Molecular responses of plants to solar UV-B and UV-A radiation

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Academic dissertation

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1. Table of Contents

2. Original publications

This thesis is based on the following original publications, which are referred to in the text by their roman numerals (I-IV). The articles are reprinted with the kind permission of their copyright holders.

I. **Morales, L.O**., Tegelberg, R., Brosché, M., Keinänen, M., Lindfors, A., and Aphalo, P.J. (2010) Effects of solar UV-A and UV-B radiation on gene expression and phenolic accumulation in *Betula pendula* leaves. Tree Physiology 30: 923-934.

II. **Morales, L.O**., Tegelberg, R., Brosché, M., Lindfors, A., Siipola, S., and Aphalo, P.J. (2011) Temporal variation in epidermal flavonoids due to altered solar UV radiation is moderated by the leaf position in *Betula pendula*. Physiologia Plantarum 143: 261-270.

III. **Morales, L.O**., Brosché, M., Vainonen, J., Jenkins, G.I, Wargent, J.J., Sipari, N, Strid, Å., Lindfors, A.V., Tegelberg, R., Aphalo, P.J. (2013) Multiple Roles for UV RESISTANCE LOCUS 8 in Regulating Gene Expression and Metabolite Accumulation in *Arabidopsis* under Solar UV Radiation. Plant Physiology 161 (2): 744-759.

IV. **Morales, L.O**., Brosché, M., Vainonen, J.P., Sipari, N., Lindfors, A.V., Strid, Å., Aphalo, P.J. Are solar UV-B- and UV-A-dependent gene expression and metabolite accumulation in *Arabidopsis* mediated by the stress response regulator RADICAL-INDUCED CELL DEATH1?. Manuscript submitted to Plant Cell and Environment.

Author´s contributions

I. L.O.M designed and performed the experiments, analysed the data and wrote the manuscript with advice from M.B, R.T and P.J.A.

II. L.O.M designed the experiment with contributions by P.J.A. L.O.M. performed the experiments. L.O.M analysed the data and wrote the manuscript with advice from R.T, M.B and P.J.A.

III. L.O.M designed the experiments with contributions by M.B. and P.J.A. L.O.M. performed the microarray analyses, western blots and HPLC-MS analyses with support from M.B, J.V and N.S. respectively. L.O.M. designed and performed the q-PCR experiments and Dualex measurements. L.O.M analysed the data and wrote the manuscript with advice from M.B, J.J.W, G.I.J, Å.S and P.J.A.

IV. L.O.M designed and performed q-PCR experiments and Dualex measurements. L.O.M. performed the western blots and HPLC-MS analyses with support from J.V and N.S, respectively. L.O.M analysed the data with contributions by P.J.A. and wrote the manuscript with advice from M.B and P.J.A.

3. Abbreviations

ANOVA	Analysis of variance
BR	Brassinosteroid
Chl	Chlorophyll
COP1	CONSTITUTIVE PHOTOMORPHOGENIC1
CRY	CRYPTOCHROME
Dualex	Dual excitation of chlorophyll fluorescence
HY5	ELONGATED HYPOCOTYL5
HPLC	High pressure liquid chromatography
LME	Linear mixed effects models
MS	Mass spectrometry
JA	Jasmonic acid
O_3	Ozone
PAR	Photosynthetically active radiation
PDX1	PYRIDOXINE BIOSYNTHESIS1
PHOT	PHOTOTROPIN
PHY	PHYTOCHROME
qPCR	Quantitative real time PCR
RCD1	RADICAL-INDUCED CELL DEATH1
ROS	Reactive oxygen species
SA	Salicylic acid
UV	Ultraviolet
UV-B_{BE}	Biologically effective UV-B radiation
UVR8	UV RESISTANT LOCUS8

4. Abstract

Plant responses to solar ultraviolet radiation (UV, 280-400 nm) were assessed at different molecular levels using *Betula pendula* Roth (silver birch) and *Arabidopsis thaliana* (*Arabidopsis*) as model species in outdoor experiments to assess the possibly interacting roles of the UV-B and UV-A wavebands in acclimation to sunlight. Solar UV-B (280-315 nm) and UV-A (315-400 nm) irradiance was attenuated with plastic films. Both solar UV-B and UV-A promoted the acclimation of silver birch and *Arabidopsis* to UV in sunlight by regulating the expression of genes with functions in UV protection and also by inducing the accumulation of phenolic compounds in the leaves. Solar UV also regulated transcript accumulation of genes involved in the signaling and biosynthesis of auxin, brassinosteroids and jasmonic acid (JA) in *Arabidopsis*. A new role of *Arabidopsis* UV-B photoreceptor UV RESISTANCE LOCUS8 (UVR8) in the regulation of some responses to solar UV-A radiation was observed in addition to its previously described role in UV-B perception. High UV-A irradiance as present in sunlight, had a large effect on plant metabolism and modulated some of the previously characterized UV-B responses most probably through interaction between UVR8 and CRY pathways. In contrast to UVR8, under UV-B irradiation conditions not inducing stress, RADICAL-INDUCED CELL DEATH1 (RCD1) played no active role in UV signaling and acclimation, but rather modulated UV responses under sunlight. We demonstrated that solar UV-A makes an important contribution to acclimation of plants to sunlight, independently and interacting with UV-B.

5. Introduction

Solar radiation is of great importance for plants not only as a source of energy for photosynthesis but also as an environmental signal that regulates growth and development. Plants sense light signals through multiple protein photoreceptors that accurately perceive fluctuations in the intensity, spectral quality, direction, timing and periodicity of incoming sunlight (Frankhauser and Staiger, 2002). Photoreceptors are not directly involved in photosynthesis; however, their functionality may be a prerequisite for normal development of the photosynthetic apparatus (Lambers et al., 2008). The largest family of characterized plant photoreceptors comprises in *Arabidopsis thaliana* (*Arabidopsis*) five members of phytochromes (PHY A-E) which mediate responses to red and far red light (600-750 nm). Blue light (400-500 nm) and ultraviolet-A radiation (UV-A, 315-400 nm) are perceived by cryptochromes (CRY1 and CRY2), phototropins (PHOT1 and PHOT2) and the zeitlupe proteins (ZTLs), while ultraviolet-B radiation (UV-B, 280-315 nm) is sensed by UV RESISTANT LOCUS 8 (UVR8) (Figure 1) (Heijde and Ulm, 2012). Each individual photoreceptor has its own adsorption spectral properties that allow it to mediate the perception of specific environmental sources of information and play specific functions, usually different from those of other photoreceptors (Smith, 1982). Photoreceptors preferentially trigger responses to certain regions of the spectrum; however, they are also sensitive towards other wavelengths (Figure 1). The absorption of light photons by photoreceptors initiates signaling cascades that typically culminate in the regulation of the expression of genes that enable the plant to respond at the physiological level (Gyula et al., 2003). Morphological, developmental, and physiological responses driven by the light environment are defined as photomorphogenic and examples include seed germination, de-etiolation, growth, phototropisms and expression of associated genes. Photomorphogenic events regulated by UV-B have been not as well understood as those triggered by longer wavelengths of the spectrum under the control of phytochromes, cryptochromes and phototropins. Plant responses to UV-B are complex because numerous macromolecules absorb UV-B photons (nucleic acids, aromatic amino acids, proteins, lipids and phenolic compounds) (Jordan, 2002; Ulm et al., 2004; Jenkins, 2009). In addition, no UV-B photoreceptor had been characterized until

recently (Rizzini et al., 2011). In this thesis, molecular responses of plants to solar UV-B and UV-A are studied under natural sunlight to assess the possibly interacting roles of these two wavebands in acclimation to sunlight.

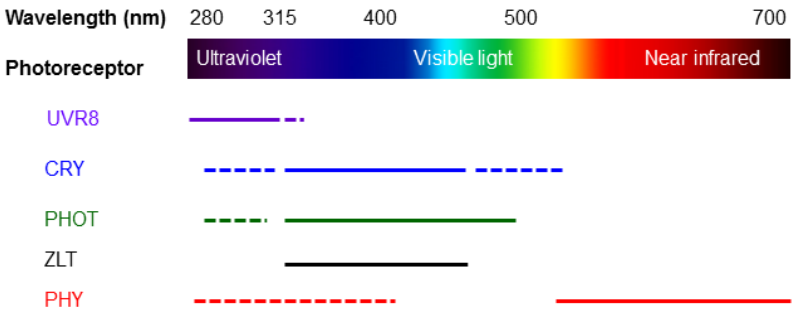

Figure 1. Plant photoreceptors and their absorption in the solar spectrum. Regions of maximal absorption are indicated by solid lines while sensitivity towards other wavelengths is indicated with dash lines. For more detailed information on absorption spectra of different photoreceptors see (Briggs and Christie, 2002; Chen et al., 2004; Christie et al., 2012).

5.1 Solar ultraviolet (UV) radiation and plant responses

Ultraviolet radiation (100-400 nm) is by convention divided into three spectral regions, UV-C (100-280 nm), UV-B and UV-A. Solar radiation of wavelengths in the UV-C region and the short-wavelength UV-B (280-293 nm) are almost completely absorbed by atmospheric oxygen (O_2) and ozone (O_3). While UV-A is weakly absorbed by O_3 and thus transmitted almost completely to the surface of the Earth, the levels of UV-B are more variable (Madronich et al., 1998). Terrestrial UV-B levels are influenced by solar zenith angle, latitude, altitude, variability in cloud cover, time of the day and season of the year, shade, aerosols and surface reflectivity (McKenzie et al., 2003). UV-B and UV-A represent approximately 0.15% and 6%, respectively of the energy in solar radiation at ground level (Frederick et al., 1989). However, solar UV is of particular importance because several plant biomacromolecules including DNA, RNA, lipids and proteins absorb in this region of the spectrum. Furthermore, UV-B photons have the highest energy of all wavelengths in sunlight and thus have the potential to cause cellular damage through photochemical reactions (Caldwell and Flint, 1994; Jansen et al., 1998; Ballaré, 2003).

In the middle of the 1980s there was great concern about the biological impacts of the predicted increase of UV-B irradiance at ground level as a consequence of O_3 depletion (Caldwell and Flint, 1994). Many studies addressed this question by exposing plants to high levels of UV-B mimicking O_3 depletion scenarios both under controlled environments and field conditions. From studies evaluating the effects of increased UV-B irradiance in plants, we have learned that high energy UV-B photons can produce mutagenic lesions in DNA, mainly cyclobutane-pyrimidine (CPDs) and pyrimidine (6, 4) pyrimidone dimers (6-4 PPs). These photoproducts can have negative effects in DNA replication and transcription since DNA and RNA polymerases cannot read through CPDs and 6-4 PPs (Jansen et al., 1998; Frohnmeyer and Staiger, 2003). Plants efficiently repair UV-B-induced DNA damage by a photoreactivation mechanism mediated by UV-A and blue light where the enzyme photolyase breaks the chemical bonds of cyclobutane rings and reverts the damage. CPDs and 6-4 PPs are also removed through nucleotide excision repair mechanisms in darkness, and by homologous recombination (Frohnmeyer and Staiger, 2003). The production of reactive oxygen species (ROS) and associated oxidative damage have been also observed in plants exposed to high UV-B doses. Hydroxyl radicals, singlet oxygen, superoxide radicals and hydrogen peroxide are amongst the main ROS produced by UV-B (A-H-Mackerness, 2000; Hideg et al., 2002). ROS scavenging in plants is mediated by enzymes with high antioxidant activity including glutathione, ascorbate, vitamin C, D and pyridoxine (vitamin B6) (Chen and Xiong, 2005). However, ROS are not only a source of cellular damage but also important signaling molecules that regulate the expression of several UV-B responsive genes (A-H-Mackerness et al., 2001). Previous studies have also indicated that UV-B can have a negative impact in the photosynthesis of higher plants by down-regulating the expression of photosynthetic genes, lowering enzyme activity, damaging D1 proteins and photosystem II, reducing chlorophyll (Chl) contents and CO_2 uptake (reviewed by Teramura and Sullivan, 1994). However, there is evidence that UV-B within the ambient range has little effect on photosynthesis (Allen et al., 1998; Brosché and Strid, 2003; Ballaré et al., 2011). Other observed negative effects of UV-B include reduction of growth through inhibition of cell division, decrease plant biomass and seed production (Jordan, 2002; A-H-Mackerness et al., 2001).

It is generally accepted that acute stress responses caused by high UV-B levels are initiated through DNA-damage and ROS signaling pathways that do not involve the UV-B photoreceptor. UV-B-induced stress responses have also parallels with those induced by other abiotic stress and pathogen attack (A-H-Mackerness et al., 1999, 2001; Brosché and Strid, 2003; Hideg et al., 2013). It should be noted that the magnitude of the UV-B damage is influenced by plant species and geographical region of origin (Sullivan et al., 1992; Ballaré et al., 2001; Searles et al., 2001), developmental stage (Jordan, 2002; Laitinen et al., 2002; Casati and Walbolt, 2004), daily UV-B dose and spectral distribution within UV-B (Ulm et al., 2004; Kalbina et al., 2008), length of UV-B exposure and exposure history of the plant (Jenkins, 2009), and UV-B:UV-A and UV-B:PAR ratios (Krizek, 2004). Experiments addressing O_3 depletion and UV-B stress carried out in controlled environments have been particularly useful in identifying the mechanisms by which UV-B can exert damage in plants. However, the interpretation of results from these studies should be cautious. These type of experiments have been often criticized for using unrealistically high UV-B doses and high UV-B:UV-A and UV-B:PAR ratios (Ballaré, 2003). This sometimes has produced exaggerated responses because the sensitivity of plants to UV-B is increased under low UV-A and PAR given the protective mechanisms induced by wavelengths in these regions of the spectrum (Ballaré, 2003; Paul and Gwynn-Jones, 2003). As mentioned above, UV-A and PAR ameliorate UV-B damage by inducing photoreactivation mechanisms, the expression of genes conferring UV-B protection, the synthesis of phenolic compounds and other antioxidants, and help plants to avoid UV-B exposure through altered leaf morphology (Allen et al., 1998; Ballaré, 2003).

Recent research has also shown that UV-B does not only cause damage but that it is also an environmental signal that induces a range of photomorphogenic responses that allow plant acclimation to UV and thus minimize UV-B damage (Jansen, 2002; Potters et al., 2007; Jenkins, 2009). Low levels of UV-B modify plant morphology by increasing leaf thickness, promoting axillary branching and reducing hypocotyl length; these responses are thought to help plants reduce exposure to UV-B (Jansen, 2002). The expression of hundreds of genes with important functions in UV protection and acclimation is controlled by UV-B most likely through transcriptional regulation (Jordan, 2002; Brosché and Strid, 2003; Jenkins, 2009). Previous studies

have shown that UV-B induces transcript accumulation of genes involved in the synthesis of phenolic compounds, antioxidant enzymes and repair of UVB damage (Brown et al., 2005; Hectors et al., 2007; Favory et al., 2009). The synthesis and accumulation of phenolic compounds is the most frequently observed response in plants exposed to UV-B radiation (Caldwell et al., 1983; Stapleton, 1992). Phenolic compounds accumulate mainly in the cuticles, vacuoles or cell wall of the epidermis, and efficiently attenuate harmful UV radiation while transmitting PAR to mesophyll cells (Day et al., 1992; Bilger et al., 2001). The most effective UV-B screening phenolics in plants are hydroxycinnamic acid derivatives (*p*-coumaric, caffeic and ferulic acids) and flavonoids, especially derivatives of the flavonols quercetin and kaempferol (Burchard et al., 2000; Agati and Tattini, 2010). In addition, to their role in UV protection, flavonoids play multiple physiological functions in plant including growth regulation, reproduction, antioxidant activity and defence against attack from herbivores and pathogens (Harborne and Willians, 2000; Hernández et al., 2009; Agati, 2012).

5.2 Perception of UV-B by UVR8 and signaling in plants

During recent years we have learned that UV-B-induced photomorphogenic responses in plants are at least partially mediated by the UV-B photoreceptor UVR8. This seven bladed β-propeller protein of 440 amino acids has sequence similarity to the human REGULATOR OF CHROMATIN CONDENSATION 1 (RCC1) (Kliebenstein et al., 2002; Christie et al., 2012). However, UVR8 and RCC1 differ in activity and function (Rizzini et al., 2011) and also in their monomeric topology (Wu et al., 2012; Christie et al., 2012). The lack of any external co-factor (chromophore) in UVR8 to perceive UV-B is quite different from all other known plant photoreceptors, where an external chormophore is needed for light absorption. For example, to perceive UV-A and blue light, CRYs bind flavin adenine dinucleotide (FAD) and methenyltetrahydrofolate (MTH), PHOT and the ZTLs bind flavin mononucleotide (FMN), and PHYs bind to phytochromobilin (reviewed by Heijde and Ulm, 2012). Analysis of the crystal structure of the UVR8 protein has revealed that the amino acid tryptophan (Trp) is the chromophore that absorbs UV-B as opposed to an external co-factor. Tryptophans Trp 285 and Trp 233 play major roles in UV-B

perception while Trp 337, Trp 198 and Trp 94 play auxiliary roles (Christie et al., 2012; Wu et al., 2012).

The combined model proposed here (Figure 2) summarizes the most recent findings related to UV-B perception and signaling mediated by UVR8 as determined in experiments under controlled environmental conditions. Under visible light (400-750 nm) or conditions devoid of UV-B, UVR8 appears in plants as homodimer. After UV-B perception by Trp 285 and Trp 233, the salt bridges joining the dimer break splitting UVR8 into monomers (Christie et al., 2012; Wu et al., 2012). The active UVR8 monomers interact with the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) (Rizzini et al., 2011). The interaction between UVR8-COP1 occurs through the C terminal region of UVR8 and WD40 domain of COP1 (Cloix et al., 2012). Under photomorphogenic UV-B, UVR8 monomers are thought to sequester COP1-SUPRESSOR OF PHY A (SPA) (COP1-SPA) complexes from its association with CULLIN4–DAMAGED DNA BINDING PROTEIN 1 (CUL4-DB1) (CUL4-DB1-COP1-SPA-CUL4–DDB1) which mediate repression of photomorphogenessis in darkness (Huang et al., 2013). The UVR8-COP1-SPA are required for the induction and stability of the transcription factor ELONGATED HYPOCOTYL5 (HY5) (Huang et al., 2013) and for relaying the signal that activates gene expression and UV-B acclimation in plants (Favory et al., 2009). HY5 and the HY5 HOMOLOG (HYH) act redundantly to regulate the expression of most of the genes involved in the UVR8 photoregulatory pathway (Brown and Jenkins, 2008). HY5 and the transcription factor FAR-RED ELONGATED HYPOCOTYL 3 (FHY3) are positive regulators of the UVR8 pathway by regulating COP1 expression under UV-B in a UVR8-dependent manner (Huang et al., 2012; Tilbrook et al., 2013). The UV-B induction of the proteins REPRESSOR OF PHOTOMORPHOGENESIS 1 and 2 (RUP1 and 2) is dependent of UVR8-COP1-HY5 (Gruber et al., 2010). RUPs interact with UVR8 and act as negative regulators of UV-B signaling by promoting UVR8 redimerization post UV-B exposure (Heijde and Ulm, 2013; Heilmann and Jenkins, 2013; Tilbrook et al., 2013). The transcription factor SALT TOLERANCE (STO/BBX24) and the stress regulator RADICAL-INDUCED CELL DEATH1 (RCD1) are also proposed negative regulators of UV-B signaling by impinging on HY5 (Jiang et al., 2012; Jiang et al., 2009).

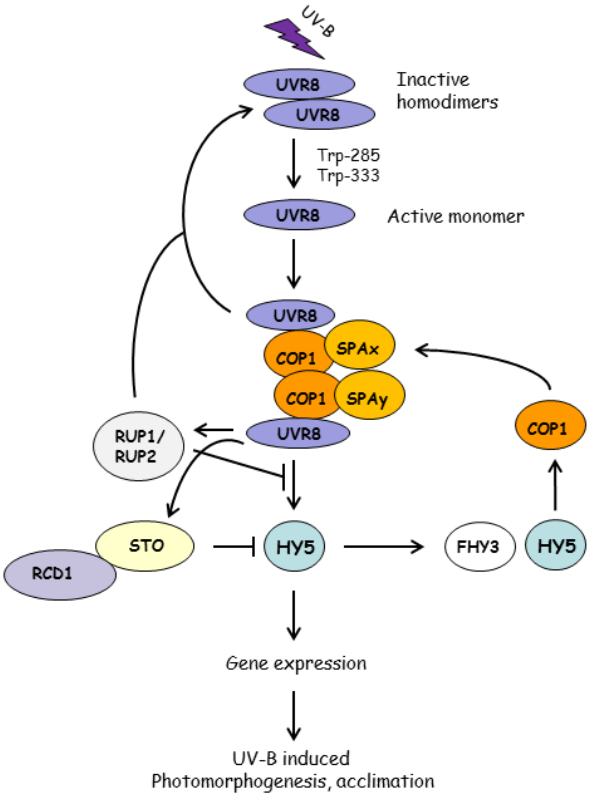

Figure 2. Proposed integrative model describing UV-B perception and signaling mediated by UVR8 in *Arabidopsis* as determined in experiments under controlled environmental conditions. After UV-B perception, UVR8 homodimers are monomerized and the active UVR8 monomers interact with COP1 and stabilize HY5 which relays the UV-B signal that promotes altered gene expression and UV-B acclimation. The proteins RUP1 and RUP2 disrupt UVR8-COP1, inactivate the signaling pathway and promote the regeneration of UVR8 homodimers. STO and RCD1 are proposed negative regulators of the UVR8 pathway while FHY3 and HY5 play positive roles, see Tilbrook et al. (2013) for more detailed information on UV-B signaling mediated by UVR8.

Despite recent advances in our understanding of UV-B signaling mediated by UVR8, the mechanisms by which UVR8 regulates gene expression are not clearly understood. Furthermore, there is limited understanding on how the UVR8 pathway functions under sunlight and how it interacts with other pathways under the control of other photoreceptors (Tilbrook et al., 2013). Another question mark in UV-B signaling in plants is the presence of other UV-B photoreceptors, and what other pathways could mediate UV-B responses (Ulm et al., 2004; Kalbina et al., 2008; Brown and Jenkins, 2008).

Most of the available information related to molecular events regulated by UV-B and signaling in plants comes from experiments under controlled environments using artificial light conditions. These studies on the regulatory role of UV-B radiation have

substantially expanded our mechanistic understanding of plant responses to UV-B by identifying major regulators in UV-B signaling pathways and elucidating gene functions. However, because there is limited information on how these pathways operate under sunlight, our understanding on the molecular mechanisms behind plant responses to UV-B and UV-A in the natural environment is still limited. At the level of gene expression, only a few studies have measured transcript accumulation in plants exposed to UV-B under field conditions (Casati and Walbot, 2003; 2004; Casati et al., 2011; Izaguirre et al., 2003). Growth conditions used in almost all experiments indoors are temporarily and spatially less variable than those plants experience in natural environments, under which selection has been taking place for millions of years (Frenkel et al., 2008).

6. Aims of the study

The aims of this study were: 1) to assess solar UV-B and UV-A-induced changes in gene expression and metabolite profiles with relevance for plant acclimation to solar UV, and, 2) to investigate the role of the UV-B photoreceptor UVR8 and the stress regulator RCD1 in regulating gene expression and metabolite accumulation under full spectrum sunlight. These aims were pursued by using silver birch and *Arabidopsis* in outdoor experiments designed to manipulate solar UV-B and UV-A irradiance using plastic films.

It was hypothesized that:

1) In addition to solar UV-B, solar UV-A triggers changes in gene expression and metabolite accumulation that promote plant acclimation to sunlight.

2) UV-A modifies UV-B-induced responses in plants exposed to full spectrum sunlight.

3) The dynamics of the accumulation of phenolic compounds depends on the direction of step changes in solar UV-B and UV-A irradiance and the developmental stage of leaves.

4) The acclimation and survival of *Arabidopsis* under solar UV are not dependent solely on UV-B perception by the photoreceptor UVR8.

5) Under natural sunlight, RCD1 is a negative regulator of the UV-B induced gene expression and metabolite accumulation in *Arabidopsis*.

7. Materials and Methods

All experiments were conducted in the greenhouse and field area of the Viikki campus of Helsinki University, Finland (60º13´ N, 25º1´ E) during the summers 2007-2010 (Figure 3). The plant material and methods used in this study are described in the respective publications I-IV as indicated in Tables 1 and 2. Silver birch seeds used in publications I and II were obtained from the Finnish Forest Research Institute (Suonenjoki Station, Seed orchard 379, Ey/FIN M29-93-0001). The seeds of *uvr8-2* and *rcd1-1* mutants were kindly provided by Professors Gareth Jenkins, University of Glasgow and Jaakko Kangasjärvi, University of Helsinki, respectively. The same plastic films were used to create the UV treatments in all experiments (publications I-IV). Exclusion of solar UV-A+B was provided by the theatrical “gel” (Rosco E+# 226), exclusion of solar UV-B with polyester film (0.125 mm thick, Autostat CT5, Thermoplast, Helsinki, Finland), and near-ambient solar UV-A+B was provided by polythene film (0.05 mm thick, 04 PE-LD, Etola, Jyväskylä) (Figure 4). The UV treatments were named according to journal styles (publications I-III).

Figure 3. Pictures of the outdoor experiments. The year of realization and respective publication are indicated in parenthesis, A (2007, Publication I), B (2008, Publication II) and C (2010, Publications III and IV).

The effects of solar UV-B and UV-A on gene expression, metabolite accumulation and growth parameters were assessed in the presence of solar UV-A and ambient PAR. This approach is quite different from those used in most experiments indoors dealing with UV-B induced molecular responses. The same methods were used to determine the effects of solar UV on growth, epidermal UV absorbance, Chl contents and metabolite profiles in publications I, III and IV. The analysis of qPCR data in experiment I was done according to (Livak and Schmittgen, 2001) using one reference gene. In experiments III and IV three reference genes were used to normalize the data using qbase^{PLUS} 2.0 (www.biogazelle.com) and the statistical analysis was done on log transformed data.

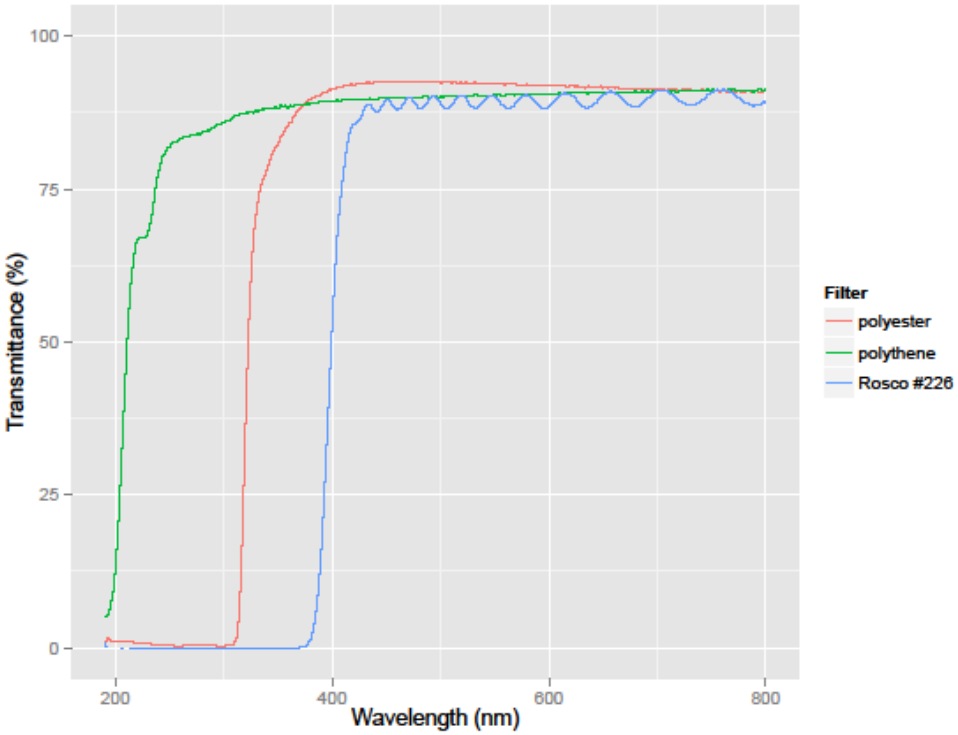

Figure 4. Transmittance of the films used in this study as measured with a spectrophotometer equipped with an integrating sphere (Shimadzu UV-2501 PC UV–VIS, Kyoto, Japan).

Table 1. Plant material and methods used in publications I-IV.

Measurement	Method	Species	Publication
Gene expression	Quantitative real time PCR	silver birch, Arabidopsis	I, III, IV
Gene expression	Microarray hybridization	Arabidopsis	III
Protein expression	Western hybridization	Arabidopsis	III, IV
Epidermal UV absorbance	Dualex FLAV	silver birch, Arabidopsis	I-IV
Epidermal UV absorbance	Dualex HCA	Arabidopsis	III, IV
Chl content	SPAD Chl meter	silver birch, Arabidopsis	I-IV
Growth	Height, dry weight, number of leaves	silver birch	I, II
Plant performance	Number of flowers, time of flowering	Arabidopsis	III
Metabolite profiles	UPLC-MS/MS	silver birch, Arabidopsis	I, III, IV

Table 2. List of Arabidopsis ecotypes and mutants used in this study.

Genotype	Annotation	Reference	Publication
Ler	Landsberg *erecta*		III
uvr8-2	*UV resistant locus8-2*	Brown et al. 2005	III
Col-0	Columbia		IV
rcd1-1	*radical-induced cell death1-1*	Overmyer et al. 2000	IV

8. Results and Discussion

8.1 Effects of solar UV-B and UV-A on gene expression

The regulation of gene expression is one of the earliest responses observed in plants exposed to UV-B (Jenkins, 2009). UV-B induced changes in gene expression as determined indoors have often been obtained using irradiation protocols where plants do not receive any UV-B before the actual UV-B treatment. These irradiation conditions are very different from those present in the natural environment where plants are constantly exposed to UV-B and therefore get acclimated (Casati et al., 2011). There is evidence that most UV-B regulated genes are transiently expressed (Brosché et al., 2002; Ulm et al., 2004; Kilian et al., 2007; Favory et al., 2009) and that after UV-B acclimation fewer genes are expressed to maintain this state (Hectors et al., 2007; Jenkins, 2009). Only during emergence from the soil, seedlings are exposed to a drastic step change in UV-B and afterwards acclimation adjustment depends on gradual changes in UV-B. Therefore after emergence, for plants growing in sunlight, long-term acclimation is the most important response for coping with UV exposure. Thus, in two of the experiments, patterns of gene expression with relevance for long term acclimation of plants to solar UV were determined in the presence of solar UV-A and high PAR (Publications I, III).

Silver birch has been used as model in studies targeting UV-B responses primarily at the metabolite level (Lavola et al., 1997; Kostina et al., 2001; de la Rosa et al., 2001; Tegelberg et al., 2001; Kotilainen et al., 2009). However, the molecular mechanisms of metabolite regulation by UV-B in the species are poorly understood, especially at the level of transcription. We show for the first time that transcripts of the phenylpropanoid gene *PAL* and the transcription factor *HYH* were accumulated in a linear relationship with increasing solar UV-B doses in young and expanding leaves of silver birch growing outdoors for one month (Publication I). Thus, silver birch seedlings growing in open fields may keep steady expression of *PAL* to fuel the UV-B induction of general phenylpropanoid pathway (Publication I). This agrees with the correlation observed in expression of phenylpropanoid genes and the accumulation of phenolic compounds after short time exposure to solar UV-B (Publication III). More generally, the transcriptional regulation of a large array of genes involved in UV acclimation is controlled by *HYH* (Brown et al., 2005; Brown and Jenkins, 2008). In

contrast to *PAL* and *HYH,* effects of solar UV-B and UV-A on the expression of other genes involved in the flavonoid pathway were small (less than 1.5 fold increase) and not significant (Figure 5) (Publication I). In *Arabidopsis* expression of many flavonoid genes including *CHI* and *DFR* was significantly increased (between 5 and 10 fold) by solar UV after 12 h outdoors (Publication III, Figure 2, Supplemental Table S1). Thus, it is possible that long exposure to a range of other environmental factors such as high PAR could have masked the UV regulation of these genes in silver birch after one month (Figure 5) and in *Arabidopsis* after three weeks (less than 2 fold increase) (Publication III, Figure 6) (Winkel-Shirley, 2002; Kleine et., al 2007).

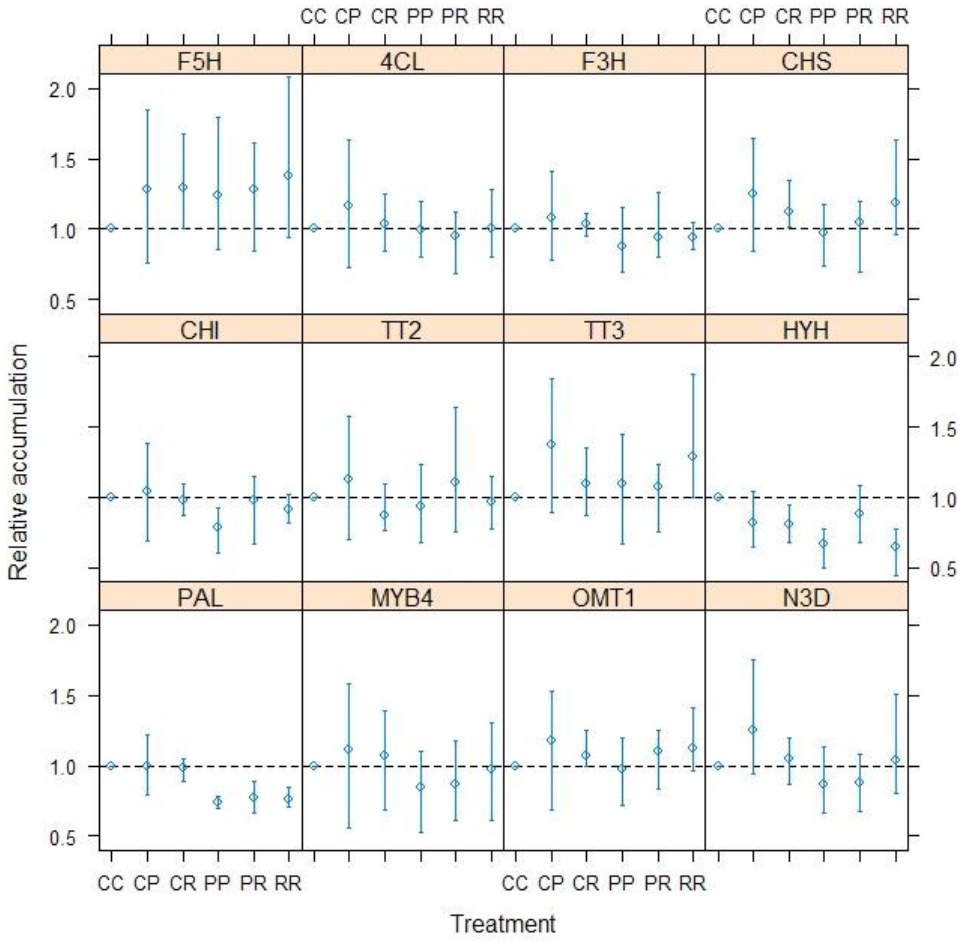

Figure 5. Relative transcript accumulation of genes analysed in silver birch leaves exposed for one month to several solar UV treatments (I). Mean values of five replicates per treatment are plotted with the standard errors. Treatment legend: CC (UV-A 100% UV-B 100%), CP (UV-A 100% UV-B 50%), CR (UV-A 50% UV-B 50%), PP (UV-A100% UV-B 0%), PR (UV-A 50% UV-B 0%) and RR (UV-A 0% UV-B 0%).

Arabidopsis has been used as model in studies analyzing UV-B induced global changes at the transcriptome (Ulm et al., 2004; Brown et al., 2005; Oravecz et al., 2006; Hectors et al., 2007; Favory et al., 2009) and metabolome levels (Lake et al., 2009; Kusano et al., 2011). These two types of approaches when combined bring useful information on plant regulation of metabolism in response to environmental signals. However, the number of such combined approaches addressing plant responses to UV-B remains limited (Kusano et al., 2011). Here, wild-type *Arabidopsis* (Ler and Col-0) and the mutants *uvr8-2* and *rcd1-1* were used in outdoor experiments to study the roles of UVR8 and RCD1, respectively, in regulating gene expression and metabolite accumulation in *Arabidopsis* (Publications III, IV). Our microarray analysis identified 96 genes showing differential expression in wild-type Ler exposed for 12 h to solar UV-B plus UV-A (Publication III). Most of these genes (67) had overlap in previous transcriptome analyses with wild-type plants irradiated with supplementary UV-B under controlled environments (Ulm et al., 2004; Brown et al., 2005; Oravecz et al., 2006; Hectors et al., 2007; Kilian et al., 2007; Favory et al., 2009). While these previous experiments under controlled environments have made substantial contributions to our understanding of how UV-B modifies gene expression, they may fail to identify the whole array of genes capable of responding to UV under sunlight (Publication III). This is supported by the fact that, after 12 h exposure, 29 genes showing differential expression under solar UV-B plus UV-A compared to no UV had not been previously identified as UV-B regulated by any of the studies mentioned above (Publication III). Thus, this group of genes may play specific roles mediating UV responses in *Arabidopsis* under sunlight, either through UV-A or through a distinct response to solar UV-B.

Accessions of *Arabidopsis* have earlier shown differences in their sensitivity to UV-B radiation (Cooley et al., 2001; Kalbina and Strid, 2006; Jansen et al., 2010). In agreement with these previous studies, Ler and Col-0 had different acclimation responses to solar UV (Publications III, IV). We observed differences between these two accessions in the solar UV regulation of the expression of several genes (Figure 6, Publications III, IV). ANOVA detected main significant effects of the genotype for the expression of *CHS*, *TT7*, *ATR4*, *F7A740* (At5g01520), *LOX*, *AOC3*, *AIF1*, *PMI2*, *SIGE*, *STO* and *PR1* after 12 h and *RUP2*, *VSP1*, *HAT2* after 36h ($P < 0.05$). The interaction UV treatment × genotype was also significant for *TT7* after 12 h ($P <$

0.05) indicating a UV-B effect on the expression of this gene in Col-0 but not in Ler (Figure 6).

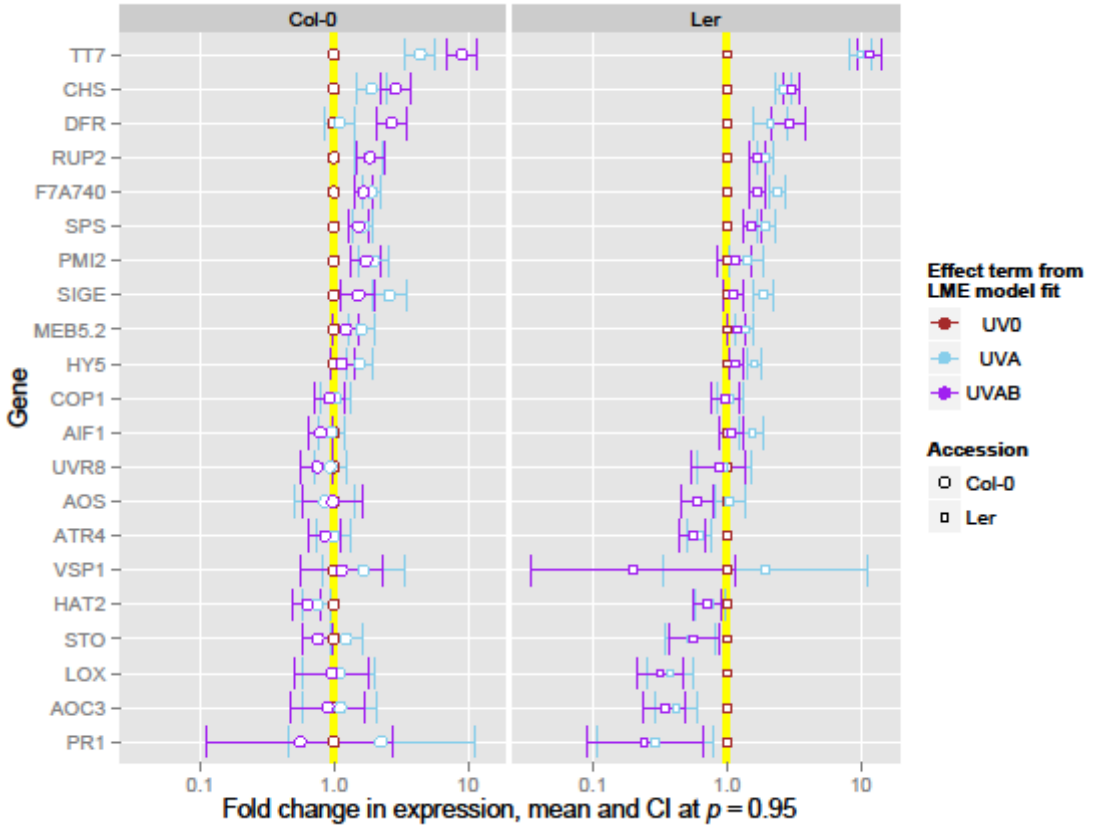

Figure 6. Effects of UV radiation on gene expression as measured by qPCR after 12 h of exposure to solar radiation in two accessions of *Arabidopsis*. Data points show mean effects of treatments on expression, as estimated from LME models fitted separately for each individual gene. Error bars show confidence intervals. Note the logarithmic scale on x-axis.

The UV-B photoreceptor UVR8 plays key roles in UV acclimation of plants growing in sunlight by regulating transcript accumulation of genes involved in the biosynthesis of flavonoids and anthocyanins (*DFR*, *LDOX*, *AT5MAT* and *PAP1*) and defence and responses (At4g12490, *LURP1*, *JAZ1*, *SYR1*, *WRKY70, JR1*, At1g16850, *AOC3, ANNAT1* and *MKK4*). In addition, UVR8 regulates the expression of genes involved in the biosynthesis and signaling of jasmonic acid (JA) (*AOS*, *AOC1*, *AOC3, WRKY70*, *JAZ1*, *SYR1* and *GRX480* and *OPR3*), glucosinolate biosynthesis (*ATR4* and *SOT17*), and auxin (*HAT2*) and brassinosteroid (*AIF1*) signaling (Publication III). Furthermore, the role of UVR8 in regulating gene expression in sunlight is more

complex than that determined by experiments under controlled environments and may involve the action of other light photoreceptors (Publication III).

The mechanism of UV-B signal transduction has been elucidated from experiments indoors where plants have been often grown in steady conditions of low PAR and devoid of UV-A (Ulm et al., 2004; Brown et al., 2005; Oravecz et al., 2006; Favory et al., 2009; Gruber et al., 2010; Liang et al., 2012; Huang et al., 2013). This type of experimental approach is needed to avoid complex interaction between UV-B signaling pathways with those triggered by UV-A and visible light and, thus, dissect mechanistically UV-B specific responses (Jenkins, 2009). However, plants growing in natural environments are exposed to much higher PAR than that used in controlled experiments, and also inevitable receive UV-B in the presence of UV-A. In fact, solar UV-A as present in sunlight makes approximately 98 % of the total UV irradiance as measured under clear sky at noon during sunny summer days (Publications III, IV).

Solar UV-A had a major role in regulating the expression of most UV-B regulated genes (Publications I, III, IV). Furthermore, our results may also indicate that UVR8 could mediate solar UV-A responses under natural sunlight given the impact of UVR8 on solar UV-A-mediated gene expression, acting as a positive and negative regulator of transcript accumulation of several genes (Publication III, Figure 2). Despite having maximal absorption in the UV-B, UVR8 also absorbs in the UV-A region of the spectrum (Figure 1, Christie et al., 2012). At 315 nm, the absorbance of UVR8 is approximately 10% of that at 300 nm (Christie et al., 2012). Based on the high UV-A irradiance present in sunlight together with a moderate absorbance of UVR8 in this band of the spectrum we propose that the UVR8-dependent response observed (*TT7* and *DFR* in Figure 9, Publication III) could indicate direct perception of solar UV-A through UVR8. Another role for solar UV-A radiation could be as a modulator of UV-B response. Under natural radiation various photoreceptors are simultaneously activated, and the control of several physiological events can occur through multiple interactions between photoreceptors (Casal, 2000; Jenkins et al., 2001; Chen et al., 2004). To test the possible roles of plant photoreceptors on the regulation of genes induced by solar UV in our conditions, the expression of genes up-regulated by solar UV-B plus UV-A in wild-type (Ler) after 12 h outdoors

(Publication III, Supplemental Table S1) was compared with available transcriptome data from experiments with photoreceptor mutants in *Arabidopsis* (Figure 7). This comparison shows that genes induced under our conditions were also up-regulated in wild-type Col-0 exposed to UV-B indoors. In addition, UV-B-treated *uvr8* and *cop1* mutants had lower expression of these genes than wild-type plants (Figure 7). Furthermore, most of solar UV induced genes in Ler are also regulated by CRY and PHY (Figure 7). This highlights that plants exposed to full spectrum sunlight integrate information from several pathways to convey UV acclimation.

Based on our observations in sunlight (Publication III) and those in the literature in controlled environments (Fuglevand et al., 1996; Boccalandro et al., 2004; Wade et al., 2001; Kleine et al., 2007) we hypothesize that under sunlight, the interaction between the UV-B and UV-A signaling pathways has a visible impact on the overall responses to solar UV. A possible mechanism for the interaction is proposed as a graphical model (Figure 8). This interaction most probably occurs through COP1 which plays a central role in both signaling pathways (Liu et al., 2011; Huang et al., 2013). After excitation by radiation, UVR8 monomers sequester COP1 as discussed in the introduction, while excited CRY can also interact with the COP1-SPA1 complex (Rizzini et al., 2011; Huang et al., 2013; Liu et al., 2011). As mentioned in publication III the WD40 domain of COP1 is a common point of interaction for several photoreceptors including UVR8 monomers and CRY (Heijde and Ulm, 2012). A recent study by Huang et al. (2013) supports our interaction hypothesis through COP1 (Publication III) by suggesting that UVR8 monomers compete with DDB1 for binding the WD40 domain of COP1. The interaction observed under sunlight's very low UV-B:UV-A photon ratio might be mediated by COP1 as depicted in the model; however, further research is required to unequivocally demonstrate the role of COP1 in mediating this interaction between the two signaling pathways.

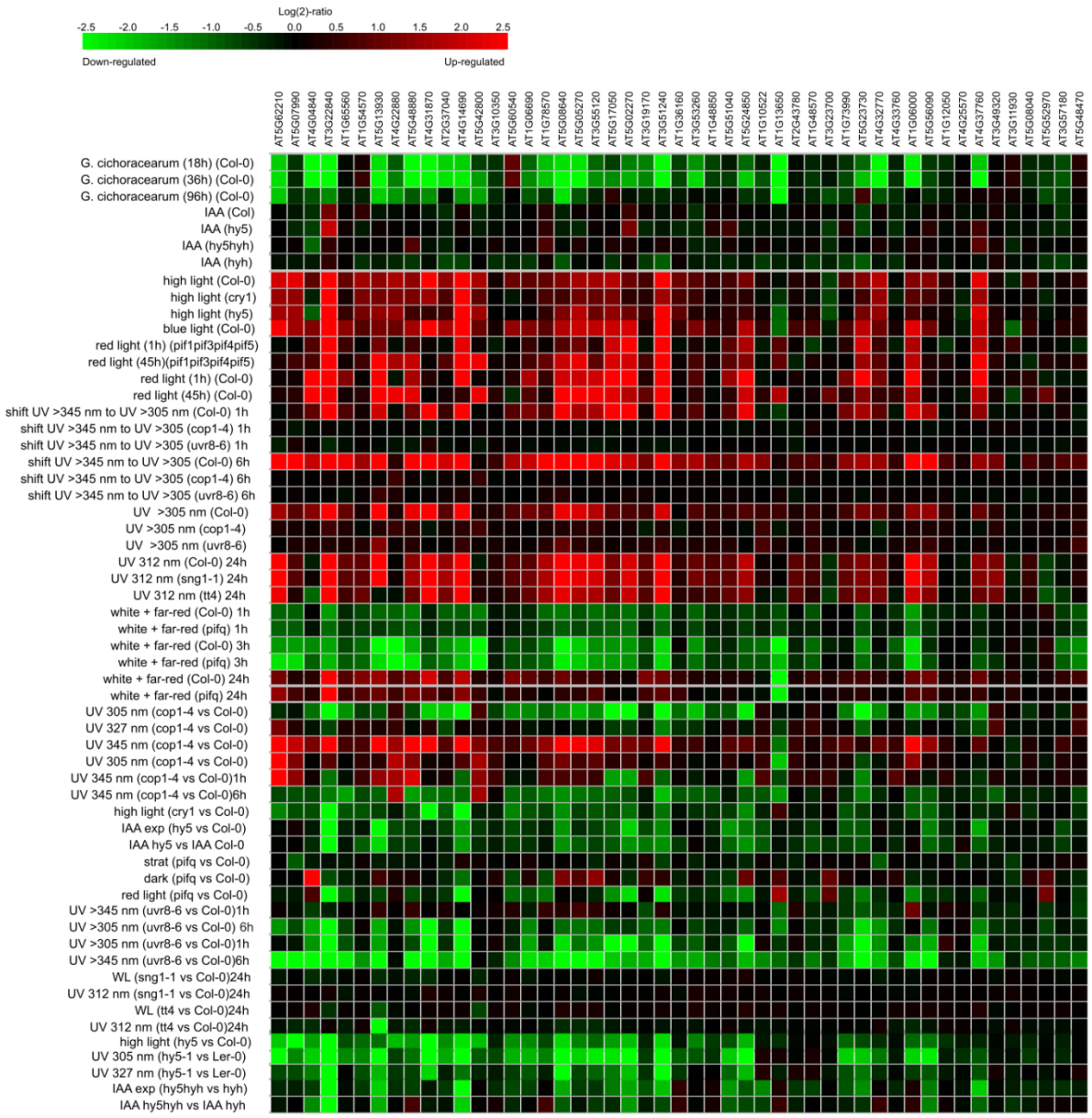

Figure 7. Heat map comparing the expression of solar UV induced genes in wild-type Ler exposed for 12h to solar UV with microarray data of photoreceptor mutants available in Genevestigator database (Hruz et al., 2008). The gene expression responses are calculated as $\log_2$-ratios between the signal intensities from treated genotypes vs controls. Red and green colours are used to indicate up-regulation and down-regulation of genes, respectively. Detailed information for the genes selected can be found in paper III, Supplemental Table S1. Expression profiles of the genotypes used in the figure were obtained from Genevestigator database IDs: AT-00246, AT-00390, AT-00516, AT-00528, AT-00254, AT-00257, AT-00528 and AT-00616.

We also propose that HY5, HYH and other unidentified transcription factors under the control of UVR8 and CRY could mediate or regulate the expression of genes involved in acclimation to solar UV in sunlight (Figure 8).

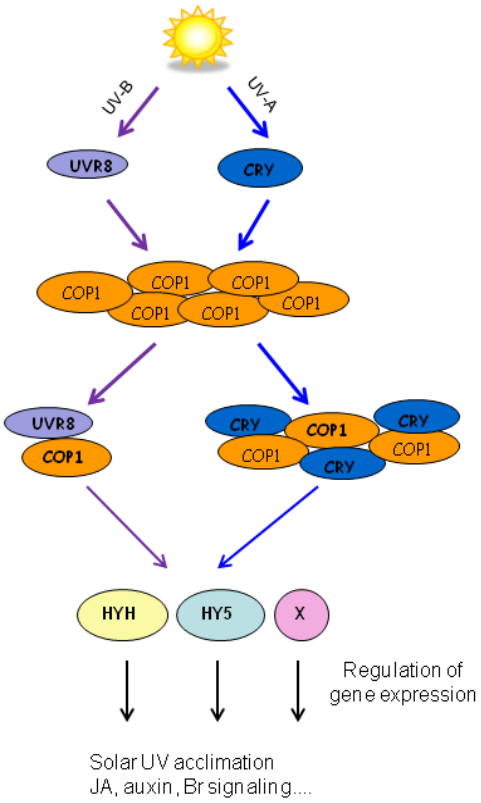

Figure 8. Proposed model for the interaction between UVR8 and CRY mediated UV-signaling in *Arabidopsis* under sunlight.

One striking observation from Figure 7 is the similar pattern of expression induced by infection of Col-0 with powdery mildew (*Golovinomyces cichoracearum*) (Christiansen et al., 2011) and the HY5 regulation of solar UV induced genes in high light and UV-B experiments (Figure 7). Thus, it may be possible that HY5 is a connecting link between UV signaling pathways and pathways regulating defense against biotic stressors. However, further research using *hy5* mutants in factorial experiments including both UV-B and biotic stressors is needed to clarify this possible interaction.

In contrast to UVR8, we could not assign a direct role to RCD1 in UV-B signaling under natural sunlight (Publication IV). This is supported by similar patterns of gene expression observed in Col-0 and *rcd-1* (Publication IV, Supporting information Fig. S1). For the same set of genes we show clear differences between *uvr8-2* and Ler under identical experimental conditions (Figure 9, Publication III). It has been proposed that RCD1 mediates stress responses in plants (Jaspers et al., 2009). However, under our conditions, genes related to stress were down-regulated by

solar UV indicating that plants preferentially expressed pathways related to acclimation rather than response to stress (Publications III, IV).

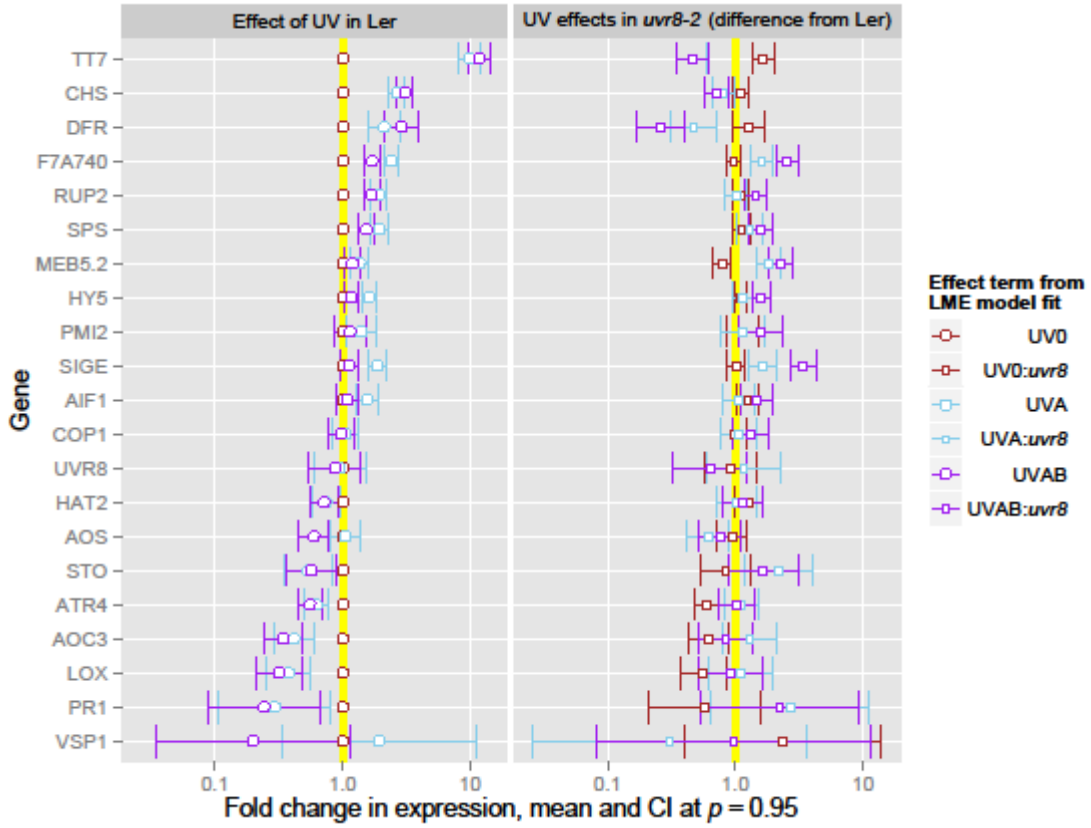

Figure 9. Effects of UV radiation on gene expression as measured by qPCR after 12 h of exposure to solar radiation in wild-type (Ler) and *uvr8-2*. Data points show mean effects of treatments on expression, as estimated from LME models fitted separately for each individual gene. Error bars show confidence intervals. Note the logarithmic scale on x-axis.

8.2 Solar UV-B and UV-A modify leaf chemistry

Solar UV-B and UV-A induced the accumulation of phenolics in leaves of silver birch and *Arabidopsis* (Publications I-IV). In *Arabidopsis,* accumulation of phenolic compounds after 12 h under solar UV-B plus UV-A was tightly regulated by coordinated expression of enzymes involved in the phenylpropanoid and flavonoid pathways (*PAL*, *CHS*, *CHI*, *TT7*, *DFR*, *FLS1*, *F3'H*, *LDOX* and *UGT78D2*) (Publication III). The most common phenolics detected in both species included derivatives of the flavonols quercetin and kaempferol (Publications I, III, IV). However, quercetin derivatives were the flavonols most strongly regulated by solar UV (Publications I, III, IV). This was also correlated with enhanced transcript

accumulation of enzymes involved in the conversion of dyhydrokaempferols to dyhydroquercetins (*F3'H*) (Publication III) and *TT7* (Publications III, IV). The dihydroxy B-ring-substitution and the catechol group present in quercetin derivatives are thought to improve their antioxidant capacity compared to that of monohydroxy B-ring kaempferols (Agati, 2012). In this context, quercetins have been shown to be more efficient than kaempferols in forming complexes with Cu and Fe ions which help to inhibit ROS generation. In addition, quercetin derivatives modulate the activity of several proteins involved in cell growth and differentiation (reviewed by Agati, 2012). In agreement with previous observations indoors (Ryan et al., 1998; Götz et al., 2010), our findings in sunlight highlight that quercetin derivatives may be the primary flavonoids conferring acclimation of silver birch and *Arabidopsis* to solar UV. Furthermore, the UV acclimation role of quercetins might vary in time as indicated by the changes in concentration observed for several quercetin derivatives (Publication III, Figure 4). Neither accumulation of *p*-coumaric and chlorogenic acids nor induction of UV-stress regulated genes were affected by solar UV under our conditions (Publications I, III, IV), indicating that these compounds may play a more important role in more stressful UV-B conditions (Lake et al., 2009).

In agreement with previous studies with silver birch (Lavola et al., 1997; Kostina et al., 2001; de la Rosa et al., 2001; Tegelberg et al., 2001; Kotilainen et al., 2009), we show that young and expanding leaves of seedlings growing outdoors for one month had enhanced accumulation of flavonoids by solar UV-B (Publication I). Furthermore, flavonoid accumulation in the leaves had a linear relationship with the solar UV-B doses received and appeared to be regulated by steady expression of *PAL* and *HYH* (Publication I). In agreement with the gene expression data, solar UV-A had also a regulatory role in the accumulation of flavonoids in the leaves. Solar UV-A induced the accumulation of quercetin-3-galactoside and quercetin-3-arabinopyranoside in a non-linear relationship (Publication I). We also show that solar UV regulates the accumulation of flavonoids in the leaf epidermis of silver birch seedlings as indicated by Dualex measurements (Publications I, II). Silver birch leaves at different stages of leaf expansion had similar sensitivities towards UV-B and showed increased levels of epidermal flavonoids when exposed to solar UV-B (Publication II). Moreover, young unfolded leaves alter their contents of epidermal flavonoids during their expansion following step changes in solar UV-B and UV-A irradiance in the

environment. Plants not previously exposed to UV showed a faster accumulation of epidermal flavonoids in the leaves when exposed to solar UV-B and UV-A than those previously acclimated to UV (Publication II). This response is most likely mediated by a quick UV induction of genes involved in the flavonoid pathway (Publication III). We also show that other environmental factors such as high PAR (Barnes et al., 2013) could play a role inducing UV protection in plants growing under low levels of solar UV (Publication II).

We also confirmed at the metabolite level the role of UVR8 conferring acclimation of *Arabidopsis* to solar UV. Leaves of *uvr8-2* exposed to solar UV-B failed to accumulate several phenolics compared to the wild-type (Publication III). This was observed for quercetin and kaempferol derivatives and for epidermal flavonoids and hydroxycinnamic acids estimated with Dualex (Publication III, Figures 4 and 5). This suggests that functional UVR8 may regulate UV-protection in different tissues of the leaves. However, the localization and function of UVR8 in different tissues has not yet been described. In agreement with the findings at the gene expression level presented and discussed above, our metabolite analysis shows that UVR8 had also an impact on the UV-A regulation of several metabolites (Publication III, Figure 4). In agreement with changes observed in gene expression, RCD1, in contrast to UVR8, modulates the accumulation of specific compounds independently of UV radiation (Publication IV). Based on these contrasting responses we conclude that in absence of stress, as in our experiment, only those phenolic compounds whose accumulation is regulated through photoreceptor signaling can respond to solar UV radiation.

The observed changes in leaf phenolics induced by solar UV-B and UV-A could play a significant role in the protection of silver birch and *Arabidopsis* in the natural environment against abiotic stress and the attack of herbivores and other pathogens (Izaguirre et al., 2003; Paul and Gwynn-Jones, 2003; Demkura et al., 2010; Demkura and Ballaré, 2012). In addition to phenolics, we also show that PDX1, a protein involved in vitamin B6 biosynthesis (Denslow et al., 2007) may play key roles in the acclimation of plants growing in sunlight (Publications III, IV). Quick accumulation of PDX1 was detected in leaves of *Arabidopsis* exposed to solar UV and ambient PAR (Publications III, IV). PDX1 levels in the leaves are more likely to be regulated by solar UV-A under our experimental conditions given the high UV-A irradiance in

sunlight (Publication III). However, solar UV-B trough UVR8 may modulate the solar UV-A induction of PDX1 in plants exposed to both solar UV-B and UV-A (Publication III). Consistent with gene expression and phenolic data, RCD1 was not involved in the solar UV induction of PDX1 (Publication IV).

8.3 Plant growth and morphological responses to solar UV

Although the effects of UV-B on plant growth reported in the literature are variable, it is generally accepted that growth regulation of perennial species is less sensitive to ambient levels of UV-B than that of herbaceous and annual plants (Ballaré et al., 2001; Searles et al., 2001; Li et al., 2010). Here, in agreement with the previous statement and other reports in silver birch (Kostina et al., 2001; Tegelberg et al., 2001; Kotilainen et al., 2009) and other tree species (Hunt and McNeil, 1999; Sullivan, 2005), we show that different doses of solar UV-B and UV-A within the ambient range had a negligible effect on the growth, and biomass production of silver birch seedlings (Publication I). In addition, photosynthesis was unlikely to be affected by solar UV since chlorophyll (Chl) concentrations estimated in the leaves with SPAD remained unchanged (Publication I, Tegelberg et al., 2001: Kotilainen et al., 2009). In the review by Allen et al. (1998) it was concluded that UV-B-induced reduction of biomass is likely a result of reduced leaf area rather than direct damage of UV-B to photosynthesis. It has been reported that *Arabidopsis* plants exposed to UV-B have reduced growth and leaf expansion (Cooley et al., 2001; Hectors et al., 2007; Wargent et al., 2009). Technical issues prevented us to measure accurately growth parameters in *Arabidopsis* germinated and grown outdoors for three weeks (Publication III). However, in agreement with Hectors et al., (2007) *Arabidopsis* exposed to solar UV-B plus UV-A for 12h had decreased expression of genes involved in auxin (*HAT2* and *SAUR6*) and brassinosteroid (*AIF1*) signaling and cell expansion (Publications III, IV). Furthermore, the expression of *HAT2*, which is required for a positive regulation of auxin signaling in shoot tissues (Sawa et al., 2002), was lower in *uvr8-2* than in the wild-type after 12 h and three weeks outdoors under solar UV-B plus UV-A (Publication III). It is also known that under UV-B, UVR8 is required for normal leaf growth of *Arabidopsis* by increasing cell area, density of stomata and the regulation of endopoliploidy (Wargent et al., 2009). Taken together,

solar UV through UVR8 and auxin signaling might modify the growth of *Arabidopsis* in sunlight, especially at early stages of development (Publication III).

9. Conclusions

After assessing solar UV-B and UV-A-induced changes in gene expression and phenolic metabolite, and investigating the role of the photoreceptor UVR8 and the stress regulator RCD1 in their regulation, we conclude for each of the hypotheses given above:

1) Both solar UV-B and UV-A trigger changes in gene expression and metabolite accumulation that promote plant acclimation to sunlight (Publications I - IV). At the level of transcription, solar UV-B plus UV-A induce transcript accumulation of genes involved in UV protection (Publications I, III, IV), oxidative stress, defence against herbivores, and the signaling and biosynthesis of several plant hormones including auxin, brassinosteroids and JA in *Arabidopsis* (Publications III, IV). At the metabolite level, the accumulation of phenolics was strongly regulated by solar UV-B and UV-A in the leaves of both species (Publications I- IV), and this response was correlated with changes observed in gene expression (Publications I, III, IV).

2) Under full spectrum sunlight, UV-A modifies plant responses to UV-B most likely through complex interactions between UVR8 and CRY (section 8.1, Publication III).

3) The accumulation of epidermal flavonoids in silver birch leaves depends on leaf age and the direction of step changes in solar UV-B and UV-A irradiance. Solar UV-B induces the accumulation of epidermal flavonoids irrespective of leaf age. However, the youngest leaves are more capable of adjusting their flavonoid contents under dynamic fluxes of UV-B and UV-A during their expansion than older leaves, and this response is affected by their previous UV exposure history (Publication II).

4) The UV acclimation of *Arabidopsis* dependent on UVR8 is important at early stages of plant development for the normal growth of plants and for the expression of genes promoting long term acclimation to sunlight. However, survival of

Arabidopsis under sunlight is not dependent solely on UV-B perception by the photoreceptor UVR8 (Publication III).

5) Under sunlight, the stress regulator RCD1 plays no active role in UV signaling and acclimation in *Arabidopsis*. RCD1 is not involved in the regulation of transcript accumulation of genes with functions in UV acclimation, but modulates the solar UV-induction of flavonoid accumulation in the leaves (Publication IV).

The findings presented in this thesis represent a step forward in our understanding of the molecular mechanisms behind solar UV-B and UV-A perception and signaling responses that these wavebands mediate under sunlight. The results obtained highlight the complex interactions between responses to UV-B and UV-A and the dependence of the overall response to sunlight on the balance between these two wavebands. This knowledge will be applicable in horticultural practices that aim to improve produce quality through better nutritional and pharmacological properties such as increased concentration of phenolic compounds.

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