figure 3.1(C) and Figure 3.11 (B) clearly showed that *elip1/2* mutant is tolerant of UV-B. Taken together, the impairment and reduction of Fv/Fm values in *uvr8-1* mutant apparently was not caused by *ELIP1* deficiency.

4.2. Different response of *psbA* and *psbD*-BLRP genes to UV-B may indicate SIG5-related and SIG5-unrelated mechanisms

That *SIG5* activates *psbD*-BLRP and recognizes *psbA* promoter has been proposed by several groups of researchers (Tsunoyama *et al*., 2002; Onda *et al*., 2008). Therefore, it is possible that these genes may respond differently to light-stress

conditions. Consistent with this idea, results found in this study showed that these two genes differ in response to UV-B. This observation was clearly shown for wt L*er*.

In wt L*er* plants exposed to UV-B at different fluence rates, *psbD*-BLRP transcripts accumulated linearly with increasing intensities and time of exposure **(Figure 3.5)**. This indicates that expression of this gene in UV-B was dependent on fluence rate and time. In earlier works, *psbD*-BLRP transcripts were found to increase linearly to intensities in response specifically to blue light treatment (Tsunoyama *et al*., 2002; Mochizuki *et al*., 2004). A response to UV-B has not been reported previously and to date nothing is known how *psbD*-BLRP transcript accumulated in response to UV-B. According to gel-doc photographs **(Figure 3.6)** the *psbD*-BLRP transcript increases in response to UV-B. On the other hand, *psbA* transcript in wt L*er* was little affected by UV-B.

As mentioned above, the different response of these two genes to UV-B may be related to activation of *SIG5* gene. In a previous study, Tsunoyama *et al* (2004) were using the *sig5.1* knock out mutant. The *psbD*-BLRP transcript reduced severely compared to wild type in high light, whilst *psbA* transcript reduction was detected only slightly. The author suggested that activation of *psbD*-BLRP specifically requires *SIG5*. Furthermore, the study also showed that *SIG2* gene recognized *psbA* and over expression of *SIG2* gene enhanced transcription of *psbA* and *trnE* operon (Tsunoyama *et al*., 2004). Onda *et al* (2008) noted that *psbA* promoter is recognized by *SIG1, SIG2, SIG5* and *SIG6*. Moreover, they proposed that *SIG5* has dual functions in plastid promoter recognition and recognized *psbD*-BLRP and *psbA* differently.

Furthermore, our finding provides evidence that UVR8 is involved in transcription of *psbD*-BLRP since *uvr8-1* mutant failed to show strong expression in response to UV-B compared to wild type. This pattern was not obviously found in *psbA* transcript. The failure to see an involvement of UVR8 in *psbA* transcript accumulation in UV-B is perhaps because activation of this gene is not specific to *SIG5*. There is no report that UVR8 also regulates other sigma factors which recognize *psbA* promoter. In fact, *SIG1*, *SIG2* and *SIG6* were not assessed in this study.

4.3. UV-B may differentially affect transcript and translation level of D1 protein

The failure to see any effect of UVR8 on the *psbA* transcript level in UV-B illumination leads to several questions to be addressed in correlation with *psbA* transcripts, D1 and UVR8 proteins. Is there any UV-B photoreceptor strongly regulating *psbA*? Does UVR8 also regulate *SIG1*, *SIG2* and *SIG6* in *Arabidopsis thaliana*? In the presence of UV-B, why did D1 protein diminish rapidly? Is D1 synthesis regulated in transcript, translation or post-translational level?

As shown in Figure 3.7, D1 protein diminished after 14 hours exposure in both genotypes. Consistent with Fv/Fm measurement **(Figure 3.2 (B))**, after 14 hours exposure both wild type and *uvr8-1* mutant had decreased levels. In addition, the decrease in Fv/Fm and possibly also D1 protein in *uvr8-1* appeared to be more severe than in wild type. This implies that UV-B could harm photosynthetic apparatus, D1 protein particularly. This finding agreed to earlier works that D1 protein degrades rapidly either in UV-B alone or in mixture with PAR (Jansen *et al*., 1996b; Babu *et al*., 1999; Booij-james *et al*., 2000). Furthermore, deficiency in UVR8 protein may cause D1 protein to diminish more rapidly under UV-B exposure and this needs to be studied further.

The results failed to provide any evidence that the reduction of D1 protein was a consequence of a reduction of *psbA* transcript. It is thus difficult to conclude that D1 protein level in UV-B environment is regulated at the transcript level. As shown in Figure 3.6(A) and (B) no sharp increase of *psbA* transcripts was detected when wild type plants were shifted to UV-B. Observation during treatment duration time also showed that *psbA* transcript did not strongly accumulate in UV-B-treated *uvr8-1* compared to untreated plant.

Kettunen and co-workers (1997) reported that transcript and translation of *psbA* gene in pea (*Pisum sativum*) was adjusted during photoinhibitory condition. The accumulation of *psbA* transcript was followed by the increasing of D1 synthesis which indicated the turnover process had happened to maintain PSII activity. *In vivo* and *in vitro* studies in *Spirodella* mature chloroplast concluded that synthesis of D1 (*psbA* gene

product) was regulated mainly at transcription level (Fromm *et al*., 1985). In contrary to these studies, Baena-González and co-workers (2001) as quoted by Nagashima *et al* (2004) provided evidence that synthesis of D1 protein was mainly controlled at the translation level. Once again, there is no evidence from this study that the reduction of D1 protein and photoinhibition of PSII is related to *psbA* transcripts under UV-B exposure. The reduction in D1 protein could be the result of inhibition of translation or destruction of the protein. This study failed to find an involvement of UVR8 in transcript regulation of D1 protein and leads to the possibility that UVR8 may act differentially in transcript level and translation or proteolysis of D1 protein.

4.4. UVR8 is involved in *psbD*-BLRP transcript accumulation under UV-B

Assessment of *psbD*-BLRP transcripts in wt L*er* and *uvr8-1* mutant indicated that *psbD*-BLRP transcripts were not accumulated in mutant as strongly as wild type **(Figure 3.5 and 3.6)**. Among six σ-factors in *Arabidopsis thaliana*, only *SIG5* is required for activation of *psbD*-BLRP gene (Nagashima *et al*., 2004). According to Brown and co-workers (2005) and result shown here **(Figure 3.4)**, *SIG5* is significantly regulated by UVR8. Thus it is suggested that activation of *psbD*-BLRP is dependent on *SIG5* and regulated by the UVR8 pathway.

When plants were shifted from growth condition to UV-B, accumulation of *psbD*-BLRP transcripts was increased and both wild type and mutant showed reduction in Fv/Fm values. However, no indication of photoinhibition was detected at least until 7 hours exposure **(Figure 3.2).** This implies that *psbD*-BLRP transcripts are involved in repairing PSII system under photodamage condition.

By extended contact with UV-B, reduction in Fv/Fm values was shown in plants subjected to ambient and high level of UV-B **(Figure 3.2 (B) and (C))**. However, in wild type Fv/Fm values were higher than mutant. As mentioned in Chapter 3, a significant difference between wild type and mutant was found in ambient level of UV-B. Thus analysis of UVR8 and photosynthetic activity was conducted at 3 ± 0.5 μmol

$m^{-2}s^{-1}$ of UV-B. Here, it is clearly shown that extended contact with UV-B did not cause damage or photoinhibition along with the increasing amount of *psbD*-BLRP transcript in wt L*er*.

On the other hand, deficiency in UVR8 produced less amount of *psbD*-BLRP transcript and reduced plant ability to maintain PSII activity. This result further suggested that *psbD*-BLRP transcript was controlled by UVR8 in UV-B irradiation. However, there was no evidence that lacking *psbD*-BLRP transcripts in *uvr8-1* mutant caused reduction in D2 protein and directly affected PSII activity. The experiments failed to show a consistent trend in D2 protein.

4.5. Statistical analysis on transcripts and protein level

All the above analysis is descriptive analysis, based on images. However, analysis was applied to convert images to quantitative data. Some discrepancies were found which will be presented in this section.

Gel images showed that in *uvr8-1* mutant the *psbD*-BLRP transcripts consistently had less expression compared to wild type. However, standard error bars on scanned gel bands indicate that the transcript level of *psbD*-BLRP in *uvr8-1* mutant was similar to wild type except for 7 hours UV-B treatment. It is likely that variability in the data is caused by scanning the bands, adjusting to the adjacent background level and normalizing against *ACTIN2*. It would be better to use quantitative Real-Time RT-PCR to measure transcript levels in future experiments.

As mentioned previously, *psbD* encodes D2 protein in PSII Reaction Center. However, whether the blue light promoter of this gene was activated to regulate D2 protein under UV-B irradiation is still not clear. Four independent experiments were done and analyzed but no consistent trend in the D2 protein was observed. Whether the D2 protein was regulated at transcript level remains unclear. Furthermore, whether the decrease in Fv/Fm values and UV-B sensitivity in *uvr8-1* mutant was related to

regulation either of the *psbD*-BLRP transcript level or D2 protein needs further research.

In previous section (**Figure 3.7 (A)**), the D1 protein (*psbA* product) apparent reduced under UV-B particularly in *uvr8-1* mutant. Even though western blotting images of D1 protein showed increased reduction in response to UV-B in *uvr8-1* mutant in two experiments, the statistical analysis provided a different result. The scanned band of D1 protein was normalized to rubisco large sub unit (rbcL) and analysis of standard error bars showed there is no significant difference between wild type and *uvr8-1* mutant **(Histogram in Figure 3.7 (B))**. However, there was a significant difference between UV-B and non UV-B treatment, indicating that UV-B caused reduction in the amount of D1 protein. This finding agreed with previous researches as mentioned in previous chapter. Even though UV-B is known to cause the damage of D1 protein, the mechanism remains unclear. According to Semi-Quantitative RT-PCR analysis and western blotting photographs, the *psbA* transcripts and D1 protein were regulated differently.

The discrepancy between imaging observation and statistical analysis is perhaps because the statistical analysis data were calculated from sqRT-PCR and western blotting analysis which were converted to quantitative data. The weakness of this method is that the results are not purely quantitative data. Some error during quantification processes might happen. Since all the bands must be subtracted to background, different background could provide different result. In different experiments, different gels provide different values of background. Some are darker than the other. In case of protein analysis, some possibilities might cause different values of background that can affect the overall result. First, in the result scans of ponceau staining of rbcL, some membranes have more pink color than the other. More pink color of the membrane will cause higher values of background and less subtracted-band values. Second, the result scans of western blotting analysis were taken in not exactly the same developing time in the UV cassette. Some experiments showed bands after being developed for a few seconds whilst the others needed a few minutes to be developed. The intensities of the bands will depend on subjectivity assumption which

developing time was the best in particular experiment. For example bands recorded after being developed for 2 minutes gave stronger expression than those developed after 10 second. Example for the calculation is shown in Appendix 3. For all the reasons above, quantification method might not provide accurate amount of transcripts and protein. Repeating experiments both in transcripts and protein level, using quantitative method is highly suggested. In transcripts level, quantitative Real Time RT-PCR method can be used whilst in protein analysis, labeling protein with radioisotope may provide more accurate result.

Conclusion and Suggestions

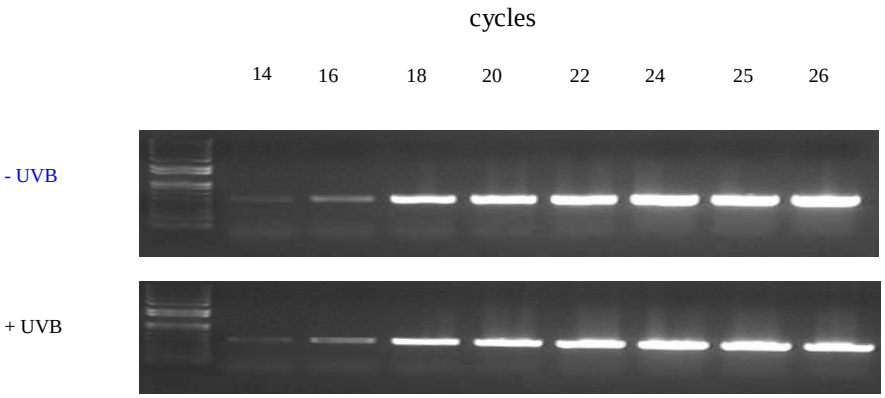
Conclusions for all the experiments as described in previous chapters are:

1. The *uvr8-1* mutant is more sensitive to UV-B compared to wild type and shows a greater reduction in photosynthetic efficiency (Fv/Fm) after UV-B exposure.
2. According to analysis on Gel-doc images, UV-B stimulates accumulation of *psbD*-BLRP transcripts and the *psbD*-BLRP activation in response to UV-B depends on *SIG5* and is regulated by UVR8.
3. UV-B has little effects in *psbA* transcript
4. D2 protein regulation under UV-B irradiation remains unsolved.
5. According to western blotting scan result, UV-B cause damages to D1 protein consistent with reduced PSII efficiency.
6. The reduction in D1 protein was not regulated at the transcripts level since *psbA* transcripts showed little change in all treatments.
7. SIG5 was not important in photosynthetic efficiency regulation under UV-B
8. *ELIP1* and *ELIP2* were not important in PSII efficiency in UV-B.

Suggestions:

1. Since there were discrepancies between descriptive and statistical analysis repeating experiment using most quantitative methods is highly recommended to obtain clear explanation.
2. Further research may be conducted to observe relationship between UVR8 and other Sigma factors that recognize *psbA* promoter, i.e. *SIG1*, *SIG2* and *SIG6*.
3. Further research needs to be focused on D2 protein assay to obtain knowledge whether UVR8 also regulates D2 protein.

Appendix 1. Semi-quantitative RT-PCR of *psbA* transcripts, amplified in several cycles to find the optimum cycle to be used in the experiments. Samples are cDNA of wt L*er* either untreated or exposed at 3 µmol $m^{-2}s^{-1}$ of UV-B for 4 hours.



Primers :

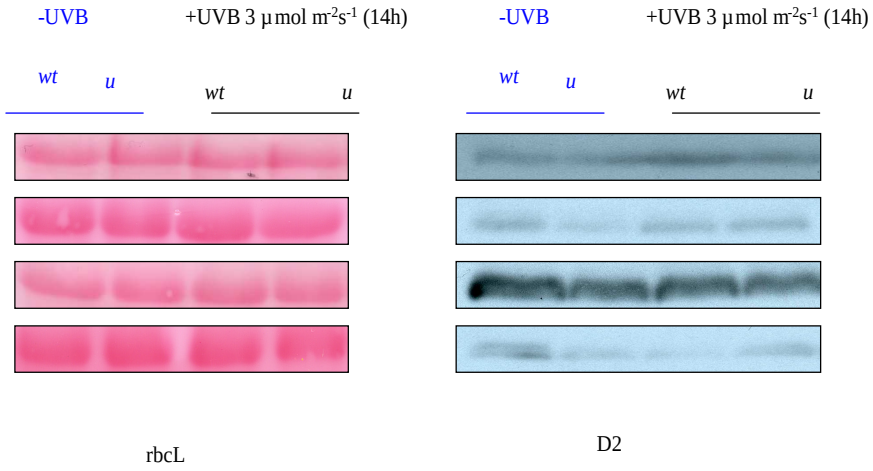
psbA R : 5’ GAA AAT CAA TCG GCC AAA AT-3’
psbA F : 5’ TTA CCC AAT CTG GGA AGC TG-3’

Reference :

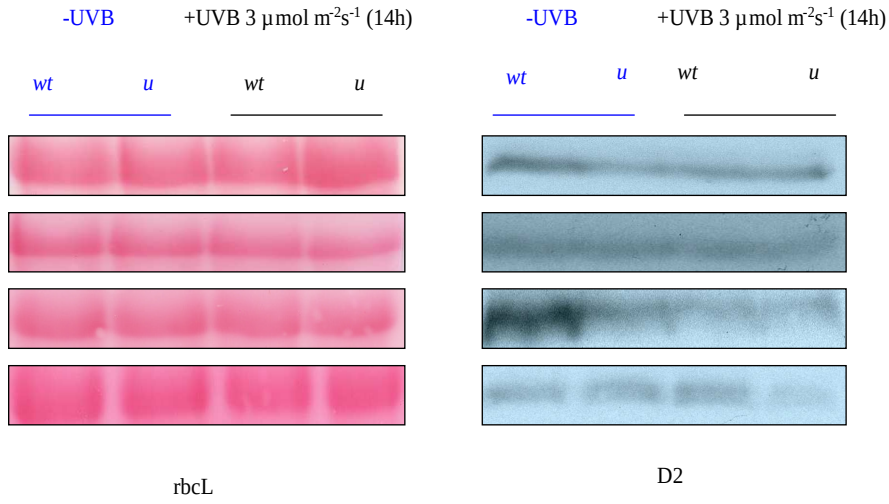
Wormuth, D., Baier, M., Kandlbinder, A., Scheibe, R., Hartung, W., and Dietz, K-J. 2001. Regulation of gene expression by photosynthetic signals triggered through modified CO2 availability. BMC Plant Biology.

Appendix 2. Western Blot Analysis of D2 protein in wt L*er* (*wt*) and *uvr8-1* (*u*) from 4 independent experiments. Ponceau staining of ribulose-1,5-biphosphate carboxylase large subunit (rbcL) was used as a loading control. Equal amount of 20 µg of total protein was loaded for each lane.

D2 in experiment 1

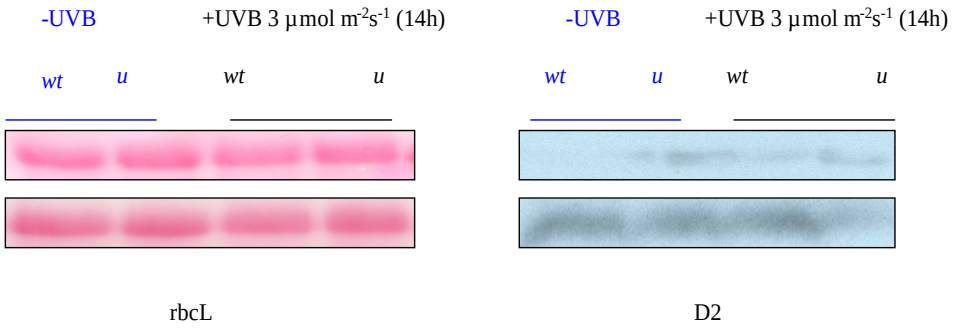


D2 in experiment 2

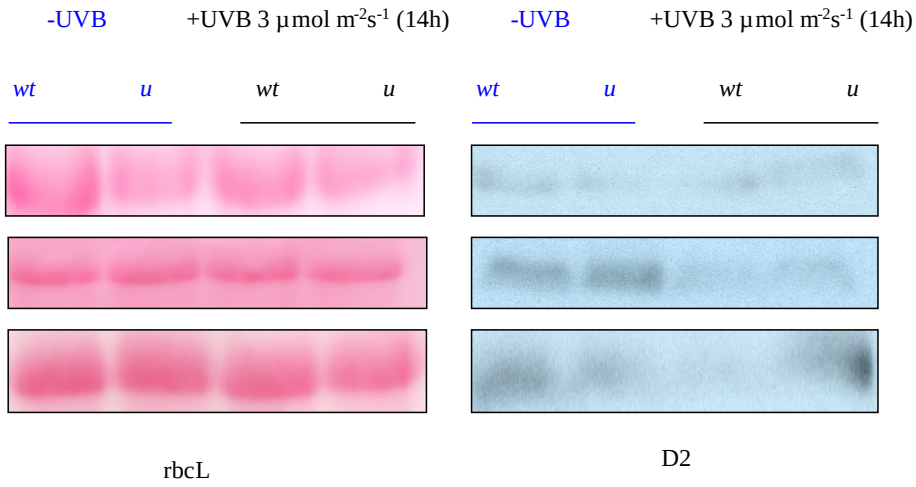


Appendix 2 (Cont..)

D2 in experiment 3



D2 in experiment 4



Appendix 3. Comparison calculation of rbcL and D1 protein of untreated wt L*er* band from two independent experiments. All experiments undertaken in same condition. For D1 western blot analysis, equal amount of 10 µg of total protein was loaded in SDS/PAGE gel. Scanned images were quantified using *Quantity One* software (BIORAD).

Membrane	1	2
rbcL band background (INT*mm2)	1007.36 879.83	1267.48 778.84
Adj. Vol. to background (INT*mm2)	58.93	245.64
D1 band background (INT*mm2)	1586.91 852.70	4357.72 1244.45
Adj. Vol. to background (INT*mm2)	378.52	2400.35
Normalized D1 to rbcL (D1/rbcL)	**6.42**	**9.77**

Appendix 4. Recipe for SDS/PAGE

Reagents
40% Acrylamide
1.5M Tris Buffer
DH_2O
10% SDS
0.1% SDS
10% APS
TEMED
0.5M Tris Buffer

SDS Running Buffer (for 5 litres total volume):
151.5 g Tris HCl
720 g Glycine
50 g SDS

10× Transfer Buffer (for 5 litres total volume):
151.5 g Tris HCl
720 g Glycine

1× Transfer Buffer (for 1 litres total volume):
100 ml 10× Transfer Buffer
200 ml Methanol
700 ml DH_2O

Separating gel:
1.5 ml 40% Acrylamide
1.5 ml 1.5 M Tris Buffer
2.9 ml DH_2O
60 µl 10% SDS
30 µl 10% APS
4 µl TEMED

Stacking Gel:
250 µl 40% acrylamide
660 µl 0.5 M Tris Buffer
1.6 ml DH2O
25 µl 10% SDS
12.5 µl 10% APS
4 µl TEMED

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