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Molecular genetic analysis of the UV-B response in *Arabidopsis thaliana*: the positive regulatory roles of the E3 ubiquitin ligase COP1 and the bZIP transcription factor HY5

Ph.D. Dissertation

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

35S	promoter of the CaMV 35S RNS gene
6-4PP	6-4 photoproducts
BER	base excision repair
bHLH	basic helix-loop-helix motif
bps	base pairs
bZIP	basic leucine-zipper motif
CaMV	cauliflower mosaic virus
CDD	COP10-DDB1-DET1 complex
CHS	CHALCONE SYNTHASE
Col	*Arabidopsis thaliana* Columbia accession
Cop	constitutive photomorphogenic
COP1	CONSTITUTIVE PHOTOMORPHOGENIC 1
CPD	cyclobutane pyrimidine dimers
Cry1/2	Cryptochrome 1/2 (holoproteins)
CSN	COP9 SIGNALOSOME
CUL	CULLIN
DDB1	UV-DAMAGED DNA BINDING PROTEIN 1
DET1	DE-ETIOLATED 1
DREB2A	*DROUGHT RESPONSIVE ELEMENT-BINDING PROTEIN 2A*
eid6	*empfindlicher im dunkelroten licht 6*
ELIP2	*EARLY LIGHT INDUCED PROTEIN 2*
FUS	FUSCA
GFP	GREEN FLUORESCENT PROTEIN
HFR1	LONG HYPOCOTYL IN FAR-RED LIGHT 1
HY5	ELONGATED HYPOCOTYL 5
HYH	HY5-HOMOLOG
icx1	*increased chalcone synthase expression 1*
kb	kilobasepair
kDa	kilodalton
LAF1	LONG AFTER FAR-RED LIGHT 1
Ler	*Arabidopsis thaliana* Landsberg *erecta* accession
NAM-like	*NO APICAL MERISTEM-like*
NER	nucleotide excision repair

NES	nuclear export signal
NIS	nuclear import signal
PAL	PHENYLALANINE AMMONIUM LYASE
Phot1/2	Phototropin 1/2 (holoproteins)
PhyA/B/C/D/E	Phytochrome A/B/C/D/E (holoproteins)
Pro	promoter
RBX1	RING-BOX 1
RCC1	REGULATOR OF CHROMATIN CONDENSATION 1
RING	REALLY INTERESTING NEW GENE
ROS	reactive oxygen species
rRNA	ribosomal RNA
SPA	SUPPRESSOR OF PHYA
TT4/5	TRANSPARENT TESTA 4/5
ULI3	UV-B LIGHT INSENSITIVE 1
UV-A/B/C	ultraviolet-A/B/C
UVR8	UV-RESISTANCE LOCUS 8
WL	white light
Ws	*Arabidopsis thaliana* Wassilewskija accession
XP	xeroderma pigmentosum
YFP	YELLOW FLUORESCENT PROTEIN

1. INTRODUCTION AND AIMS OF THE WORK

Light is the vital source of energy on Earth. The light eradiated by the Sun provides the ultimate energy for virtually all living organisms, among which, however, only plants and some bacteria are able to directly absorb and convert this radiation into bio-utilizable chemical energy. Light, on the other hand, is not exclusively a source of energy that drives photosynthesis, but is also one of the most important environmental cues conveying essential information about the surroundings. The ability to accurately sense and respond to the ever-changing environment, including changes in light quality, quantity and duration, is essential for the optimal performance of plants. Therefore, plants have evolved sophisticated photosensory systems to continuously monitor their light environment. Phytochromes are photoreceptors specialized in the sensing of red and far-red light ranges of the light spectrum, whereas cryptochromes and phototropins absorb photons of the blue and UV-A wavelength ranges. Light perception by these photoreceptors triggers complex transcriptional cascades and signal transduction relays to govern a multitude of morphological and physiological responses over the entire life-cycle of plants.

An intrinsic part of the electromagnetic radiation of the Sun that reaches the biosphere is UV-B (Ultraviolet-B) light. Owing to their sessile lifestyle and to their absolute dependence on light, plants inevitably have to face exposure to UV-B. High levels of UV radiation can evoke a diverse range of cellular stresses including oxidative stress, damage to the lipid and protein components of virtually all cell compartments and, most importantly, DNA and RNA damage. UV-B however is not a mere stress agent. Similarly to other regions of the light spectrum, low levels of UV-B may also act as an environmental signal conveying essential information about the ambient light settings. Investigations of recent years revealed that plants respond to low levels of UV-B irradiation by a set of physiological responses, presumably under the regulation of UV-B specific photoreceptor(s). Such responses include photomorphogenesis and prophylactic responses such as the induction of the biosynthesis of secondary metabolites.

In contrast to the perception and signal transduction of visible light and UV-A, the components mediating non-damage-related UV-B specific responses have remained largely unknown so far. This notion is best exemplified by the lack of a molecularly identified UV-B photoreceptor(s). Therefore the aim of our work is to widen our understanding of the molecular mechanisms underlying UV-B perception and signal transduction, first by assessing the specific transcriptional changes triggered by low-level UV-B irradiation. This knowledge provides a further base for the identification and analysis of potential new

components of UV-B responses, and the comparison of signalling mechanisms driven by visible light and UV-B.

According to this, and based on our first result the following goals were pursued in this study:

1. The characterisation of low level UV-B induced transcriptional responses in white light-grown *Arabidopsis* seedlings. The investigation of the known photoreceptors in these responses using biochemical and genetic tools.
2. The analysis of potential roles of HY5 and HYH transcription factors in UV-B responses by genetic, recombinant genetic, biochemical, cell biological and physiological means.
3. The assessment of the function of COP1, a major negative regulator of HY5 and HYH in light signal transduction, in UV-B signalling by different genetic, recombinant genetic, biochemical, cell biological and physiological approaches.
4. The investigation of COP1-HY5 relation in UV-B signalling with regard to HY5 protein stability using biochemical and a pharmacological approach.
5. The assessment of the potential roles of known COP1-regulators in UV-B signalling based on the analysis of transcriptional responses.

2. LITERATURE OVERVIEW

The survival of all living organisms depends on their ability to accurately sense and respond to the ambient environment. Among all environmental cues, light is of utmost importance for plants that, beside some bacteria, are the sole living entities being able to directly absorb and convert sunlight into bio-utilisable chemical energy. The perception and the signal transduction of light have pivotal role in the regulation of a wide range of physiological responses throughout the plant life-cycle, including seed germination, seedling photomorphogenesis, photo- and gravitropism, chloroplast movement, shade avoidance, circadian rhythms and flower induction (Jiao et al., 2007).

The profound effect of light is perhaps most saliently demonstrated during the early development of seedlings that occur in between the time of germination and the advance of the first true leafs. This process, whereby plants mature from an endosperm-dependent embryo to a self-sufficient photoautotroph is termed photomorphogenesis (e.g., Nemhauser and Chory, 2002). Under conditions when light is limiting or is not present at all, seedlings exhibit etiolated or so called skotomorphogenic development that is characterized by the lack of pigmentation, elongated hypocotyls topped by tightly closed and underdeveloped cotyledons protected by an apical hook, and a limited root system (Figure 2). The main purpose of such a growth pattern is to ensure seedling emergence through the soil out to the light a process that uses the nutrient supplies stored in the seed endosperm. When seedlings become exposed to light, their development switches from the skoto- to the photomorphogenic program, also termed as de-etiolation, during which hypocotyl elongation ceases, the cotyledons open up, enlarge and turn green and roots develop (Figure 2). This fundamental change in growth and

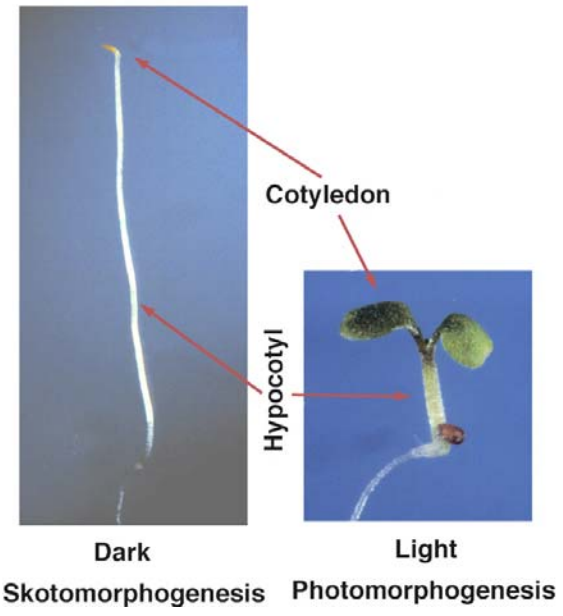

Figure 2. The opposing phenotypes of dark and light grown *Arabidopsis* seedlings
Dark grown seedlings undergo skotomorphogenesis or etiolation characterized by elongated hypocotyls, closed and underdeveloped cotyledons and apical hook. Light grown seedlings undergo photomorphogenesis or de-etiolation and are characterized by short hypocotyls, open and expanded green cotyledons (from Wang and Deng, 2002).

developmental pattern requires sophisticated detection and interpretation of a wide range of environmental signals, of which light perception has utmost importance, and not only in the seedling stage, but over the entire life of a plant.

2.1. Light perception and signalling

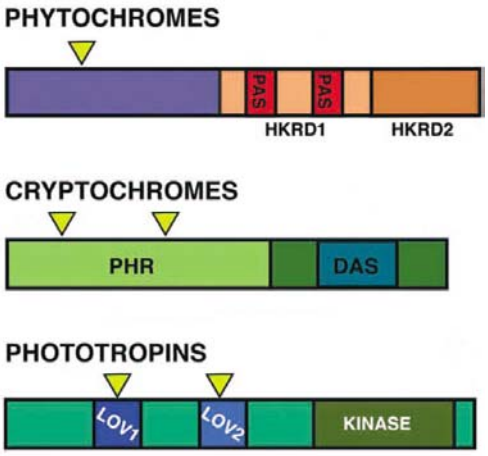

Figure 2.1. The schematic structures of the three classes of plant photoreceptors
The red/far red light-sensing phytochromes contain two carboxy-terminal histidine kinase domains (HKRD) and two Per-Amt-Sim domains (PAS) within the HKRD1 domain. The blue/UV-A-sensing cryptochromes have an amino-terminal photolyase related domain (PHR) and a DQXVP-acidic-STATES (DAS) motif at their C-terminus. The also blue/UV-A-sensing phototropins contain two chromophores binding domains (LOV1 and LOV2) and a C-terminal kinase domain. Yellow triangles represent the chromophores attachment sites in each of the photoreceptos (modified after Sullivan and Deng, 2003).

Light is the ultimate source of biological energy for plant life and therefore it is arguably the most important environmental parameter, which conveys essential information for survival. Being sessile and photoautotrophic, plants are extremely sensitive to this vital environmental signal. Accordingly, they adopted sophisticated photosensory systems in order to continuously monitor the absence or presence, colour (wavelength), intensity (fluence rate), directionality and diurnal duration of the incident light. Three classes of plant photoreceptors have been described that track almost the entire spectrum of the terrestrial sunlight. Four classes of photoreceptors monitor the light spectrum in plants: the phytochromes detect far-red/red region, the cryptochromes and phototropins the blue/UV-A part of the spectrum (Figure 2.1.). The nature of the photoreceptor(s) that sense the UV-B spectral region is still elusive (Nagy and Schafer, 2002; Quail, 2002a; Wang and Deng, 2002; Gyula et al., 2003; Chen et al., 2004; Ulm, 2006).

2.1.1. The photoreceptors

2.1.1.1. Phytochromes

Phytochromes are dimeric chromoproteins of two ~125 kDa polypeptides, each containing a covalently linked linear tetrapyrrol chromophore, phytochromobilin that is synthesized in the chloroplasts from heme. The phytochrome protein consists of two structural domains connected by a flexible hinge region. The N-terminal is the photosensory domain that contains a bilin lyase subdomain with a conserved cysteine that forms a thioether linkage with

the A ring of the phytochromobilin. The C-terminal half of the protein is believed to be the regulatory or signal-output domain. It contains the regulatory core sequence, termed as Quail-box that overlaps with a pair of PAS (PER-ARNT-SIM) domains which either serve as platforms for protein-protein interactions or for response modules for small ligands. The phytochrome carboxy-terminal domain further contains two dimerization motifs and two histidine kinase-related domains (Figure 2.1.) (Quail, 2002b; Wang and Deng, 2002; Chen et al., 2004). Phytochromes mainly absorb red/far-red light, however they also have a smaller absorption peak in blue light (Wang and Deng, 2002). The photosensory properties of phytochromes reside on their capacity to undergo reversible, light induced interconversion between two conformers. Phytochromes are synthesized in the cytoplasm in their physiologically inactive red-light absorbing (P_r) conformer. Absorption of a photon results in the photoconversion to the physiologically active, far-red-light absorbing (P_{fr}) conformer. On the subsequent absorption of far-red light, the P_{fr} converts back to P_r, or alternatively, in the absence of light it undergoes slow dark reversion, also resulting in the P_r form (e.g., Wang and Deng, 2002). Phytochromes are cytosolic in their P_r form but photoconversion to the P_{fr} result in their translocation to the nucleus, which is crucial to allow the interaction with transducers to initiate downstream transcriptional cascades (Nagy and Schafer, 2002; Wang and Deng, 2002; Chen et al., 2004).

Phytochromes have been classified to two types: the type I photo-labile phytochromes degrade rapidly upon light exposure whereas the type II photo-stabile phytochromes are relatively stable in light. Among the five phytochromes encoded in the *Arabidopsis* genome, PhyA is a type I, whereas PhyB to PhyE belongs to the type II class (Chen et al., 2004).

2.1.1.2. Cryptochromes

Cryptochromes (CRYs) are blue/UV-A light sensing flavoproteins commonly present in plants, animals and bacteria. Although they are structurally related to DNA photolyases, cryptochromes do not possess any DNA repair activity (Sancar, 2003). Their amino-terminal photolyase homology region (PHR) carries two, noncovalently bound chromophores: a primary or catalytic flavin adenine dinucleotide (FAD) and a second light-harvesting chromophore, a pterin or deazaflavin (Figure 2.1.) (Lin and Shalitin, 2003; Liscum et al., 2003). The photochemical mechanism of signal capture and transfer has remained undefined so far, however, as in case of the photolyases it likely involves a redox reaction (Chen et al., 2004). Beside the PHR domain, the carboxy-terminal parts of most plant cryptochromes contain a variable extension that is not present in photolyases but is essential for Cry function, at least in the case of the two cryptochromes present in *Arabidopsis*, Cry1 and Cry2 (Gyula et al., 2003; Lin and Shalitin, 2003; Liscum et al., 2003). The two cryptochromes exhibit distinct

subcellular localization depending on light conditions: Cry1 is primarily found in the nucleus in dark but largely cytoplasmic in light, whereas Cry2 exhibit constitutively localized to the nucleus (Chen et al., 2004; Jiao et al., 2007). Nuclear localised cryptochromes closely interact with the chromatin (Lin and Shalitin, 2003). Similar to the different classes of phytochromes, the two cryptochromes likewise display distinct light stability: Cry1 is light-stable, whereas Cry2 is rapidly degraded upon blue-light exposure (Gyula et al., 2003; Lin and Shalitin, 2003). *Arabidopsis* has a third cryptochrome, Cry3, that differ significantly from the other two as it lacks the C-terminal domain but has an N-terminal transient peptide sequence targeting the protein to both chloroplasts and mitochondria. This suggests a potential role for Cry3 in the regulation of transcription in the organelles (Kleine et al., 2003).

2.1.1.3. Phototropins

Phototropins are also blue/UV-A light-absorbing flavoproteins, of which two can be found in *Arabidopsis*: Phot1 and Phot2 (Briggs and Christie, 2002; Liscum et al., 2003). The C-terminal portions of phototropins contain a classical Ser/Thr kinase domain, whereas the N-terminal halves carry two LOV (LIGHT, OXIGEN, VOLTAGE) domains, which are structurally related to the PAS domains (Figure 2.1.). Both LOV domains, of which LOV2 is the primary light sensing motif, tightly associates with a flavin adenine mononucleotide (FMN) chromophore that potentiate light sensing (Liscum et al., 2003). Light absorption triggers the transient covalent binding of the FMN to a conserved cysteine residue in the core of the LOV domain, which is followed by the relatively slow return to the ground state (Chen et al., 2004). Upon signal capture, both Phot1 and Phot2 undergo autophosphorylation, which is likely the initial step of downstream signalling events. Both phototropins are primarily localised to the plasma membrane, however upon light activation a fraction of Phot1 is released to the cytosol (Chen et al., 2004).

2.1.2. Photoreceptor-mediated light responses

Light perception by each of the three classes of photoreceptors may trigger specific responses. However, for most of the developmental processes more than one photoreceptor can contribute to the perception of light signals resulting in a complex network of interactions among the different photosensory pathways. For instance, seed germination and shade avoidance are solely controlled by phytochromes, partly through the very low fluence response (VLFR) activity of PhyA and the low fluence response (LFR) activity of PhyB and PhyE (Wang and Deng, 2002; Chen et al., 2004). Furthermore, Cry1 is the primary photoreceptor under high blue light fluences, whereas Cry2 is more important under low fluence rates of blue light. The blue light mediated remodelling of the transcriptional program

is mostly achieved through Cry1 and Cry2, albeit to a lesser extent both phototropins and PhyA contribute to this activity (Chen et al., 2004). The two phototropins are the primary photoreceptors that mediate light induced curvature. Phot1 is specialized for low blue light fluences and, in addition to phototropism, mediates the chloroplast accumulation response (redundantly with Phot2) to maximise light capture in low light. Phot2 on the contrary is more important for high light responses that mediate chloroplast avoidance response to minimize chloroplast photodamage in high light (Briggs and Christie, 2002). However, the amplitude of both chloroplast movement and phototropism responses is modulated by both cryptochromes and phytochromes (Chen et al., 2004). Phototropins also control blue-light driven stomatal opening, a response that is also mediated by other photosensory systems including UV-B receptor(s) (Briggs and Christie, 2002; Eisinger et al., 2003; Ulm, 2006). Additionally, Phot1 transiently controls light-mediated inhibition of hypocotyl growth in concert with other photoreceptors, and the phototropins redundantly control cotyledon and leaf expansion (Briggs and Christie, 2002; Chen et al., 2004).

The complexity of photoreceptor regulatory mechanisms is further exemplified by the fact that different members of the same photoreceptor family may monitor essentially the same light signals while regulating predominantly distinct physiological responses, and conversely, detection of different light qualities by different receptors of either common or distinct families, may mediate the same responses (Quail, 2002b). For example PhyB mainly regulates seedling establishment, typically through red light high irradiance response (R-HIR) activity, whereas PhyE is rather confined to regulate stem elongation in red light. On the contrary, the de-etiolation process is controlled both by the far-red-light-sensing PhyA and the red-light-sensing PhyB and PhyC of the phytochrome family through FR-HIR and R-HIR activity, respectively, and also by redundantly the blue-light sensing Cry1 and Cry2 (Quail, 2002b; Wang and Deng, 2002; Chen et al., 2004). Beside seedling establishment, photoperiod induced flowering and the entrainment of the circadian clock are also processes being controlled by interconnected networks of phytochromes and cryptochromes (Casal, 2000; Yanovsky and Kay, 2003). Furthermore, an additional level of complexity is indicated by the direct physical interaction between the two families of phytochromes and cryptochromes (Ahmad et al., 1998; Mas et al., 2000). Moreover, light and other environmental signals often act together to mediate specific developmental responses indicating the existence of integration points between these signalling pathways as well.

2.1.3. Light induces complex signalling pathways and transcriptional networks

Consistent with the profound change of morphology and development, light induces the extensive reprogramming of plant transcriptome that in *Arabidopsis* results in the differential expression of at least 20% of the whole genome and that ultimately leads to the coordinate regulation of most of the major biochemical pathways upon dark-to-light transition (Ma et al., 2001; Tepperman et al., 2001; Jiao et al., 2005). Transcriptional regulatory networks have fundamental role in the control of light responses through the orchestrated activation and repression of downstream genes. For example, 44% and 25% of early far-red and red light induced genes, respectively, are putative or established transcriptional regulators (Lee et al., 2007). Some of such regulators are specific for a given light quality, while others participate in the regulation of signalling pathways downstream of several photoreceptors. These signal relays modulate highly overlapping set of genes, indicating the presence of signal integration points and shared signalling components among them.

Most of our understanding on photoreceptor functions and downstream regulatory processes come from the investigation of the initial major developmental transition of plants, the de-etiolation process. Conventional genetic screens aimed at the identification of signalling intermediates of light responses revealed two classes of mutants (e.g. Quail, 2002a). One is the photodefective class, encompassing mutants that develop normally in darkness but exhibit reduced or enhanced responsiveness to various light inputs. Those having reduced responsiveness represent mutants of positive regulators, whereas those with enhanced light-responsiveness represent negatively acting effectors. The former group include the photoreceptor mutants and mutants being defective in the biosynthesis of chromophores (Parks and Quail, 1991, 1993; Reed et al., 1993; Whitelam et al., 1993). Transcriptional regulators downstream of the photoreceptors may regulate specifically either PhyA or PhyB, or phytochrome versus cryptochrome and phototropin signalling (Quail, 2002a). For example, the putative transcription factors FAR1 (FAR-RED IMPAIRED RESPONSE) and FHY3 (LONG HYPOCOTYL IN FAR-RED LIGHT 3), and the R2R3-myb transcription factor LAF1 (LONG AFTER FAR-RED LIGHT 1) are PhyA specific positive regulators of far-red light signalling (Quail, 2002a; Jiao et al., 2007). Light induces the translocation of the active phytochrome conformers (P_{fr}) into the nucleus, where they can interact with transcription factors and thus can directly initiate and regulate light-induced signalling and gene expression (Nagy and Schafer, 2002). The bHLH transcription factors PIFs (PHYTOCHROME INTERACTING FACTORs) act mainly as negative regulators of phytochrome signalling, and they represent such a direct link between photoreceptors and transcriptional regulation. Another phytochrome-response-regulator, the Daf family transcription factor COG1,

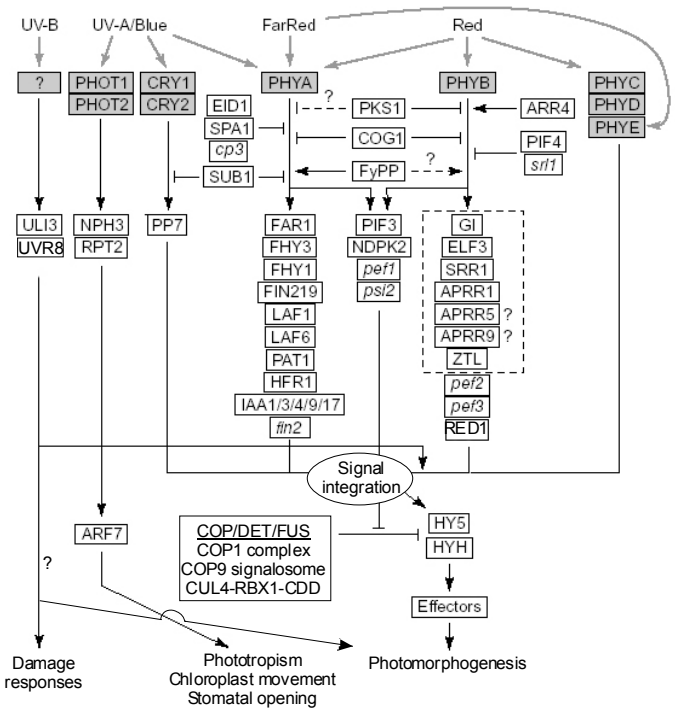

Figure 2.1.3. Schematic overview of photosensory signalling pathways

Cloned components are capitalised; genetically identified but not yet cloned components are in lower case and italics. Shaded boxes represent the photoreceptors. Components that are common to light-input pathways and the circadian clock are framed in a dashed-outline box. Arrows indicate positive, blocked arrows inhibitory correlation. See text for details. Abbreviation not present in the text are follows: APRR1/5/9, ARABIDOPSIS PSEUDO-RESPONSE REGULATOR1/5/9; ARF7, AUXIN RESPONSE FACTOR7; COG1, COGWHEEL1; cp3, compacta3; ELF3, EARLY FLOWERING3; FHY1/2, LONG HYPOCOTYL IN FAR-RED LIGHT1/2; FIN219, FAR-RED INSENSITIVE219; GI, GIGANTEA; IAA1/3/4/9/17, INDOLE-3-ACETIC ACID RESPONSE FACTOR1/3/4/9/17; LAF1/6, LONG AFTER FAR-RED LIGHT1/6; NDPK2, NUCLEOSIDE DIPHOSPHATE KINASE2; PAT1, PHYA SIGNAL TRANSDUCTION1; PSI2, PHYTOCHROME SIGNALLING2; RED1, RED LIGHT ELONGATED 1; srl1, short hypocotyl in red light; SRR1, SENSITIVITY TO RED LIGHT REDUCED1; SUB1, SHORT UNDER BLUE LIGHT1, UVR8, UV RESISTANCE LOCUS 8. (modified after Quail, 2002a; Gyula et al., 2003; Brown et al., 2005)

negatively regulates both PhyA and PhyB responses, whereas SUB1 is a negative regulator of cryptochrome and PhyA signalling. On the contrary, HFR1 (LONG HYPOCOTYL IN FAR-RED LIGHT 1), a bHLH transcription factor, is a positive regulator of both cryptochrome and PhyA responses (e.g. Chen et al., 2004; Jiao et al., 2007). However, not only transcription factors have been identified as regulators of photoreceptor mediated signal transduction. The SPA1 (SUPPRESSOR OF PHYA 1) proteins and the F-box protein EID1 (EMPFINDLICHER IM DUNKELROTEN LICHT 1) are factors that negatively regulate PhyA signalling possibly through the regulation of the proteolytic elimination of components of the PhyA pathway (Gyula et al., 2003). Most of these, and also other components in the photoreceptor pathways suggest that early events in each pathway involve intermediates dedicated to specific photoreceptors and that these separate pathways converge downstream in a signal integration process to drive common output responses (Figure 2.1.3.).

The other class of mutants revealed by classical genetic screens are those that exhibit light-grown phenotypes even when kept in complete darkness. These mutants are more or less pleiotropic, and the corresponding components have been implicated as negative regulators of photomorphogenic development that act downstream of the convergence of the phytochrome and cryptochrome pathways (Figure 2.1.3.). The 11 genes represented by these mutants

comprise the COP/DET/FUS loci (for CONSTITUTIVE PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA). In fact, 9 out of these 11 genes encode components of multisubunit protein complexes implicated in the controlled degradation of a number of regulatory intermediates in the light signalling pathways (Hardtke and Deng, 2000; Wei and Deng, 2003; Yi and Deng, 2005; Bernhardt et al., 2006; Chen et al., 2006) (see later in section 2.1.7.3.).

2.1.4. HY5 as a main signal integrator and promoter of photomorphogenesis

The fact that the perception of distinct light qualities through different photoreceptors results in the regulation of overlapping sets of genes is indicative of the presence of integration points for these different light signalling pathways. Accordingly, some light-regulated transcription factors have been identified as key regulators during photomorphogenesis. HY5 (for ELONGATED HYPOCOTYL 5) is the first known and the most extensively studied positive regulator of photomorphogenesis. *hy5* mutants exhibit partially etiolated phenotype under all light conditions indicating that HY5 acts downstream of multiple families of the photoreceptors (Oyama et al., 1997; Ang et al., 1998). Consistent with its key-regulatory role, 20% of the light induced genes are in fact regulated by HY5 (Ma et al., 2002). This regulation is most likely performed through binding directly to the promoter regions of a large number of genes (Lee et al., 2007). A typical regulatory region of such type is the G-box elements of light responsive promoters commonly found in a wide range of light regulated genes, such as CHS and RBCS-1A (Ang et al., 1998; Chattopadhyay et al., 1998). HY5 has also been suggested to act as an integration point for light and hormone signalling (Cluis et al., 2004).

HY5 protein levels are dynamically regulated in response to light by both transcriptional and post-translational mechanisms, the letter including phosphorylation, and also proteasomal degradation mediated through the COP/DET/FUS protein-degradation machinery (see also section 2.1.6.). CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), a member of the COP/DET/FUS proteins, interacts with HY5 and promotes its degradation through the Ubiquitin/26S proteasome system in darkness (Osterlund et al., 2000). Light perception, on one hand, inactivates this process and thus stabilizes the HY5 protein, while on the other hand results in the rapid transcriptional activation of the *HY5* gene (Tepperman et al., 2001; Jiao et al., 2007). Additionally, HY5 protein is also phosphorylated through a light regulated kinase activity that is more pronounced in dark grown seedlings. Phosphorylation of a serine residue in a consensus CASEIN KINASE II (CKII) site in the COP1-interacting domain weakens the COP1-HY5 interaction and thus stabilizes HY5. On the other hand, the phosphorylated HY5 protein is less effective in binding to its target promoters. Thus, on one side, regulated HY5

phosphorylation in concert with light induced transcription provides abundant and physiologically more active HY5 protein in light, whereas on the other side it helps to maintain a small pool of less active but more stable phosphorylated HY5 protein in darkness, which could support rapid initial responses upon light perception (Hardtke et al., 2000).

Consistent with its role in promoting photomorphogenesis in early seedling development, HY5 protein abundance peaks at 2-3 days after germination, after which it ceases to nearly undetectable levels. Interestingly, it resumes in inflorescence suggesting a possible role for HY5 activity in this developmental stage that is further supported by the finding that *hy5* mutants display early flowering phenotype under long-day conditions (Hardtke et al., 2000; Holm et al., 2002).

2.1.5. COP1 as a main signal integrator and repressor of photomorphogenesis

COP1 is one of the earliest identified and most extensively studied light signalling component. It functions as a main repressor of photomorphogenic development under dark conditions. More than 20% of the genome, representing 28 pathways is regulated by COP1 in

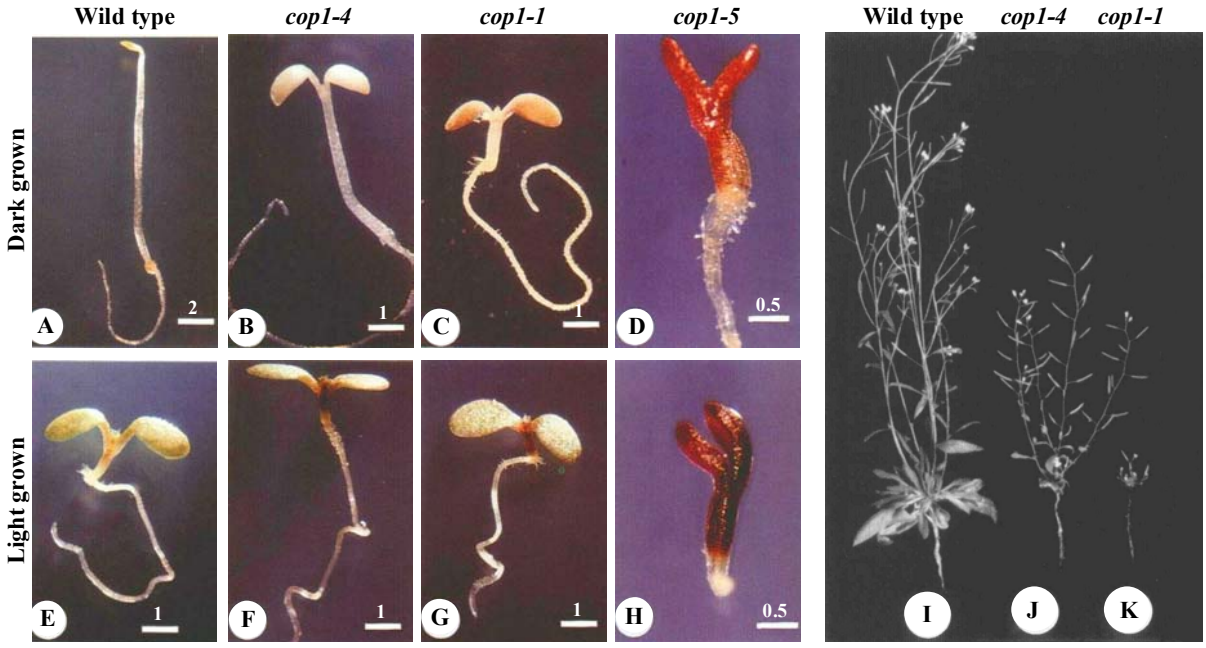

Figure 2.1.5. Phenotypes of different *cop1* mutants
Comparison of wild-type *Arabidopsis* plants (A, E and I) to the weak *cop1-4* (B, F and J), the strong *cop1-1* (C, G and I) and the seedling-lethal *cop1-5* (D and H) mutants. Corresponding seedlings grown in light (E-H) and darkness (A-D) are shown. Scale bars are indicated in mm. Adult plants grown for 1 month under long-day conditions are also shown (I-K). (modified after Ang and Deng, 1994)

the dark (Ma et al 2002). This includes 20% of all putative transcription factors of *Arabidopsis*. Therefore it is likely that most light-activated gene expression is repressed directly or indirectly by COP1. The extensive and essential role of COP1 in plant development is further exemplified by the fact, that *cop1* null alleles are seedling lethal. The strong and weak *cop1* alleles, however, are viable, but they still display a set of severe

developmental defects. These seedlings exhibit photomorphogenic development even in complete darkness (Deng and Quail, 1992 and Figure 2.1.5.), hence the name constitutively photomorphogenic (cop). Adult *cop1* mutants grown in light are dwarfs and have strongly decreased fertility compared to wild-types (Deng and Quail, 1992; Ang and Deng, 1994 and Figure 2.1.5.). *cop1*^{eid6} represents a somewhat unique, so called non-constitutive photomorphogenic *cop1* allele, that has a mutation that changes the conserved histidine-69 residue of the RING domain to a tyrosine. Interestingly, unlike other *cop1* mutants, *cop1*^{eid6} displays normal etiolated growth in dark. On the contrary, these mutants are strongly hypersensitive to visible light resulting in a mutant phenotype comparable to *cop1-4* (Dieterle et al., 2003).

COP1 protein contains three functional domains. A RING-finger motif is located at the N-terminus that is followed by a coiled-coil domain. Furthermore, a WD40 domain consisting of seven WD40 repeats is positioned at the C-terminus of the protein. Both nuclear import and export signal sequences (NIS and NES, respectively) are present in COP1 that mediate regulated nucleocytoplasmic partitioning (Yi and Deng, 2005). The RING domain is a typical element of E3 ubiquitin ligases and it is known to mediate the interaction with E2 enzymes. In fact, COP1 interacts through the RING domain with an E2 enzyme variant, COP10 (Suzuki et al., 2002). The coiled-coil domain mediates self-dimerization, as well as the interaction with other proteins, such as the four SPAs (SUPPRESSOR OF PHYA) (Hoecker and Quail, 2001; Bianchi et al., 2003; Laubinger and Hoecker, 2003; Laubinger et al., 2004). Similarly, the WD40 domain also plays a role in the interaction with several COP1-interacting proteins, including for example photoreceptors (PhyA, PhyB, Cry1 and Cry2) and both established and putative transcription factors (HY5, HYH, HFR1, STO [SALT TOLERANCE] and STH [STO HOMOLOGUE]) (Ang et al., 1998; Holm et al., 2001; Wang et al., 2001; Yang et al., 2001; Holm et al., 2002; Seo et al., 2004; Jang et al., 2005). Regarding its biochemical function, COP1 belongs to the RING domain containing single-subunit E3 ubiquitin ligases.

2.1.6. COP1 controls photomorphogenesis through regulated proteolysis

COP1 suppresses photomorphogenesis in darkness by targeting several positive regulators of the light perception and signalling pathways for proteasomal degradation (Hoecker, 2005; Yi and Deng, 2005). One of the first identified targets of COP1 was the bZIP transcription HY5 (Osterlund et al., 2000). HY5 protein accumulates to high levels in light grown seedlings, but upon light-to-dark transition it is degraded through proteasome-mediated proteolysis. This later process is impaired in *cop1* mutants indicating that functional COP1 is required for the HY5 destabilisation in darkness. Consistently, it was shown that the two

proteins directly interact through the COP1-interacting domain of HY5 and the WD40 domain of COP1, and mutations in both of these domains affecting the association of the two proteins results in HY5 stabilisation (Ang et al., 1998; Osterlund et al., 2000; Holm et al., 2001). Moreover, COP1 was shown to have intrinsic E3 ligase activity, and to ubiquitylate HY5 *in vitro* (Saijo et al., 2003). The physiological relevance of the COP1-HY5 interaction was further supported by their colocalization into subnuclear speckles in onion epidermis cells (Ang et al., 1998). And finally, HY5 is stabilised in white light under conditions when COP1 is excluded from the nucleus (von Arnim and Deng, 1994; Osterlund et al., 2000). Another bZIP transcription factor known as HYH, which forms heterodimers with HY5, was also shown to bind COP1 WD40 domain through its COP1-interaction motif, and is likewise degraded in darkness in a COP1 dependent manner (Holm et al., 2002).

The degradation-promoting function of COP1 that regulates the abundance of light signalling components is not restricted to bZIP transcription factors. For example, LAF1, a MYC-type transcription factor that participates as a positive regulator in PhyA-mediated far-red light signalling has also been demonstrated as a physiological target of COP1 (Ballesteros et al., 2001; Seo et al., 2003). Furthermore, a bHLH transcription factor, HFR1, another positively acting regulator of blue and far-red light signal transduction pathways, is likewise targeted by COP1 mediated ubiquitylation for proteasomal degradation in darkness (Duek et al., 2004; Jang et al., 2005; Yang et al., 2005). Moreover, the photoreceptors PhyA, PhyB, Cry1 and Cry2 have also been shown to directly interact with COP1 (Wang et al., 2001; Yang et al., 2001; Seo et al., 2004). In case of the light labile PhyA and Cry2, COP1 mediates their ubiquitylation and rapid proteasomal degradation upon light perception (Shalitin et al., 2002; Seo et al., 2004). This may have significant physiological role in the desensitization of the photoreceptors presumably preventing over-activation of the light-signalling pathways (Yi and Deng, 2005). On the other hand, COP1 also interacts with the more stable PhyB and Cry1, but does not seem to effect their stability (Wang et al., 2001; Yang et al., 2001). The exact role of this interaction remains to be determined, however, a likely explanation may be that COP1 regulates these photoreceptors by affecting their interactions with downstream signalling components (Yi and Deng, 2005). Another aspect of COP1 and cryptochrome interaction is that light-activated cryptochromes may directly turn off COP1 activity to release COP1-mediated suppression of photomorphogenesis genes (see below).

These findings demonstrate that COP1 mainly acts as a master switch to shut down photomorphogenesis in dark, by targeting positive regulators of specific light responses for destruction through the ubiquitin/26S proteasome pathway. However, mechanisms other than protein degradation may also apply to COP1 function, as suggested by its interaction with the

relatively light stable photoreceptors PhyB and Cry1. Additionally, a recent study demonstrated that COP1 is in fact required for the nuclear accumulation but not for the red and far-red light triggered rapid degradation of the bHLH transcription factor PIF3 that is a proposed negative regulator of phytochrome-mediated light responses (Kim et al., 2003; Bauer et al., 2004; Jiao et al., 2007). Moreover, a positive regulatory function of COP1 in PhyB-mediated red light signalling has recently been proposed, suggesting that COP1 might play opposing roles depending on light conditions (Boccalandro et al., 2004).

2.1.7. Mechanisms regulating COP1 function

2.1.7.1. Nucleocytoplasmic partitioning

Nucleocytoplasmic translocation is thought to play an important role in the regulation of COP1 function. COP1 is mainly localised to the nucleus of dark grown seedlings where it presumably ubiquitylates and thus promotes the degradation of the light development-promoting transcription factors and thereby hindering the activation of photomorphogenesis genes. Light exposure results in the drastic reduction of nuclear COP1 levels allowing these transcriptional regulators to re-accumulate and light induced transcription to occur. Such light-regulated change in nuclear COP1 levels however, is a relatively slow process of about 24 hours. This contrasts with the fast change of expression of the early light-responsive genes (detectable already less than 1 hour after light exposure) suggesting that other mechanisms may also operate to rapidly repress COP1 function upon light stimulus (Tepperman et al., 2001). Nonetheless, its nuclear-cytoplasmic partitioning still play a pivotal role in COP1 regulation. For instance, transgenic *Arabidopsis* plants expressing a NIS-mutated COP1 protein exhibit cop phenotype (Stacey et al., 1999; Stacey et al., 2000). On the contrary, NES mutations lead to increased COP1 nuclear accumulation that confers light hyposensitivity to the corresponding transgenic plants.

Interestingly, COP1 was found to co-localize with several of its interacting partners into subnuclear speckles as demonstrated by the analysis of transiently expressed fluorescent fusion proteins in onion epidermal cells (Ang et al., 1998; Holm et al., 2002; Seo et al., 2003; Duek et al., 2004; Seo et al., 2004; Jang et al., 2005; Yang et al., 2005). The signal sequence that targets COP1 to such subnuclear structures seem to partially overlap with both the coiled-coil domain, and, interestingly, with the NES (Stacey and von Arnim, 1999). Additionally, the WD40 domain through which COP1 interacts with most of its partners has also been shown to be important for speckle formation. Three separate mutations in the WD40 motif abolish subnuclear speckles, and each of the corresponding mutant seedlings develop strong cop phenotypes and die before adult stage (McNellis et al., 1994b; Stacey and von Arnim, 1999).

These findings are consistent with the notion that incorporation of COP1 into subnuclear speckles is an essential aspect of its function. However, a more definite understanding of physiological significance of these subnuclear foci, as well as the elucidation whether they represent sites of active COP1 function or storage sites for inactivated COP1 proteins, requires further investigations including combined mutant and transgenic plant studies and detailed biochemical characterisations (Stacey and von Arnim, 1999; Yi and Deng, 2005).

In addition to its relatively slow light induced nuclear exclusion, another mechanism has been proposed for the inactivation of COP1 function, which may explain the fast responsiveness of the light activated genes (Yi and Deng, 2005). Cryptochromes Cry1 and Cry2 have been shown to directly interact with of COP1 through their C-terminus and the COP1 WD40 domain in both light and darkness, and to negatively regulate COP1 activity in response to blue light (Wang et al., 2001; Yang et al., 2001). Therefore, light perception through the N-terminal domains of cryptochrome homodimers may initiate conformational changes in the C-terminus that, also involving intermolecular autophosphorylation and activation of the cryptochrome, possibly induces structural modifications of the receptor-bound COP1 that in turn releases the previously attached HY5 from COP1 (Wang et al., 2001; Yang et al., 2001; Chen et al., 2004; Yi and Deng, 2005). The issue, whether phytochromes could also utilise a similar mechanism to shut off COP1 function in red and far-red light, remains to be clarified. Notwithstanding, this rapid, phosphorylation based cryptochrome activation might represent a fast acting COP1 deactivation mechanism allowing the dynamic onset of light induced transcription. The longer-term inactivation of the COP1 function on light-responsive genes then in turn may occur by the subsequent nuclear depletion of the molecule (Osterlund et al., 1999; Yi and Deng, 2005).

Nuclear export-import mechanisms are not the sole regulatory steps that control COP1 function. In living cells, COP1 can be found in stable, high-molecular-mass protein complexes (Yi et al., 2002; Saijo et al., 2003). Genetic, yeast-two hybrid and protein complex purification approaches have identified numerous factors interacting with COP1, several of which are important physiological regulators of COP1 activity.

2.1.7.2. The SPA quartet

The SPA proteins represent a family of four, phytochrome-specific negative regulators of light responses (e.g., Hoecker, 2005; Yi and Deng, 2005). SPA1 (SUPPRESSOR OF PHYA-105) and SPA2 (SPA1-RELATED 2) are mainly involved in the suppression of photomorphogenesis in darkness, with SPA2 alone being sufficient to control this response, whereas in light grown seedlings, SPA1, SPA3 (SPA1-RELATED 3) and SPA4 (SPA1-RELATED 4) are responsible for the prevention of light-overstimulation. SPA3 and SPA4

additionally have important role in adult plants by inhibiting the development of dwarfism (Laubinger et al., 2004). These notions are in agreement with the SPA proteins having overlapping but distinct function in the regulation of plant development. Consistently, single, double or triple *spa* mutants show no phenotype at all or only restricted phenotypes under certain light conditions (Laubinger and Hoecker, 2003; Laubinger et al., 2004). On the contrary, *spa* quadruple mutants develop severe cop phenotype almost indistinguishable from *cop1* mutants (Laubinger et al., 2004). These finding strongly indicates that the SPAs are required for the suppression of photomorphogenesis in darkness, and that they work in concert with COP1 to perform this regulatory task.

The SPA proteins contain seven WD40 repeats at the C-terminal region that is highly homologous to the COP1 WD40 domain. Likewise similarly to COP1, the WD40 motifs of the SPAs are preceded by a coiled-coil domain. In addition to these, SPAs also possess an N-terminal kinase-like domains with unknown function (Hoecker et al., 1999; Laubinger and Hoecker, 2003; Laubinger et al., 2004). Consistent with the proposed joint regulatory role of the SPAs and COP1, all SPA proteins physically interact with COP1, and, as demonstrated for SPA1, this interaction is likely mediated through the mutual coiled-coil domains (Hoecker and Quail, 2001; Laubinger and Hoecker, 2003; Saijo et al., 2003; Laubinger et al., 2004). In addition, COP1 and SPA1 cofractionate in high molecular weight complexes in *Arabidopsis* seedlings and they also colocalize in subnuclear speckles, that in addition contain LAF1, in onion epidermis cells (Saijo et al., 2003; Seo et al., 2003). Furthermore, the SPA1 WD40 domain has been shown to bind HY5 in yeast two-hybrid assays, and in light grown *Arabidopsis spa1* mutants HY5 protein accumulates to higher levels than in the wild types (Saijo et al., 2003). Moreover, it was also demonstrated that SPA1 alters the E3 ubiquitin ligase activity of COP1 on both HY5 and LAF1 (Saijo et al., 2003; Seo et al., 2003). The exact mechanism by which SPA proteins modulate COP1 activity is still elusive. However, the so far accumulated data suggest that SPAs might enhance COP1 activity by helping to recruit its targets. Alternatively, SPAs may also be required for the nuclear accumulation of COP1 or they can likewise affect the stability of COP1 complexes. Control of COP1 through means of phosphorylation by SPA proteins is also an intriguing possibility given the presence of the kinase-like domains in the SPAs (Yi and Deng, 2005).

2.1.7.3. Multisubunit protein complexes regulating COP1 function

COP1 belongs to a group of genes known as the Cop/Det/Fus (Constitutive Photomorphogenic/De-etiolated/Fusca) loci, the members of which, when mutated, all share the cop phenotype. Except for COP1, all the other identified 8 members are components of

two large protein complexes: the COP9 signalosome (CSN) and the CUL4-RBX1-CDD E3 ligase (Schwechheimer, 2004; Chen et al., 2006).

The COP9 signalosome (CSN)

The eight-subunit CSN is evolutionarily conserved in both plants and animals, and six of its components are genetically defined COP/DET/FUS proteins (CSN1 to CSN4 and CSN7 and CSN8) that contain PCI-domains (for PROTEASOME, COP9, INITIATION FACTOR 3). The two other components are both encoded by two MPN (MPR1P and PAD1P N-TERMINAL) domain-containing genes (*CSN5A* and *CSN5B, CSN6A* and *CSN6B*). The CSN shows intriguing similarity to the 19S lid particle of the regulatory subunit of the 26S proteasome, and as a regulator of protein ubiquitylation and turnover, it is important for the proper development of virtually all higher eukaryotes (Serino and Deng, 2003). In plants, besides a variety of physiological and developmental processes, CSN acts as a crucial repressor of photomorphogenesis (von Arnim, 2003). CSN has been reported to interact with CUL1-, CUL3- and CUL4-containing E3 ligases from yeast, *Arabidopsis* and human (Dohmann et al., 2005 and references therein). The biochemical function of the CSN implicated in the regulation of these complexes is the cyclic conjugation and deconjugation of the ubiquitin-like RUB/NEDD8 to and from the cullin subunits (i.e. neddylation and deneddylation) (von Arnim, 2003; Moon et al., 2004). The deneddylating property of CSN is conferred by the metalloprotease activity of its CSN5 subunit. CSN5 is encoded by two genes in *Arabidopsis*, *CSN5A* and *CSN5B*, of which *CSN5A* contributes predominantly to the so far described functions of the complex. For example, *csn5a* mutants exhibit cop-like phenotype whereas *cn5b* mutants demonstrate comparably subtle defects (Dohmann et al., 2005). Interestingly, mutations in any of the PCI domain-containing subunits of the CSN result in the destabilisation of the entire complex, whereas *csn5* mutants are still able to retain stable CSN complexes (lacking CSN5) (Kwok et al., 1998; Dohmann et al., 2005). In both cases, however, mutants develop cop phenotype and pleiotropic developmental defects, indicating that it is the deneddylating activity of the complex that is required for the suppression of photomorphogenesis and the E3 regulating function of the CSN (Dohmann et al., 2005).

The CUL4-RBX1-CDD, a novel SCF-type E3 ubiquitin ligase

The CUL4-RBX1-CDD E3 ubiquitin ligase complex functions in concert with both the CSN and COP1 (Chen et al., 2006). Its CDD sub-complex was initially shown to contain COP10 and DET1, two members of the COP/DET/FUS group, and DDB1A (UV-DAMAGED DNA BINDING PROTEIN 1A), the human homologue of which is a subunit of the nucleotide excision repair enzyme that has been implicated in the severe disease xeroderma pigmentosum (Chu and Chang, 1988; Keeney et al., 1993; Yanagawa et al., 2004).

DET1 is 62-kDa nuclear protein that lacks any known functional domain and that has been shown to associate with DDB1A. Similarly to other COP/DET/FUS group members, DET1 is required for the suppression of photomorphogenesis. Additionally, it has suggested functions in chromatin remodelling to regulate light-dependent transcriptional processes, and in the control of the circadian rhythm by means of protein stability through modulating the turnover of a key regulator (Pepper et al., 1994; Benvenuto et al., 2002; Song and Carre, 2005). COP10 is an E2 enzyme variant (UEV) that has been shown to enhance the activity of several ubiquitin conjugating enzymes and to directly interact with the CSN and the RING-finger domain of COP1 (Suzuki et al., 2002). The *in vivo* interaction of COP10 with CULLIN4, and the interaction of CUL4 with RBX1, a common catalytic RING-subunit of E3 ligases, suggests that these components build up a CUL4-RBX1-CDD E3 ligase complex in *Arabidopsis* (Chen et al., 2006). In fact, the five subunit CUL4-RBX1-CDD complex could be reconstituted in vitro when expressed in baculovirus-insect cell system. Furthermore, the autoubiquitylation activity of the CUL4-RBX1 rudimentary E3 ligase was markedly enhanced by the presence of the CDD sub-complex (Chen et al., 2006). The CUL4-RBX1-CDD complex has been shown to associate with the CSN that mediates its deneddylation. It was further demonstrated that CUL4-RBX1-CDD regulates the COP1 mediated HY5 destabilization and the suppression of photomorphogenesis (Bernhardt et al., 2006; Chen et al., 2006). It is noteworthy, that COP1 does not seem to be an integrated part of this E3 ligase complex, though their interaction is evident (Chen et al., 2006). Yeast two hybrid and immunoprecipitation studies suggest that the DDB1A and perhaps COP10 subunits serve to bridge DET1 to CUL4, and the CDD may function as a substrate adaptor for CUL4-RBX1-CDD (Bernhardt et al., 2006; Chen et al., 2006). It is interesting to note, that DDB1A and DET1 are both required for the formation of a complex with COP10. Furthermore, in the *det-1* mutant the CDD complex is no longer detectable (Yanagawa et al., 2004). It is also very interesting to consider that a similar E3 ligase has been reported in mammalian cells. This complex consists of the human homologues of the CUL4, RBX1, COP1, DDB1 and DET1, in case of which the HsDET1 and HsCOP1 may form an adaptor to accomplish the HsCUL4-HsRBX1-HsDDB1-based E3 ligase that has been shown to promote the ubiquitylation and degradation of the proto-oncogenic transcription factor c-Jun (Wertz et al., 2004).

Taken all these together, it seems that the COP9 signalosome, the CUL4-RBX1-CDD E3 ubiquitin ligase and the COP1 complex act together to repress photomorphogenesis in *Arabidopsis*. The function of CUL4-RBX1-CDD E3 ligase depends on the deneddylating activity of the CSN, which function in turn could be to enhance the E3 ligase activity of COP1 by a yet unknown biochemical mechanism (Chen et al., 2006).

2.1.8. The function of the mammalian COP1

Orthologs of *COP1* are also well conserved in several non-plant multicellular organisms such as fish, amphibians, birds and mammals, however, interestingly, it has not yet been identified in the nearly fully sequenced genomes of *Drosophila melanogaster* and *Caenorhabditis elegans*, but it is present in the mosquito (Yi et al., 2002; Yi and Deng, 2005). Although the COP1 function in these organisms is less understood, recent reports indicated that animal COP1 may have important role in lipid metabolism, stress responses and tumorigenesis (Yi and Deng, 2005; Dornan et al., 2006; Qi et al., 2006). Importantly, COP1 was found to be overexpressed in breast and ovarian adenocarcinomas (Dornan et al., 2004b). The biochemical activities of COP1 as well as a set of its interacting partners seem to be conserved between plants and animals (Bianchi et al., 2003; Dornan et al., 2004b; Dornan et al., 2004a; Wertz et al., 2004; Yi and Deng, 2005; Yi et al., 2005; Qi et al., 2006).

The analysis of human COP1 by subcellular fractionation of HeLa cells and by microscopic and immunofluorescent analysis of COS7 cells expressing GFP- and FLAG-tagged fusions of huCOP1 revealed that human COP1 localises mainly to the nucleus, but also to the cytoplasm (Yi et al., 2002; Bianchi et al., 2003). Interestingly, when expressed in transgenic *Arabidopsis* seedlings, the subcellular localisation of a GUS-huCOP1 fusion protein is changed in a light-regulated manner similarly to the AtCOP1 (Wang et al., 1999). This suggests that a similar mechanism may operate for the regulation of nucleocytoplasmic partitioning of COP1 in mammalian cells. It is interesting to note, that besides the high level of similarity between the two proteins, the GUS-huCOP1 construct failed to rescue the *Arabidopsis cop1* mutant phenotype (Wang et al., 1999).

So far, two transcription factors have been shown to be substrates of COP1 in mammalian cells. COP1 serves as an E3 ubiquitin ligase for the tumour-suppressor p53, the "guardian of the genome", that facilitates the repair or elimination of severely damaged cells in response to cellular stresses. Thus COP1 inhibits p53-dependent transcription, cell-cycle arrest and apoptosis (Dornan et al., 2004a). This function of COP1 has been reported to be negatively regulated through MLF1 (MYELOID LEUKEMIA FACTOR 1), a positive upstream regulator of p53, and the CNS3 (Yoneda-Kato et al., 2005). Additionally, ATM (ATAXIA TELANGIECTASIA MUTATED) protein kinase mediated phosphorylation of COP1 in response to DNA damage has been shown to trigger COP1 autodegradation and subsequent stabilisation of p53 (Dornan et al., 2006).

Another target of COP1 is the proto-oncogene bZIP transcription factor c-Jun. Contrary to the case of p53 however, COP1 seems regulate c-Jun stability as a part of an E3 ligase complex similar to the CUL4-RBX1-CDD complex of *Arabidopsis*. This complex, dubbed as

$DCX^{hDET\text{-}hCOP1}$ contains the human homologs of the *Arabidopsis* DDB1 and DET1. X is an undefined motif called X-box that is believed to permit association between hDDB1 and the heterodimeric hDET1-hCOP1 serving as substrate adaptor (Wertz et al., 2004). A regulator of COP1 function on c-Jun activity has recently been reported in cultured kidney cells. Under unstressed condition, MVP (MAJOR VAULT PROTEIN) interacts and cooperates with COP1 to suppress c-Jun function, and thus stress-responses, whereas UV-B appears to trigger the disruption of this interaction and thus allow c-Jun activity (Yi et al., 2005). Interestingly, hCOP1 has also been reported to mediate the transcriptional activity of c-Jun independently of proteolysis (Bianchi et al., 2003).

Transcription factors, however, are not the only targets of COP1 in mammalian systems. The ubiquitylation and degradation of acetyl-coenzyme A carboxylase (ACC) is triggered through the TRB3 adaptor protein by COP1 during fasting. The elimination of this rate-limiting enzyme in fatty acid biosynthesis supports the mobilisation of lipid stores of adipocytes in mammals (Qi et al., 2006).

2.2. The ubiquitin/26S proteasome pathway and the E3 ubiquitin protein ligases

E3s are the protein ligase components of the three-step ubiquitin (Ub) conjugation cascade that very efficiently and with exquisite specificity designates proteins for degradation by the 2 MDa protease complex, the 26S proteasome. This Ub/26S proteasome (UPS) pathway has a pivotal housekeeping role by removing misfolded or abnormal proteins and maintaining the supply of free amino acids during growth and starvation (Vierstra, 1996), but is also essential for the regulation of cellular metabolism and signalling by, for example, expelling rate-limiting enzymes and short-lived regulatory proteins (Smalle and Vierstra, 2004). In *Arabidopsis*, more than 5% of the proteome represents components of the Ub/26S proteasome pathway suggesting that this catabolic mechanism plays essential role in the regulation plant cell functions (Moon et al., 2004).

2.2.1. The ubiquitin conjugation cascade

Ubiquitin is a highly conserved, 76-amino acid protein being present in all eukaryotic organisms (Hershko and Ciechanover, 1998). It has a compact globular structure with a protruding flexible C-terminal extension ending with a glycine (Smalle and Vierstra, 2004). The signal for the proteasomal degradation is typically conferred by target protein-attached lysine-linked poly-ubiquitin chains that are formed through an ATP-dependent Ub conjugation cascade. First, an ubiquitin-activating enzyme (E1) catalyses the formation of an acyl phosphoanhydride bond between the AMP moiety of ATP and the carboxyl group of the

C-terminal glycine of the Ub. Subsequently a thiol-ester linkage is formed between a cysteine of the E1 and the glycine of the Ub. This activated Ub is then transferred to a cysteine in an ubiquitin-conjugating enzyme (E2) by transesterification. The Ub-E2 intermediate delivers the Ub to an ubiquitin-protein ligase (E3) that binds the substrate and thus confers target specificity. Finally, an isopeptide bond is formed between the glycine of the Ub and the ε-amino groups of lysines in the target. The Ub polymer is built by reiterative rounds of Ub-conjugation using one of the seven lysines in the Ub (Pickart, 2001; Moon et al., 2004; Smalle and Vierstra, 2004).

The ubiquitously expressed and highly efficient E1 enzymes are commonly characterized by a positionally conserved cysteine residue that binds Ub and a nucleotide-binding motif interacting with ATP or the AMP-activated Ub intermediate (Pickart, 2001; Smalle and Vierstra, 2004). *Arabidopsis* has only two isoforms of E1s, whereas there are at least 37 E2 isoforms encoded in its genome (Smalle and Vierstra, 2004). E2s share a conserved 150-amino acid catalytic core harbouring the active-site cysteine. Interestingly, eukaryotes also possess Ub-E2 variants (UEVs) that have the conserved core domain but lack the active-site cysteine, and therefore cannot participate in Ub conjugation by itself (Pickart, 2001; Smalle and Vierstra, 2004).

E3 ligases represent as much as 90% of the UPS components encoded in the *Arabidopsis* genome, making this group a large and diverse family of proteins suitable for the elaborate regulation of the ample amount of possible targets. E3s can be classified according to the presence of a HECT (HOMOLOGY TO E6-AP C-TERMINUS) domain or a RING/U-box domain. The latter class is further divided into single subunit RING/U-box E3s and multisubunit RING E3s (Pickart, 2001; Moon et al., 2004).

2.2.1.1. HECT domain ubiquitin ligases

HECT E3s are large, typically 100-400 kDa proteins carrying a conserved C terminal HECT domain that contains both a Ub binding site and a Ub-E2 binding site. A distinctive feature of these ligases compared to other E3s is that a thiol-ester bond is formed between a unique cysteine in the HECT domain and the Ub before it is transferred to the substrate. This covalent intermediate then serves as the proximal Ub donor during the ligation reaction (Pickart, 2001). The region upstream of the HECT domain contains additional motifs that participate in target recognition. In contrast to the relatively large number of HECT E3s in animals (e.g. up to 50 is predicted in humans) (Schwarz et al., 1998), only seven of this type of E3s are encoded by the *Arabidopsis* genome (UPL1 to UPL7 [for UBIQUITIN PROTEIN LIGASE]) (Downes et al., 2003).

2.2.1.2. Single-subunit RING domain ubiquitin ligases

The characteristic of single subunit RING (for REALLY INTERESTING NEW GENE) E3s is the presence of a ~70-amino acid zinc binding motif, or RING finger, that binds the E2 (Freemont, 2000; Moon et al., 2004). U-box domains, the structurally related derivatives of RING motifs, exploit electrostatic interactions rather than metal ion chelation to stabilise the RING finger-like structure for the interaction with the E2s (Ohi et al., 2003). In both cases, docking with E2s initiates the allosteric activation of Ub transfer to the substrate (Smalle and Vierstra, 2004). Numerous other motifs may also be present in RING/U-box E3 ligases presumably endowing target specificity to these enzymes. The *Arabidopsis* genome encodes for approximately 480 RING-finger, and 64 U-box motif-containing proteins. Single subunit RING/U-box E3s have a wide range of implicated roles in plant physiology, including the regulation of photomorphogenesis, hormone responses, cold sensing, self incompatibility, wax biosynthesis and the removal of misfolded polypeptides (Smalle and Vierstra, 2004). COP1 belongs to this RING domain containing single-subunit E3 ubiquitin ligases.

2.2.1.3. Multisubunit RING domain ubiquitin ligases

Three classes of multisubunit E3 complexes can be found in plants: the SCF (for SKP1-CULLIN-F-BOX), the CUL3/BTB (for CULLIN3/BROAD-COMPLEX, TRAMTRACK, BRICK-A-BRACK) type complexes and the APC (for ANAPHASE-PROMOTING COMPLEX) (Moon et al., 2004).

The SCF class of E3 ligases are the best studied in plants. The name is derived from three out of its four core subunits: SKP1, the plant homologues of which are called ASKs for ARABIDOPSIS SKP1, Cullin or CDC53 and the F-box protein. The fourth core subunit is the RING-finger containing RBX1 (for RING-BOX 1) (Moon et al., 2004; Smalle and Vierstra, 2004). Two out of the five cullins encoded in *Arabidopsis*, CUL1 and CUL2 can be incorporated into SCF complexes. The cullin subunit serves as a scaffold and binds both RBX, that docks with the E2 enzymes, and the linker protein ASK. ASK proteins in turn bind a diverse array of F-box proteins that deliver the substrate specificity. Therefore, the Cullin-RBX1-SKP1/ASK subcomplex provides the Ub-transferase activity, and a multitude of F-box proteins confer the target specificity (Moon et al., 2004; Smalle and Vierstra, 2004). The conserved 60-amino acid F-box domain located mostly at the N terminus of the F-box protein mediates the interaction with the ASK/SKP subunits of the SCFs (Moon et al., 2004). The remainder of the protein contains the target-recruiting protein-protein interaction domains, such as leucine-rich repeats, Kelch or WD-40 domains that most probably identifies appropriate targets (Gagne et al., 2002). There are two RBX1, 21 ASK and more than 700 F-box protein coding genes in *Arabidopsis* (Moon et al., 2004). This is in sharp contrast to the

relatively low number 14 and 74 of F-box proteins in yeast and humans, respectively (Smalle and Vierstra, 2004). The remarkably high number of possible SCF E3 ligases in *Arabidopsis* suggests a fundamental role for these enzymes in plant life. Consistently, the SCF complexes described up to now play a role in the regulation of hormone responses, photomorphogenic development, circadian rhythm, floral development and senescence (Moon et al., 2004; Smalle and Vierstra, 2004).

The CUL3/BTB complexes are similar to the SCF E3s in that a cullin, CUL3 in this case, serves as a scaffold and interacts with RBX1. The SKP/ASK and F-box subunits of SCFs, however, are replaced by a single BTB subunit (Moon et al., 2004). Therefore, the substrate adapter protein directly interacts with the cullin subunit (Pintard et al., 2004). Many BTB proteins have been identified in animals (Pintard et al., 2004). In *Arabidopsis* the BTB protein ETO1 has a role in the regulation ethylene production (Wang et al., 2004).

The third member of multisubunit E3s is the highly conserved anaphase-promoting complex/cyclosome (APC/C) (Capron et al., 2003). Two out of its eleven subunits shares homology to components of SCFs: APC2 is a cullin-related protein and APC11 is a RING-protein (Tang et al., 2001). So far, mitotic cyclins have been identified as APC targets in plants, and it also controls the half-life of other factors crucial for mitotic progression and exit (Moon et al., 2004). Additionally, APC also seem to act in the control of auxin mediated responses in plants (Blilou et al., 2002).

2.2.2. Alternative roles of ubiquitylation

The 26S proteasome specifically recognizes poly-ubiquitylated proteins containing at least four Lys48-linked ubiquitins and promotes their destruction (Hoecker, 2005). However, Lys48-link poly-ubiquitin is not the sole conjugate that Ub ligases can generate. Both polyubiquitylation using lysines other than the Lys48 residues and monoubiquitylation are known functions of the Ub-conjugating cascades (Smalle and Vierstra, 2004). Lys63-linked poly-Ub chain is a common modification that is not used as a proteasome targeting signal, but rather plays an important role in, for example, postreplicative DNA repair and other processes (e.g., Weissman, 2001). In yeast, a dimer of a UEV and a real E2 (UBC13 and UBC2, respectively) in concert with two RING E3 ligases are responsible for the Lys63-linked polyubiquitylation of the DNA-repair protein PCNA (Bach and Ostendorff, 2003).

Attachment of a single Ub can also regulate the non-proteasome-coupled fate of target proteins. For instance, the monoubiquitylation of numerous receptors and transporters at the plasma membrane results in the lysosomal/vacuolar transport and turnover of these targets independently of the 26S proteasome (Hicke, 2001). Furthermore, attachment of a single Ub

to the target may result in proteolysis-independent regulation of cellular functions. The first ubiquitylated protein to be described was histone H2A (Muratani and Tansey, 2003). The monoubiquitylation of H2A and H2B subunits of the core nucleosome can promote or silence genes possibly through affecting other chromatin modifications such as acetylation and methylation (Muratani and Tansey, 2003; Smalle and Vierstra, 2004). Therefore, ubiquitylation is an integral part of the histone code that cells use to distinguish transcriptionally active and inactive chromatin, and the Ub conjugating machinery also participates in the regulation of transcription through means of chromatin remodelling (Muratani and Tansey, 2003). Chromatin modification, however, is not only one way whereby the Ub pathway is capable of modulating transcription in a proteolysis independent manner. For example, ubiquitylation of the yeast MET4 transcription factor by the SCF^{MET30} has been reported to inhibit the interaction with its co-activator CBF1, and thus the reconstitution of the active MET4 factor (Kaiser et al., 2000; Conaway et al., 2002).

2.3. The roles of UV-B irradiation on plant growth and development

Ultraviolet (UV) irradiation is an intrinsic component of the sunlight reaching the Earth. By convention, the UV spectrum is divided into UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (<280 nm) regions. As the stratospheric ozone layer very efficiently blocks the penetration of wavelength ranges below 290 nm, only UV-A and longer-wavelength UV-B reaches Earth's surface and thus have biological significance. The level of terrestrial UV-B irradiation can vary considerably depending on several large- and small-scale effects, making it a dynamically changing environmental factor (Paul and Gwynn-Jones, 2003). Though biologically effective UV-B accounts for only 0.5% of the sun's energy in general, it is at the same time the highest energetic part of the sunlight entering the biosphere (Paul and Gwynn-Jones, 2003).

UV-B is well known for the detrimental effects it can evoke in living cells, such as damaging virtually all major bio-molecules, including DNA, as well as provoking extensive cellular damage and oxidative stress (Ulm and Nagy, 2005). Though such damage is clearly significant for the highly energetic UV-B, it is only a part of a wide range of responses to this region of the electromagnetic spectrum (Paul and Gwynn-Jones, 2003). An example for the exploitation of UV irradiation unrelated to damage is the detection of UV by various animal species as part of their vision. UV-A, and to a minor extent UV-B as well, is widely used for foraging, partner selection, navigation, predator avoidance, intraspecies communication and even for the control of the circadian rhythms by both vertebrates and invertebrates (Tovee, 1995; Hunt et al., 2001; Kevan et al., 2001; Lim et al., 2007).

Owing to their absolute dependency on light and to their sessile lifestyle, plants are inevitably exposed to UV-B and to its damaging effects. UV-B, however, is not a mere stress factor for plants, but similarly to the visible and UV-A part of the spectrum, low fluences of UV-B can serve as an environmental cue conveying decisive information about the surrounding environment regulating diverse morphological and developmental processes (Kim et al., 1998; Boccalandro et al., 2001; Paul and Gwynn-Jones, 2003). In contrast to the perception and signalling of UV-A and visible light though, our understanding of specific perception of UV-B photons and the underlying signalling relays are far more limited, best exemplified by the lack of a molecularly identified UV-B photoreceptor (Ulm, 2006).

Therefore, the impacts of UV-B irradiation on plants can be broadly classified into two groups: damage (stress) and regulation (morphogenic, non-damage). The damaging effects are mainly due to the high energy per photon rate of the lower wavelength UV-radiation and to the wide range of bio-molecules that absorb it. Being sessile organisms that completely rely on sunlight for their growth and development plants have to adapt to the continuous exposure to UV-B and defend themselves against its harmful effects. In general, plants' defence include the adjustment of their growth pattern, synthesis of UV-protective phenolic pigments, production of scavengers of reactive oxygen species (ROS) and elevated potential to perform DNA repair. Defects or increased performance of these responses lead to hypersensitivity or increased tolerance to UV-B, respectively (Li et al., 1993; Landry et al., 1995; Conklin et al., 1996; Bieza and Lois, 2001; Tanaka et al., 2002; Britt and Fiscus, 2003). The UV-B radiation, on the other hand, is not only a source of stress for plants, but as part of the sunlight it also provides critical information about the prevailing light environment (Paul and Gwynn-Jones, 2003). Furthermore, due to the substantial modifying effects of environmental factors ranging from ozone column and solar angle through cloud coverage, air pollution and surface reflectance to topographical effects and plant canopy, the UV-B levels reaching an organism not necessarily correlate with other regions of the light spectrum (Caldwell and Flint, 1994; Rozema et al., 1997; McKenzie et al., 2003; Paul and Gwynn-Jones, 2003). Therefore direct perception of UV-B might be beneficial for proper responses (Ulm, 2006).

2.3.1. UV-B induced DNA damage and repair

UV irradiation can inflict a variety of damage to biomolecules absorbing it such as lipids, aromatic amino acids and nucleic acids. RNA molecules strongly absorb UV-B irradiation which may result in the crosslinking of specific ribosomal proteins with RNA that in turn may cause the inhibition of translation (Casati and Walbot, 2004). This effect is reversible, as in darkness normal translation effectiveness was restored that correlated with the disappearance

of the RNA-protein crosslinks in maize (Casati and Walbot, 2004). Moreover, despite of the general hindrance of translation, UV-B also induces the synthesis of specific ribosomal proteins (Casati and Walbot, 2004). This suggests that in response to UV-B irradiation cells increase the selective translation of transcripts of ribosomal proteins to overcome ribosome damage and thus the detrimental UV-B-impact on overall protein synthesis (Ulm and Nagy, 2005).

DNA is considered the major cellular target of genotoxic UV-B radiation, that may induce oxidative damage (pyrimidine hydrates), DNA-protein and DNA-DNA crosslinks but most prevalently the formation of various pyrimidine dimers along the genetic material. The most frequent pyrimidin dimers induced by UV-B radiation are the cyclobutane pyrimidine dimers (CPD) that make up 75% of the UV-induced DNA lesions. The majority of the remaining 25% is constituted by pyrimidine-pyrimidinon dimers (6-4 photoproducts, 6-4PP) (Figure 2.3.1. and Britt, 2004). The presence of such DNA aberrations in the genome can block

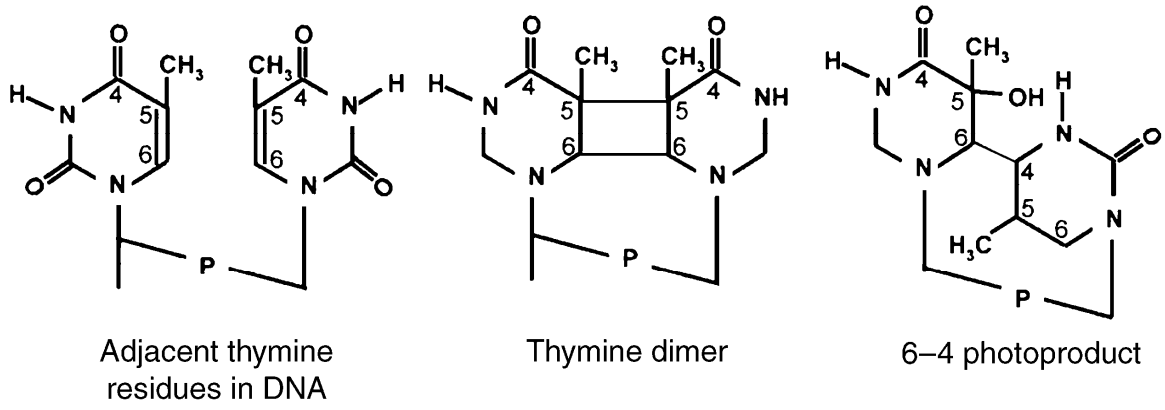

Figure 2.3.1. Structure of the two major UV-induced DNA photoproducts
Schematic structures of cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts formed between two adjacent thymine bases in a DNA strand (after Bray and West, 2005).

replication by stalling fastidious DNA polymerases that refuse to read through DNA lesions, which in turn activates cell-cycle checkpoints causing growth arrest and ultimately cell death. To overcome this, cells may perform translesion synthesis, throughout which stalled polymerases are rolled back or removed and are replaced by error-prone enzymes that can, with reduced accuracy, polymerize past dimers. Though this dimer bypass is necessary to complete the replication of a damaged template and to avoid cell death, the reduced accuracy inevitably causes mutations. Beside the interference with replication, DNA damage is likewise deleterious for transcriptional processes. Even a nonreplicating, terminally differentiated cell has to remove DNA lesions not to obstruct RNA polymerases. Thus, cells need to repair DNA lesions to restore proper transcription and error-free replication to take place. For the repair of damaged DNA three major mechanisms are employed. Pyrimidine

dimers can be repaired by photoreactivation, excision repair or recombination, whereas other DNA lesions can be resolved by the latter two mechanisms.

2.3.1.1. Photoreactivation

Photoreactivation is the process whereby pyrimidine dimers are directly reversed by using the energy of photoreactivating UV-A/blue light (350-450 nm). The reaction is performed by 55-70 kDa monomeric enzymes called photolyases that contain two prosthetic groups, a flavin adenine dinucleotide (FAD) and a pterin, typically methenyltetrahydrofolate (MTHF) or 8-deazaflavin. Photolyases specifically bind either CPDs or 6-4PPs and by a cyclic electron transfer mechanism catalyse the breakdown of the bonds holding the dimers together. The FAD cofactor is both required for the binding of the damaged DNA and for catalysis while the second chromophore, the pterin functions as a photoantenna and transfers the excitation energy to the catalytic $FADH^-$ (Sancar, 2003). It is interesting to note that placental mammals, including humans have lost this repair pathway during evolution (Britt, 2004). In *Arabidopsis* two photolyases are known, the CPD-specific PHR1/UVR2 and the 6-4PP-specific UVR3 (Ahmad et al., 1997; Nakajima et al., 1998). PHR1/UVR2 is induced transcriptionally by both white light and UV-B, whereas UVR3 is expressed constitutively (Ahmad et al., 1997; Tanaka et al., 2002; Waterworth et al., 2002; Britt, 2004). In agreement with their function as UV-B-induced DNA-damage-repair enzymes, both *uvr2* and *uvr3* mutants are distinctly sensitive to UV-B (Ahmad et al., 1997; Nakajima et al., 1998). In addition, analysis of double mutants of both photolyases with *uvr1* that is deficient in the dark-repair of 6-4PPs (see below) demonstrated that *uvr1uvr2* mutants unable to repair CPDs are more sensitive to UV-B under photoreactivating conditions than *uvr1uvr3* that accumulates the less prominent 6-4PPs (Jiang et al., 1997).

2.3.1.2. Excision repair mechanisms

Most of the other types of DNA damages cannot be directly reversed, but are rather excised and the resulting gaps are filled according to the intact strand serving as template. Commonly occurring oxidised or alkylated bases may be repaired by a mechanism termed base excision repair (BER), which is performed by a variety of lesion specific bi- or mono-functional glycosylases (Britt, 2002). These enzymes produce an abasic (apyrimidinic or apurinic, AP) site at the DNA lesion by cleaving the damaged base from the sugar-phosphate backbone. This is then followed by the cleavage of the sugar-phosphate backbone itself either by the intrinsic lyase activity of a bi-functional glycosylase or by an AP endonuclease that follows the action of a mono-functional glycosylase. Then several processing steps take place to mend the abnormal ends usually produced after the backbone cleavage (e.g. 3' end blocked

by a fragmented deoxyribose in case of a bi-functional glycosylase or a 5' deoxyribose in case of an AP endonuclease) to enable DNA polymerase and ligase to restore the integrity of the DNA.

A wide variety of DNA damage products can be repaired by another excision mechanism termed nucleotide excision repair (NER). This mechanism involves the recognition of the DNA damage, the unwinding of the DNA on a short stretch near these lesions sites, the binding of two distinct endonucleases to the 5' and 3' ends of the damaged oligonucleotide to generate double incision, the removal of the damaged strand, the resynthesis using the undamaged strand as template and finally the ligation of the resulting 5' and 3' ends. This NER pathway, that requires the coordinate action of about a dozen of proteins, is absolutely crucial to repair UV-induced DNA damages in organisms that lack photolyase activity, such as Humans. Defects in its components results in the heritable human disease, xeroderma pigmentosum (XP). XP patients suffer from extreme UV sensitivity and predisposition to develop skin cancer (e.g. Friedberg, 2001). The seven complementation groups of *xp* mutations and certain *rad* mutants represent different components of the NER system. The NER pathway of plants is homologous to that of mammals and fungi, but is unrelated to the bacterial system. A number of UV-hypersensitive *Arabidopsis* mutants are affected in NER pathway components that are homologous to the human/yeast counterparts. The *Arabidopsis* UVH6 is a XPD/RAD3 homologue that has 5' to 3' DNA helicase activity that, as part of the TFIIH general transcription factor, is responsible for the unwinding of the damaged DNA in humans and yeast (Liu et al., 2003; Sancar et al., 2004). UVH1 and UVR7 are XPF/RAD1 and ERCC1/RAD10 homologues, respectively, that both act together to perform the 5' incision of the damaged DNA strand (Fidantsef et al., 2000; Liu et al., 2000; Hefner et al., 2003; Sancar et al., 2004). UVH3/UVR1 is a XPG/RAD2 homologue responsible for the 3' cleavage of the unwound oligonucleotide stretch (Liu et al., 2001; Sancar et al., 2004). Thus the NER pathway appears to be functionally conserved in plants as well, assuring the repair of DNA lesions including pyrimidine dimers, particularly of 6-4PPs, even in the absence of photoreactivating light, therefore it is frequently referred to as the "dark repair" (Britt, 2004).

2.3.1.3. Recombinational repair

Another detrimental effect of genotoxic UV-B is the generation of DNA double strand breaks (DSB) that is in part due to the elevated formation of reactive oxygen species, particularly hydroxyl radicals, triggered by the irradiation. Furthermore, the co-incidence of single-strand nicks or gaps on the two DNA strand as a result of the activity of NER or BER during the repair of UV-B induced DNA lesions, or similar discontinuities on one strand of the replicating DNA near the replication fork can likewise result in DSBs (Bleuyard et al.,

2006). Cellular death resulting from a single DSB clearly demonstrates the deleterious nature of this DNA aberration (Bennett et al., 1993) that can only be repaired through recombination mechanisms. Most of the DBSs are fixed through the error-prone mechanism of nonhomologous end joining (NHEJ), however homologous recombination (HR) also plays critical role in the precise restoration of DNA integrity after DSBs (Bray and West, 2005; Schuermann et al., 2005). In fact, somatic homologous recombination was shown to be increased under elevated solar UV-B doses in *Arabidopsis* as measured with a recombination reporter gene, which indicates that HR may be utilised to purge UV-B induced DNA lesions in this model plant as well (Ries et al., 2000).

2.3.2. Non-damage related photomorphogenic UV-B responses and UV-B perception

It has already been emphasized that UV-B radiation is not only a mere stress signal, but it also can evoke diverse, non-damage-related phenotypic responses in higher plants. A number of these are characterized by low thresholds of the responses and by action spectra with optimum wavelengths that are different from the optimum for DNA damage, supporting the idea that these phenotypes are rather mediated by damage-independent perception pathways. Such photomorphogenic responses are hypocotyl growth inhibition, cotyledon expansion, phototropic growth, alterations in stomata aperture, leaf and tendril curling and the induction of UV-protective pigment production (Li et al., 1993; Wilson and Greenberg, 1993; Beggs and Wellmann, 1994; Ballare et al., 1995b; Brosche and Strid, 2000; Mazza et al., 2000; Eisinger et al., 2003; Shinkle et al., 2004). In line with these, several UV-B photomorphogenic phenotypes were identified in *Arabidopsis* (Ulm, 2006).

The spectral optima of some UV-B responses have been found to be at about 300 nm in the UV-B range and not in the germicidal UV-C. For instance the hypocotyl growth inhibition of tomato in response to UV-B shows maximum effectiveness at around 300 nm that, in addition, precedes the onset of UV-protective pigment production making it a relatively fast process in this plant (Ballare et al., 1995a; Ballare et al., 1995b). In cucumber, the same response is reversible by the application of gibberellic acid, is not resulting in concomitant change in dry matter production and is due to the UV-B perception in the cotyledons, which all excludes direct damage to the hypocotyls as a cause, and furthermore the UV-B response is also independent of stable phytochromes (Ballare et al., 1991). Additionally, cotyledon expansion, a typically photomorphogenic phenotype, is promoted by low fluence-rates of UV-B but not by damaging high fluence-rates (Kim et al., 1998). Similarly, a daily 2.5 hours low fluence-rates UV-B irradiation preceding a short red light pulse results in enhanced cotyledon

opening that is inhibited by higher fluence-rates of irradiation, resulting in a bell-shaped fluence rate response curve (Boccalandro et al., 2001).

Both the hypocotyl growth inhibition and cotyledon opening/expansion phenotypes of *Arabidopsis* demonstrate the regulatory and damaging aspects of UV-B radiation on the same phenotypes. The low fluence responses of the biphasic fluence response curves of hypoctyl growth inhibition represent the photomorphogenic, whereas the high fluence responses may be indicative of the stress responses resulting from DNA damage (Kim et al., 1998; Boccalandro et al., 2001). In agreement with this, the high fluence hypocotyl responses as well as the inhibition of cotyledon opening by high fluence-rates of UV-B were found to be enhanced in the compound DNA repair mutants *uvr1uvr2* and *uvr1uvr3* (Boccalandro et al., 2001). Therefore the irradiation conditions where DNA repair mutants do not show increased sensitivity denote UV-B levels that induce regulatory UV-B responses without considerable DNA damage.

These patterns of fluence rate dependencies are supportive of real photomorphogenic responses that also seem to be independent of the known photoreceptors, as demonstrated by the hypocotyl growth inhibition phenotypes of mutants defective in photoreceptors PhyA, PhyB, Cry1, Cry2 and Phot1 (Suesslin and Frohnmeyer, 2003). However, there is an apparent controversy regarding PhyA and PhyB involvement, as in similar experiments *phyAphyB* double mutants exhibited longer hypocotyls than wild-type seedlings under low fluences of UV-B (Kim et al., 1998). The UV-B induced enhancement of cotyledon opening response to a subsequent red light pulse was dependent of PhyB, but was independent of other known photoreceptors (Boccalandro et al., 2001). This may indicate that the PhyB mediated opening response is synergistically enhanced by separate receptor system perceiving UV-B (Ulm, 2006).

Part of the plant defence mechanisms against UV-B radiation is the synthesis of UV-protective “sunscreen” pigments. This is achieved by the activation of the general phenylpropanoid biosynthesis pathway resulting in the production of diverse flavonoids and sinapic esters (Figure 2.3.2.) (Winkel-Shirley, 2002). These compounds mostly accumulate in the epidermal cell layer and due to their absorption properties in the UV-B wavelength range they protect the underlying tissues. However, while filtering out the harmful parts of the sunlight they allow the penetration of photosynthetically active radiation supporting photosynthesis in mesophyll tissues.

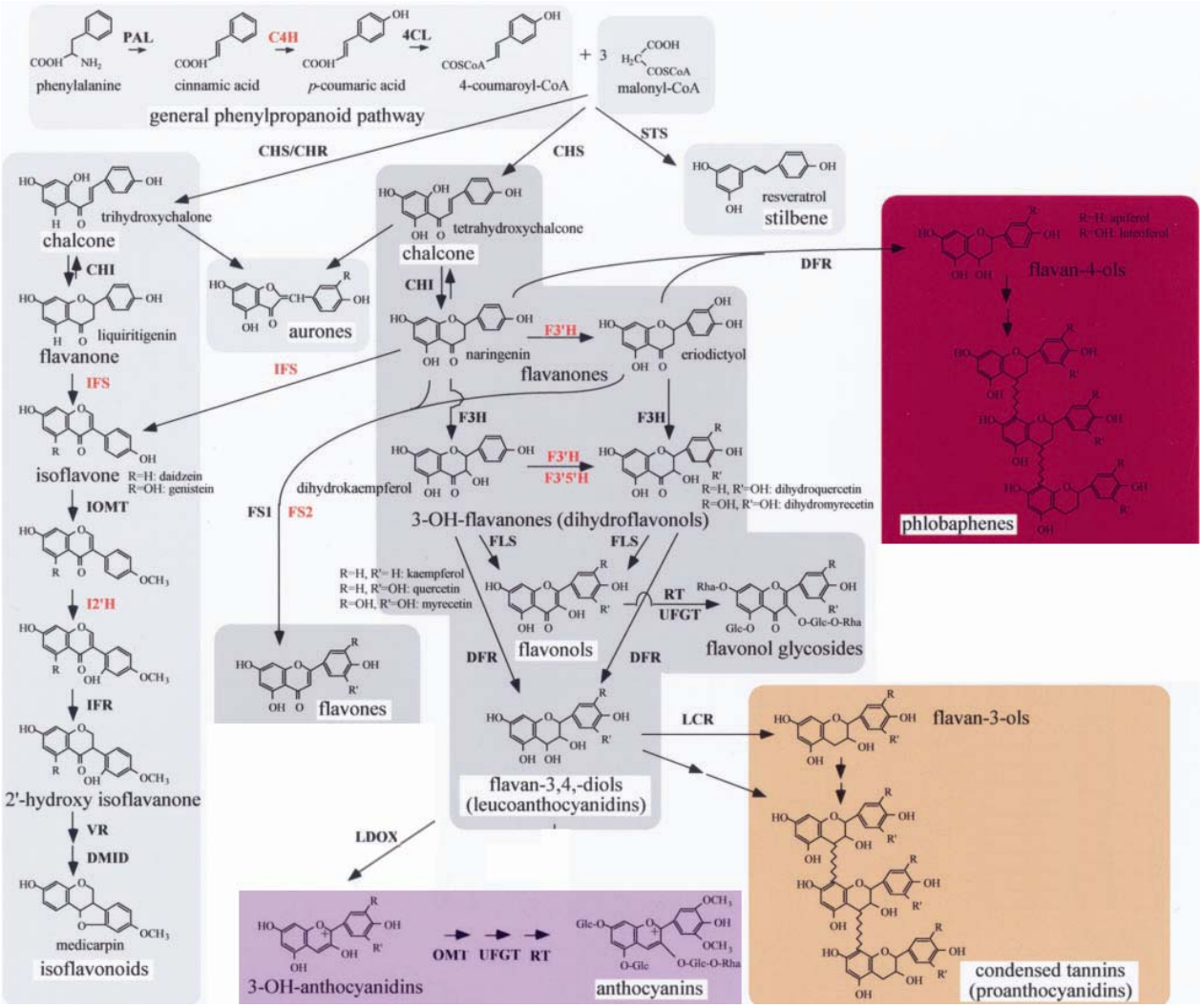

Figure 2.3.2. Schematic representation of the major branches of flavonoid biosynthesis pathway
The nine major subgroups: the colorless chalcones, aurones, isoflavonoids, flavones, flavonols, and flavandiols (gray boxes), and the anthocyanins, condensed tannins, and phlobaphene pigments (colored boxes) are shown. P450 hydoxylases are indicated in red. The first committed step is catalyzed by chalcone synthase (CHS). Enzyme names are abbreviated as follows: cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone reductase (CHR), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), 7,29-dihydroxy, 49-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F3H), flavone synthase (FSI and FSII), flavonoid 39 hydroxylase (F39H) or flavonoid 3959 hydroxylase (F3959H), isoflavone O-methyltransferase (IOMT), isoflavone reductase (IFR), isoflavone 29- hydroxylase (I29H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), O-methyltransferase (OMT), Phe ammonia-lyase (PAL), rhamnosyl transferase (RT), stilbene synthase (STS), UDPGflavonoid glucosyl transferase (UFGT), and vestitone reductase (VR). (modified after Winkel-Shirley, 2001)

It is of note that flavonoid accumulation is a characteristic feature of plant stress in general, and as a diverse group of secondary products they posses a vast array of biological functions. Beside the protection against UV-B radiation, flavonoids may also act as antimicrobials, antioxidants, regulators of auxin transport and may also have essential role in reproduction by attracting pollinators and seed dispensers (Winkel-Shirley, 2002).

The UV-B triggered flavonoid production is an extensively studied UV-B induced photomorphogenic effect, and is a well established UV-B response mediated by the postulated UV-B photoreceptor (Beggs and Wellmann, 1994; Björn, 1999). In parsley cell suspension

culture and seedlings, and also in several other plant systems as well, the UV-B induced flavonoid accumulation showed an action spectrum with a maximum effectiveness at about 300 nm, similar to the hypocotyl growth inhibition and cotyledon expansion responses described above (Beggs and Wellmann, 1994; Kucera et al., 2003). Phenylpropanoid production is also elevated in response to UV-B irradiation in *Arabidopsis* (Lois, 1994; Suesslin and Frohnmeyer, 2003). Furthermore, defects in this response or enhanced proficiency in it results in UV-B hyper- or hyposensitivity, respectively (Li et al., 1993; Landry et al., 1995; Jin et al., 2000; Bieza and Lois, 2001; Kliebenstein et al., 2002). In line with these, one of the key biosynthetic enzymes of the phenylpropanoid pathway, CHALCONE SYNTHASE (CHS, Figure 2.3.2.), was found to be transcriptionally induced by milliseconds of UV-B pulses in parsley cells (Frohnmeyer et al., 1999). It is important to note that under these conditions formation of DNA damage is minimised, and additionally *CHS* induction could be separated from pyrimidine dimer formation (Frohnmeyer et al., 1999). Likewise, pyrimidine dimer levels did not correlate with transcript levels of *CHS* in UV-B treated *Argenteum* pea (Kalbin et al., 2001). It is also known that the presence of photoreactivating UV-A and blue light results in the hyperstimulation of UV-B induced *CHS* expression (Wade et al., 2001). These findings demonstrate the lack of correlation between DNA damage and gene expression resulting from UV-B irradiation. Beside UV-B, red and blue/UV-A light also regulates the expression of *CHS* and thus interaction within network of phytochrome-, cryptochrome- and UV-B signalling pathways mediate the transcriptional regulation of this key component of “sunscreen” pigment production. Thus the regulation of *CHS* expression provides the opportunity to investigate the interplay of UV-B triggered responses with processes induced by other parts of the light spectrum (Jenkins et al., 2001).

Taken all together, a number of findings point to the existence of a UV-B specific perception system that is neither responding to damage nor is mediated by the known photoreceptors for visible light. However, as no UV-B photoreceptor is known at present, UV-B photobiology relies on defining UV-B treatments that induce specific responses separable from those resulting from damage (Ulm, 2006). One tempting possibility regarding the nature of the morphogenic UV-B-perception is that it would be perceived by a protein-pigment complex, similar to the light photoreceptors. Possible chromophores may include a tetrahydropterin derivative (Galland and Senger, 1988b; Björn, 1999) or a flavin (Galland and Senger, 1988a; Ensminger and Schäfer, 1992; Ballare et al., 1995a). In support of this, the absorbance maxima of such compounds correspond to the action spectra of several UV-B responses that has highest effectiveness at around 300 nm (Galland and Senger, 1988a, b). Moreover, riboflavin feeding of parsley cells enhanced the UV-B mediated increase chalcone

synthase and flavonoid levels (Ensminger and Schäfer, 1992), whereas treatment of tomato with compounds that perturb the normal chemistry of pterins and flavins resulted in the impairment of UV-B mediated inhibition of stem elongation (Ballare et al., 1995a). However, the clarification of this matter requires the identification of the UV-B receptor molecule.

2.3.3. Possible transducers of UV-B signals

The direct or indirect perception of UV-B signals should lead to the activation of specific signal relays to effectuate appropriate downstream responses such as gene activation or repression. Our understanding of such signalling pathways is only fragmentary. Furthermore, the separation of damage induced signalling events from those mediating morphogenic responses is often ambiguous. Moreover, the convergence of UV-B induced signalling cascades with other pathways triggered by a variety of distinct environmental stimuli, such as pathogen or herbivore attack, further exemplifies the complex nature of such responses. Nevertheless, pharmacological studies and analysis of diverse mutants and transgenic plants linked several signalling intermediates to UV-B responses. These include reactive oxygen species, nitric oxide, calcium/calmodulin, reversible phosphorylation events and a the plant hormones salicylic acid, jasmonic acid and ethylene (reviewed in, e.g., Brosche and Strid, 2003; Frohnmeyer and Staiger, 2003; Ulm, 2006).

2.3.4. Known genetically defined components of UV-B signalling

In contrast to the perception of visible light and the underlying signalling events our knowledge about UV-B triggered processes supported by genetic evidences is rather scant. This is in great part due to the paucity of well defined UV-B phenotypes and the confounding damaging aspects of UV-B treatments. However, some genetic screens that were based on phenotypic alterations or screens that exploited reporter gene fusion constructs revealed first components that can be linked to UV-B signal transduction. These efforts were aimed at the identification of mutants based mainly on two approaches: they followed alterations of tolerance to sustained UV-B radiation, or the development of aberrant phenotypic responses. These studies led to the identification of DNA repair mutants (Britt, 2004) (see also section 2.3.1.), and mutants which produce reduced or elevated levels of sunscreen metabolites, or show altered hypocotyl growth inhibition responses upon UV-B stimulus (Jin et al., 2000; Kliebenstein et al., 2002; Suesslin and Frohnmeyer, 2003; Wade et al., 2003; Brown et al., 2005).

2.3.4.1. Negative regulators of UV-B responses

One of the first genetically identified component of UV-B signalling in *Arabidopsis* was the R2R3 MYB transcription factor MYB4 (Jin et al., 2000). Under conditions devoid of UV-

B, MYB4 acts as a repressor to down-regulate the expression of its target gene *C4H* (cinnamate 4-hydroxylase), which encodes the enzyme that catalyzes a rate limiting step in the synthesis of sunscreen sinapate esters (Jin et al., 2000 and Figure 2.3.2.). However, UV-B irradiation results in the down-regulation of *MYB4* expression that leads to the derepression of *C4H*, and consequently to the elevated production of UV-protective sinapate esters. Accordingly, the *myb4* mutant exhibit elevated production of the protective pigments conferring enhanced UV-B tolerance to such plants, while on the contrary, MYB4 overexpression results in increased UV-B sensitivity (Jin et al., 2000).

Another negative regulator of phenylpropanoid biosynthesis was obtained by a transgene expression screen using *CHS* promoter driven GUS reporter gene (Jackson et al., 1995). The *icx1* (*increased chalcone synthase expression 1*) mutant found by this screen exhibit increased induction of flavonoid biosynthetic genes, such as *CHS, PAL* and *DFR,* in response to several environmental stimuli (Wade et al., 2003 and Figure 2.3.2.). These stimuli encompass low temperature, sucrose, cytokinin and light, including UV-B as well. Therefore, ICX1 seems to be a general and ubiquitously acting negative regulator of flavonoid biosynthesis. However, the *icx1* mutation and the gene responsible for the *icx* phenotype have not yet been identified, precluding the insight into ICX1 function on the molecular level.

2.3.4.2. Positive regulators of UV-B responses

uli3 (UV-B light insensitive) was identified as a mutant displaying reduced hypocotyl growth inhibition phenotype in response to UV-B (Suesslin and Frohnmeyer, 2003). *uli3*, and members of two other complementation groups of *uli* mutants (*uli1* and *uli2*) exhibit wild-type hypocotyl growth inhibition under red, far-red, blue and UV-A light conditions, indicating that these mutants are specifically affected in their UV-B pathways. ULI3 also acts as a positive component of the UV-B induced phenylpropanoid pathway (Suesslin and Frohnmeyer, 2003). *uli3* mutants accumulate reduced levels of anthocyanins in response to UV-B, and in agreement with this hyposensitivity, *CHS* gene activation upon UV-B treatment is also reduced in these mutants. *ULI3* itself was found to be transcriptionally induced by UV-B and preferentially expressed in the outer cell layers of aerial tissues. The ULI3 protein localises to the cytoplasm and also adjacent to the plasma membrane according to the analysis of ULI3-GFP fusion proteins in parsley protoplasts (Suesslin and Frohnmeyer, 2003). The predicted 80 kDa ULI3 protein shares 27% homology to a human diacylglycerol (DAG) kinase. It has a DAG- and two heme-binding sites in its N-terminal region, and an aspartic acid-rich domain in its C-terminus implicated in protein-protein interactions. Despite the homology to human DAG kinase, ULI3 lacks the conserved kinase domain (Suesslin and Frohnmeyer, 2003). Thus, ULI3 is a genetically defined positive regulator of

photomorphogenic UV-B responses, however, its position and role in the UV-B signalling network along with its exact biochemical function remains to be determined.

Another positive regulator of UV-B signalling was revealed according to the reduced UV-B tolerance of its mutant allele. *uvr8-1* is hypersensitive to UV-B stress, and in line with this notion this mutation also blocks the UV-B induced *CHS* gene activation and flavonoid accumulation. Thus, UVR8 (UV-resistance locus 8) positively regulates UV-B responsive phenylpropanoid biosynthesis (Kliebenstein et al., 2002). The predicted UVR8 protein has 35% sequence identity and 50% similarity to the human regulator of chromatin condensation RCC1. RCC1 functions as a guanine nucleotide exchange factor for the Ran GTPase and is implicated in fundamental cellular processes such as nucleocytoplasmic partitioning, mitotic spindle formation, nuclear envelope assembly and cell cycle progression (Dasso, 2002). *Arabidopsis* also possess Ran GTPases and several Ran interacting proteins (Haizel et al., 1997). It will be of interest to learn the exact biochemical activity as well as the precise cellular function of UVR8 in UV-B responses.

3. MATERIALS AND METHODS

3.1. Biological materials and plasmid vectors

3.1.1. Plant materials

Three *Arabidopsis thaliana* wild-type accessions were used in this study: Columbia (Col), Landsberg *erecta* (Ler) and Wassilewskija (Ws). Different mutants of these accessions used herein, along with the corresponding references are listed in Table 3.1.

Genotype	NASC ID	Wild type accession	Reference
cop1-1		Col	(McNellis et al., 1994b)
cop1-4		Col	(McNellis et al., 1994b)
cop1^{eid6}		Ler	(Dieterle et al., 2003)
cry1-304 cry2-1		Col	(Mockler et al., 1999)
csn5a-1	N563436	Col	(Dohmann et al., 2005)
det1-1	N6158	Col	(Pepper et al., 1994)
hy5-1	N71	Ler	(Oyama et al., 1997)
hy5-215		Col	(Rubio et al., 2005)
hy5-ks50		Ws	(Oyama et al., 1997)
hyh-1		Ws	(Holm et al., 2002)
hyh-1 hy5-Ks50		Ws	(Holm et al., 2002)
tt4	N520583	Col	(Alonso et al., 2003)
phot1-5 phot2-1		Col	(Kinoshita et al., 2001)
phyA-201 phyB-5		Ler	(Reed et al., 1994)
spa1-3 spa2-1 spa3-1 spa4-1		Col	(Laubinger et al., 2004)
uvr1-1 uvr2-2		Ler	(Jiang et al., 1997)
uvr1-1 uvr3-1		Ler	(Jiang et al., 1997)

Table 3.1. List of *Arabidopsis thaliana* mutants used in this study.

3.1.1.2. Transgenic Arabidopsis thaliana lines used in this study

hy5-1/Pro$_{HY5}$::HY5-YFP: *hy5-1* mutant plants expressing the HY5 protein fused to the N-terminus of the YFP protein under the control of the endogenous *HY5* promoter.

cop1-4/Pro$_{35S}$::YFP-COP1: *cop1-4* mutant plants expressing the COP1 protein fused to the C-terminus of the YFP protein under the control of the strong constitutive 35S RNA gene promoter of cauliflower mosaic virus (CaMV).

hy5-215/Pro$_{35S}$::HY5: *hy5-215* mutant plants expressing wild-type HY5 protein under the control of the CaMV 35S promoter.

3.1.2. Microorganisms

The *Echerichia coli* strains TOP10 [F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*Z ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] and DH5α [F$^-$

ϕ80*lac*ZΔM15 Δ(*f*v*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-] were purchased from Invitrogen and were used for molecular cloning purposes. The *Echerichia coli* strain DB3.1 [F^- *gyr*A462 *end*A1 Δ(sr1-*rec*A) *mcr*B *mrr hsd*S20(r_B-, m_B-) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^R) *xyl*-5 λ- l*eu mtl*1] from Invitrogen was used for the propagation of the Gatway vectors. *Echerichia coli* strain S17-1 (Sm^R, Sp^R, *Tra*$^+$, *pro thi rec*A *hsd*R *(RP4-2 kan::Tn7 tet::Mu), λpir lizogen*) was used to introduce binary vectors to *Agrobacterium* by conjugation. For plant transformations the *Agrobacterium tumefaciens* GV3101 (pMP90RK) strain was used (Koncz and Schell, 1986).

3.1.3. Plasmid vectors

YFP-NOS pPCV

This vector is based on pPCV812 (Plant Cloning Vector) binary plasmid used for the *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* (Koncz et al., 1994). The YFP-NOS pPCV is a 9 kb Ti-plasmid-based vector that has the left- and right-border (LB and RB) integration sequences of the *Agrobacterium* T-DNA. The following elements are placed between these: a ColE1 bacterial origin of replication; a β-lactamase gene that confers ampicillin/carbenicillin resistance for bacterial selection; a hygromycin-phosphotransferase gene under the regulation of the *nopaline synthase* gene promoter and terminator sequences that results in constitutive expression in plants conferring the hygromycin resistance for plant selection; a multiple cloning site after which a *yellow fluorescent protein*-coding DNA sequence is placed followed by the 3' polyA signal of the *nopaline synthase* gene.

pENTR3C, pDONR207 and pENTR207C

pENTR3C and pDONR207 are Gateway-based cloning vectors (Invitrogen). pENTR207C was derived from pENTR3C and pDONR207 as described in section 3.2.1.and was used to introduce *HY5* gene into the pMDC32 binary vector through recombination-based cloning.

pMDC32

pMDC32 is Gateway-based, binary destination vector (Curtis and Grossniklaus, 2003). The Gateway-cassette is inserted after the CaMV 35S promoter allowing the expression of the inserts introduced from pENTR vectors. It also has a hygromycin-phosphotransferase gene cassette. These two elements are placed between the left- and right-border (LB and RB) integration sequences of the *Agrobacterium* T-DNA. Replication in bacteria is ensured by a ColE1 origin of replication, and selection by a kanamycin resistance gene.

3.2. Molecular methods

3.2.1. Molecular cloning

Cloning was performed according to Sambrook and Russel (2001)

Pro$_{HY5}$:HY5-YFP in YFP-NOS pPCV

To express HY5-YFP fusion protein under the control of the endogenous *HY5* promoter in plants, a genomic fragment containing the *HY5* gene (without the STOP codon) and the upstream genomic region of 1071 bps was amplified from genomic DNA of Col plants by polymerase chain reaction (PCR) using Pfu polymerase (Promega) and the following oligonucleotides: forward 5'- CG*GAATTC*ATGTGATGCAACAGAGATGG -3' reverse 5'-A*GCGGCCGC*AAGGCTTGCATCAGCATTAG - 3' (italic characters indicate the introduced *Eco*RI and *Not*I restriction sites). The resulting fragment was cut by *Eco*RI and cloned into the *Eco*RI-*Sma*I site of YFP-NOS pPCV.

Pro$_{35S}$::HY5 in pMDC32-HY5

For the overexpression of HY5 protein in *Arabidopsis*, the pMDC32-HY5 construct was generated. The full length *HY5* coding sequence was amplified by PCR from cDNA of the Col accession using the following oligonucleotides: forward 5'-CGC*GGATCC*ATGCAGGAACAAGCGACT-3', reverse 5'-ACGC*GTCGAC*TCAAAGGCTTGCATCAGC-3' (italic characters indicate the introduced *Bam*HI and *Sal*I restriction sites). The resulting *Bam*HI-*Sal*I fragment was cloned into the *Bam*HI-*Xho*I site of the pENTR207C Gateway vector (pENTR207C-HY5). The pENTR207C vector, that carries a gentamicin resistance gene and the pENTR3C Gateway cassette, was generated as following: the *att*P1-*ccd*B-Cm^R-*att*P2 cassette of pDONR207 (Invitrogen) flanked by the *Nhe*I and *Pst*I sites was replaced by the *Nhe*I-*Pst*I fragment carrying the *att*L1-*ccd*B-*att*L2 Gateway cassette from the pENTR3C vector (Invitrogen). The pENTR207C-HY5 was used to integrate *HY5* into the pMDC32 vector behind the strong constitutive CaMV 35S promoter by LR recombination using Gateway LR Clonase Enzyme Mix (Invitrogen) according to the manufacturer's instruction.

Pro$_{35S}$:YFP-COP1

This construct was kindly provided by Dr. Éva Ádám (Biological Research Center, Szeged, Hungary). The COP1 open reading frame was cloned in frame to the C terminus of the YFP coding sequence under the control of the CaMV 35S promoter in a pPCV-based binary vector conferring ampicillin/carbenicillin resistance for bacterial and hygromycin resistance fro plant selection.

All clones were verified by sequencing.

3.2.2. *Agrobacterium tumefaciens* conjugation

E. coli strain S17-1 was transformed with the appropriate binary vectors and cultured overnight at 37°C in LB medium supplemented with 100 µg/ml ampicillin (for Pro_{HY5}*:HY5-YFP* and Pro_{35S}*:YFP-COP1* constructs) or 50 µg/ml kanamycin (for the Pro_{35S}*:HY5* construct). In parallel, *Agrobacterium tumefaciens* GV3101 culture was grown at 28°C overnight in YEB medium (0.5 % [w/v] beef extract; 0.1 % [w/v] yeast extract; 0.5 % [w/v] peptone; 0.5 % [w/v] sucrose; 1 mM NaOH, 0,75 mM $MgCl_2$). 1 ml from both bacterium cultures were mixed and centrifuged at 14000 rpm for 3 minutes and washed three times in YEB medium. The resulting pellet was resuspended in 50-100 µl YEB medium and applied in drops onto YEB plates (YEB medium containing 1.5 % [w/v] agar), and was incubated overnight at 28°C. A portion of this culture was spread on YEP plates containing 100 µg/ml rifampicin, 20 µg/ml gentamycin and 100 µg/ml carbenicillin (for Pro_{HY5}*:HY5-YFP* and Pro_{35S}*:YFP-COP1* constructs) or 50 µg/ml kanamycin (for the Pro_{35S}*:HY5* construct). Resistant colonies were re-streaked three times under the above selection, before the analysis of plasmid content by restriction digestion. The correct colonies were used for plant transformation (see section 3.3.4.).

3.2.3. Genomic DNA extraction from plants

Genomic DNA from *Arabidopsis* plants was extracted using the Nucleon™ PhytoPure™ Genomic DNA Extraction Kit (Amersham) according to the manufacturer's instruction.

3.2.4. RNA extraction

50-70 *Arabidopsis* seedlings per sample were harvested and snap frozen in liquid nitrogen. Total RNA was either extracted immediately, or the deep frozen samples were stored at -80°C for longer term. Frozen tissues mixed with 5-7 glass beads were ground for 10 sec using Silamat S5 mixer (Ivoclar Vivadent). Total RNA from the grinded tissues was isolated with the Plant RNeasy kit according to the manufacturer's instructions (Qiagen, Chatsworth, CA). RNA concentration was measured in 1:50 or 1:100 dilutions in TE buffer (10 mM TRIS-HCl, pH8.0; 1 mM EDTA) at 260 nm using a BioPhotometer (Eppendorf). $A_{260} = 1$ indicates 40 µg/ml RNA. $A_{260}/A_{280} \geq 2.0$ indicates proper RNA quality.

3.2.5. cDNA preparation

2.5 µg RNA was DNase treated in 10 µl RNase-free water containing 1x DNase I Restriction Buffer and 1 µl Deoxyribonuclease I (Invitrogen) at room temperature for 15 min. Then 1 µl 25 mM EDTA was added and the mixture was incubated for 10 min at 65°C. To this, 16.5 µl RNase-free water, 5 µl 10 mM dNTP mix and 6 µl Oligo $(dT)_{15}$ primer (~80 ng/µl) was added, and incubated at 65°C for 5 min. Then 8 µl 5x cDNA Synthesis Buffer, 2.5

µl 0.1 M DTT (dithiothreitol) and 1 µl Cloned AMV Reverse Transcriptase (Invitrogen) was added and the reaction was incubated at 45°C for 1 hour, and was stopped by incubating it at 85°C for 5 min. This cDNA was used as a template for PCR amplifications.

3.2.6. RNA gel blot analysis

RNA electrophoresis and blotting

10 µg total RNA were vacuum evaporated at room temperature (RT) using Concentrator 5301 (Eppendorf). The resulting samples containing 10 µg RNA were dissolved in 20 µl RNA loading buffer (50% [v/v] formamide; 1xMOPS [20 mM MOPS; 5 mM CH_3COONa; 1 mM EDTA; pH7.0]; 0.26% [w/v] bromphenol-blue [from 1.4% {w/v} stock in 36% {v/v} glycerol]; 10 µg/ml Ethidium-bromide; in RNase free distilled water) by 10 sec vortexing and then samples were incubated at 65°C for 15 min after which they were immediately transferred to ice for 5 min. Solutions were shortly spun down in a tabletop centrifuge to collect the liquid. Samples of the 10 µg RNA were electrophoretically separated in 1% formaldehyde-agarose gels (1% [w/v] agarose; 1xMOPS; 0.64 M formaldehyde). The resulting RNA patterns were documented under a UV-transilluminator. RNA containing gels were gently washed for 10 min in 10xSSC (1.5 M NaCl; 0.15 M Sodium citrate). Capillary transfer was carried out overnight in 10xSSC to Hybond N+ nylon membranes (Amersham Biosciences) that prior to the transfer were first soaked in distilled water, and in 10xSSC. Transferred RNA was UV-cross-linked to the membrane by UV Stratalinker 2400 (Stratagene).

Probe preparation

Gene-specific probes were amplified by PCR from *Arabidopsis* cDNA using the primer-pairs depicted in Table 3.2. PCR fragments were separated by agarose gel electrophoresis and specific bands were isolated from the gels by GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), and integrated into the pCR2.1-TOPO vector (Invitrogen) according to the manufacturers' instructions, and verified by sequencing. Approximately 1 µg of plasmid DNA was digested with the appropriate restriction endonuclease (typically *EcoRI* [New England Biolabs]) to cut out the probe fragment. After agarose gel-elctrophoresis the fragments were isolated from the gels. Radioactively labelled probes were prepared by Klenow fill-in reaction with Random Primers DNA Labelling System (Invitrogen) using 50 µCi [α^{32}P]dCTP (Amersham Bioscience), and the labelled probes were cleaned through NucTrap Probe Purification Columns (Stratagene), according to the manufacturers' instructions.

Gene ID		Oligo sequence 5' - 3'
At1g32870	fw	*CGG GAT CCA TTT AAA T*GC TCA AGA GGT GTG GTG G
	rev	*GGT CTA GAG GCG CGC C*GA AGG AAC AGG GTT TAG G
At3g17610	fw	*ACG CGG ATC C*AT GTC TCT CCA ACG ACC C
	rev	*ACG CGT CGA C*TT AGT GAT TGT CAT CAG T
At4g14690	fw	*CGG GAT CCA TTT AAA T*CA ACT CCA TCT CAC TTC TC
	rev	*GGT CTA GAG GCG CGC C*TC ACA CTG TTT AAA ACT C
At4g15480	fw	CTC TGT TTC ATT CCC TAC
	rev	CAC AAT CAT CCC TTT ACC
At1g19020	fw	ATA CAA TAA TGA ACG GAA CA
	rev	AAC TCG AAC AAT TTA CCA CA
At5g05410	fw	*CGC CTA GGA TTT AAA T*AG AGG AGT TAG GCA AAG G
	rev	*GGT CTA GAG GCG CGC C*GT TGA GGC TTT GTA GCG G
At5g11260	fw	*ACG CGG ATC C*AT GCA GGA ACA AGC GAC T
	rev	*ACG CGT CGA C*TC AAA GGC TTG CAT CAG C
At5g13930	fw	*CGG GAT CCA TTT AAA TCC* TCA AGG AAA ACC CAC AC
	rev	*GGT CTA GAG GCG CGC CCA* CTG AAA AGA GCC TGA CC
At5g52250	fw	*CGG GAT CCA TTT AAA T*CT CTC TCT TTC CGC CG
	rev	*GGT CTA GAG GCG CGC CCA* CAT TTG AAC CGT TCC
At5g23730	fw	CCG GCG AAA CTT AGT AGT C
	rev	CTT GAA GAA AGT CAT TCC CA
At5g59820	fw	ATC ATC ACA ACT ACT ATC
	rev	ACA AAT CTC CAA TGC TAC

Table 3.2. List of DNA oligonucleotides used for the generation of specific probes for the corresponding genes.

Hybridisation

Membranes were pre-hybridised in hybridisation buffer (50 mM $NaHPO_4$, pH6.5; 5xSSC; 5xDenhardt's solution [0.1% {w/v} polyvinylpyrolidone {PVP40} {Sigma}; 0.1% {w/v} Ficoll PM400 {Amersham}; 0.1% {w/v} bovine serum albumin]; 50% [v/v] formamide; 0.2% [w/v] SDS; 0.1 mg/ml denatured sheared salmon sperm DNA) for 4 hours at 42°C. Hybridisation was performed overnight at 42°C in fresh hybridisation buffer containing the labelled probe, after which the following washing steps were applied: 2x10 min at 42°C in washing buffer 1 (2xSSC; 0.2% [w/v] SDS), 2x10 min at 65°C in washing buffer 1, 2x10 min at 65°C in washing buffer 2 (1xSSC; 0.2% [w/v] SDS), 10 min at 65°C in washing buffer 3 (0.5xSSC; 0.2% [w/v] SDS). Membranes were analysed by autoradiography with BioMax MS films (Kodak) using BioMax TransScreen-HE Intensifying Screens (Kodak) using appropriate exposure times (from 2 hours to 3 days) at -80°. For sequential hybridisations with different probes membranes were stripped by 10 min incubation at 85-90°C in stripping buffer (10 mM TRIS-HCl, pH7.5; 0.1% [w/v] SDS). For each blot ethidium bromide-stained ribosomal RNA (rRNA) is shown to demonstrate equal loading of the RNA samples.

3.2.7. Protein extraction

For the extraction of total cellular proteins a modified method of Duek et al (2004) was used. 30-50 *Arabidopsis* seedlings per sample were harvested and snap frozen in liquid

nitrogen. Frozen tissues mixed with 5-7 glass beads were ground for 8 sec using Silamat S5 mixer (Ivoclar Vivadent). 50-100 µl protein extraction buffer (0.1 M TRIS-HCl, pH8.0; 50 mM EDTA; 0.25 M NaCl; 0.7% [w/v] SDS; 1 mM DTT; 10 mM NaF; 15 mM β-glycerolphosphate; 15 mM p-nytrophenyl phosphate; 1xComplete EDTA-free Protease Inhibitor Cocktail [Roche]) was added to each sample on ice. The samples were thoroughly mixed using the Silamat S5 mixer and shortly spun down in a tabletop centrifuge. Samples were then incubated at 65°C for 10 min followed by centrifugation for 10 min at 4°C with 20800 g (Centrifuge 5804R; Eppendorf) and the supernatant was transferred to a pre-cooled eppendorf tube.

Protein concentration was measured the amido-black method [modified after Moser et al (2000)]. Protein samples were diluted 1:50 in 200 µl distilled water, and BSA (Bio-Rad) standard sample dilution series of 10-250 µg/ml was also prepared in 200 µl solutions. 800 µl precipitation solution (10% [v/v] acetic-acid; 90% [v/v] methanol; 0.01% [w/v] Naphtol Blue Black [Amidoblack]) was added. After mixing by vortexing samples were centrifuged for 15 min at RT with 20800 g. After washing with 1 ml washing solution (10% [v/v] acetic-acid; 90% [v/v] ethanol) and centrifugation for 15 min at RT with 20800 g, precipitants were air-dried for ~15 min at RT, and dissolved in 500 µl 0.2 N NaOH. 2x200 µl of each sample were transferred to 96-well ELISA plates and extinctions were measured at 630 nm in a MRX Microplate Reader 630 (Dynex Technologies). Protein sample concentrations were determined according to the BSA calibration curve.

3.2.8. Immunoblot analysis (Western blot)

SDS Polyacrylamid Gel Electrophoresis (SDS-PAGE)

20 µg of total cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 12% gels (except for Figure 11D top, where a 10% gel was used) according to Sambrook and Russel (2001) using the Mini Protean 3 Electrophoresis System (Bio-Rad). For protein size comparison SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was used. Gels were elecrtophoretically transferred overnight at 4°C with 40 mV to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) by wet transfer (transfer buffer: 25 mM TRIS; 192 mM glycine; 20% [v/v] methanol) in the Mini Trans-Blot Cell system (Bio-Rad) according to the manufacturer's instructions.

Antibodies

For the detection of HY5, CHS, Actin and YFP proteins the following primary antibodies were used: polyclonal anti-HY5 (Oravecz et al., 2006), polyclonal anti-CHS (aC-20) (Santa Cruz Biotechnology), polyclonal anti-Actin (I-19) (Santa Cruz Biotechnology) and

monoclonal anti-GFP (BAbCO), respectively. As secondary antibodies horseradish peroxidase-conjugated anti-rabbit (for anti-HY5), anti-goat (for anti-CHS and anti-Actin) and anti-mouse (for anti-GFP) immunoglobulins (DAKO) were used.

Antibody incubation and washing

For the detection of HY5, blots were briefly rinsed in PBS (Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich) and dried in vacuum for 20 min before incubating with 1:2000 diluted anti-HY5 antibody in PBS-T (0.1% [v/v] Tween 20 in PBS) for 1 h at room temperature (RT) on an orbital shaker. After the following washing steps (2 x rinse, 1 x 15 min, 3 x 5 min in PBS-T at RT) blots were incubated in 1:20000 diluted anti-rabbit secondary antibody in PBS-T for 45 min at RT. Then blots were washed as before and signal detection was carried out using the ECL Plus Western blotting detection system (Amersham Biosciences) according to the manufacturer's instructions using BioMax XAR films (Kodak).

For the detection of CHS, YFP and Actin, blots were blocked in PBS-T containing 5% (w/v) non-fat dried milk before a short washing (2 x 2 min in PBS-T at RT) was followed by the primary antibody incubation. Antibody incubations, washing procedures and detection were done as described above, except that for the antibody incubations 1% (w/v) non-fat dried milk containing PBS-T was used.

Blots were stripped by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% [v/v] SDS, 62.5 mM TRIS-HCl pH6.7) for 30 min at 50°C with occasional agitation and then washed in PBS-T for 2 x 15 min at RT. Stripped blots were blocked again in PBS-T 5% (w/v) non-fat dried milk.

3.2.9. Flavonoid extraction and measurement

Flavonoid measurements were performed according to Kucera et al (2003). After the fresh-weight of 30-50 four-day-old seedlings were measured, they were snap frozen in liquid nitrogen. Flavonoids were extracted in 500 µl flavonoid extraction solution (79% [v/v] ethanol; 1% [v/v] acetic acid) at 85°C for 30 min at 1200 rpm in a Thermomixer Comfort heating block (Eppendorf). Following centrifugation for 15 min at 4°C with 20800 g (Centrifuge 5804R; Eppendorf) the flavonoids from the supernatant were separated by downstream paper chromatography in 15% (v/v) acetic acid (Markham, 1982). Bands corresponding to flavonoids were identified by their purple colour under UV-A, and were cut out. Elution was performed in 1 ml of 50% (v/v) ethanol at 85°C for 20 min at 1200 rpm in a heating block after which samples were centrifuged for 15 min at 4°C with 20800 g. The absorbance values of the supernatants were measured at 360 nm and were referred to the

fresh-weights of the corresponding samples or to the number of seedlings used for the given sample.

3.3. Plant methods, growth conditions and treatments

3.3.1. Plant growth conditions

Arabidopsis seeds were surface-sterilized by incubation in 5% v/v sodium-hypochlorite containing 0.1% (v/v) Tween 20 that was followed by 3 x washing with sterile distilled water. Seeds were plated on ½ MS plates (0.215% [w/v] Murashige and Skoog basal salt mixture [Duchefa], 0.05% [w/v] MES buffer [Roth] pH5.7) containing 1% (w/v) sucrose and 0.8% (w/v) agar and were stratified for at least 2 days at 4°C in darkness. Seeds were germinated and grown aseptically at 25°C either in a standard growth chamber (MLR-350, Sanyo Electric Co., Ltd.) with a 12 h/12 h light/dark cycle (fluence rate = 69 µmole m^{-2} sec^{-1}), or under continuous irradiation in the narrowband UV-field (see below).

3.3.2. UV-B treatments

The UV-B treatment of *Arabidopsis* seedlings were performed using the following, two different UV-fields:

(I) Shot term irradiation of 7-d-old seedlings were performed under a UV-B light field designated as the "broadband UV-field" consisting of six broadband Philips TL 40W/12RS UV fluorescent tubes (λ_{max} = 310 nm, half-bandwidth = 40 nm, fluence rate = 7 W/m^2 or 18 µmole m^{-2} sec^{-1}). Different UV-B spectra were generated by filtering the emitted light through 3-mm transmission cut-off filters of the WG series with half-maximal transmission at the indicated wavelength (WG295, WG305 and WG327; Schott, Germany), or unfiltered through a 3-mm quartz plate (Figure 3.1.). WG327 serves as the minus UV-B control, WG305 as weak UV-B treatment, WG295 as stronger UV-B and the quartz filter as the strongest UV-B treatment. For figure 3.1., spectral energy distributions of UV-B sources were measured with

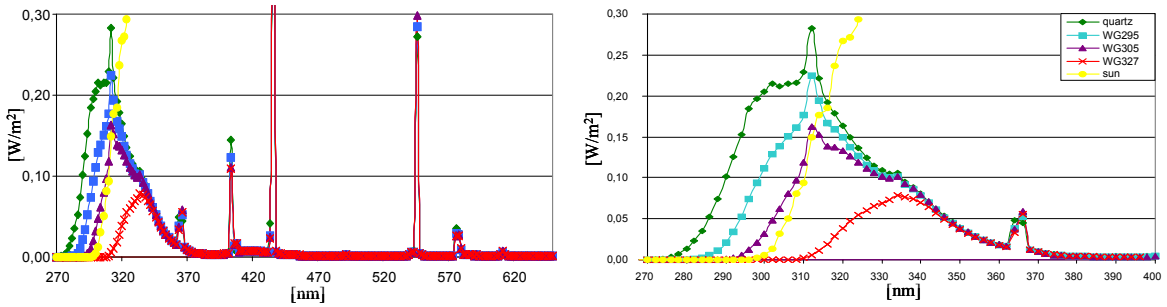

Figure 3.1. Spectral irradiance of the different UV-B light ranges in the broadband UV-field
Spectra of different UV-B scenarios demonstrated over the light spectrum (left) and over the UV-range (right). Spectral irradiance was measured in 2 nm intervals under the different cut-off filters (WG327, WG305, WG295) and quartz glass (unfiltered, representing the spectrum of the UV lamp).

an OL 754 UV-visible spectroradiometer (Optronic Laboratories, Orlando, FL). UV-B irradiance and radiant exposure were weighted with the generalized plant action spectrum, normalized at 300 nm (according to Caldwell, 1971), giving the biologically effective quantity, UVBE [W/m^2], for WG327: 0.0004, WG305: 0.12; WG295: 0.42, Quartz: 1.18. In comparison, sunlight on a sunny day of July in Freiburg at around noon was measured as UV_{BE} = 0.05 W/m^2 (Figure 3.1.).

Unless otherwise stated, the following irradiation protocol was used for UV-B treatments under this broadband UV-field: seven-day-old seedlings grown in the standard white light growth chamber in 12h light/12h dark cycles (MLR-350, Sanyo Electric Co., Ltd.) (fluence rate = 69 µmole m^{-2} sec^{-1}) were irradiated for 15 minutes at midday under the appropriate cut-off filters (WG327, WG305, WG295 or quartz), after which plates were immediately returned to the standard growth chamber for an additional 45 minutes and seedlings were than harvested. Non-irradiated controls were continuously kept in the standard growth chamber until harvesting.

(II) The continuous UV-B treatments were performed under a UV-B supplemented white light-field designated as the "narrowband UV-field" consisting of six dimmable light tubes. The white light is provided by three Osram L18W/30 tubes (3.6 µmole m^{-2} sec^{-1}; measured with a LI-250 Light Meter, LI-COR Biosciences, Lincoln, NE) that is supplemented with UV-B irradiation provided by three Philips TL20W/01RS narrowband UV-B tubes (0.56 W/m^2 or 1.5 µmole m^{-2} sec^{-1}; measured with a VLX-3W Ultraviolet Light Meter equipped with a CX-312 sensor, Vilber Lourmat, Marne-la-Vallée, France). The UV-B range was modulated by the use of 3-mm transmission cut-off filters of the WG series (WG305 and WG327; Schott Glaswerke, Mainz, Germany). Spectral energy distributions of UV-B sources were measured as described above (Figure 3.2.).

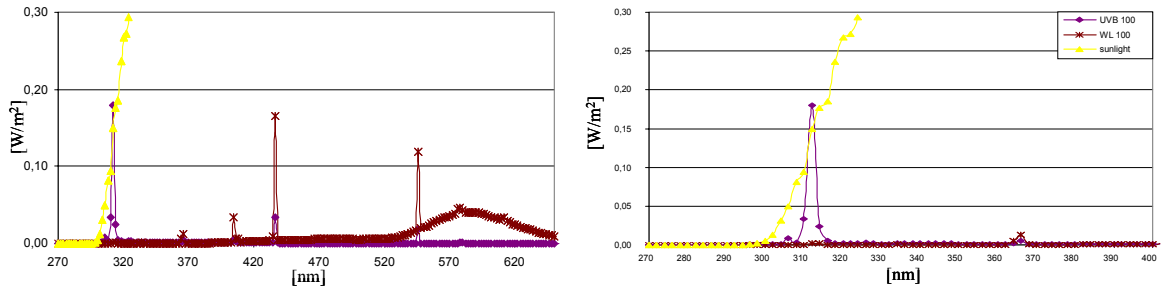

Figure 3.2. Spectral irradiance of the narrowband UV-field
Spectra of the UV-B field over the light spectrum (left) and over the UV-range (right). Spectral irradiance was measured in 2 nm intervals. Note that values represent 100% setting of the lamps (i.e. not dimmed). Settings were adjusted according to the values indicated in the text.

Unless otherwise stated, the following irradiation protocol was used for UV-B treatments under this narrowband UV-field: seeds were germinated and grown for four days in the UV-

field either only in white light under WG327 cut-off filters or in UV-B supplemented white light under the WG305 cut-off filters. Additionally, 1 and 6 hours UV-B treated samples were generated by exchanging the WG327 cut-off filters of white light-grown samples to WG305 1 and 6 hours before harvesting. All samples were harvested at the same time.

3.3.3. Epoxomicin treatment

hy5-215/Pro$_{35S}$:HY5 (line 7) seedlings were grown for three days on ½ MS agar plates containing 1% sucrose in the "narrowband UV-field" under WG305 cut-off filters. On the third day, 30-40 seedlings per treatment were transferred to wells of tissue culture test plates (TPP, Switzerland) filled with 1 ml of ½ MS 1% sucrose liquid media. On the fourth day samples were treated with 10 µM epoxomicin (BostonBiochem) (10 mM stock in DMSO) and controls were either treated with DMSO or untreated. Light treatments were performed as indicated.

3.3.4. Generation of transgenic plants

Plant transformation

Floral dip was used for the *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* plants (Clough and Bent, 1998). *Agrobacterium tumefaciens* GV3101 harbouring the appropriate binary vector was grown to stationary phase for two days at 28°C, 250 rpm in 5 ml LB medium (1% [w/v] tryptone; 0.5% [w/v] yeast extract; 0.5% [w/v] NaCl) containing 100 µg/ml rifampicin, 20 µg/ml gentamycin and 100 µg/ml carbenicillin (for *Pro$_{HY5}$:HY5-YFP* and *Pro$_{35S}$:YFP-COP1* constructs) or 50 µg/ml kanamycin (for the *Pro$_{35S}$:HY5* construct). This culture was diluted 1:100 in 500 ml LB containing 100 µg/ml carbenicillin or 50 µg/ml kanamycin and grown for 18-24 h at 28°C, 250 rpm after which cells were harvested by centrifugation at 5500 g for 20 min and resuspended in 500 ml inoculation medium (5% [w/v] sucrose; 0.05% [v/v] Silvet L-77; 10 mM $MgCl_2$). The resulting inoculum was added to a beaker and plants were inverted into it such that all inflorescence were submerged and were kept there for 5-10 seconds with gentle agitation. Dipped plants were laid down in a plastic tray covered with transparent foil to maintain humidity, and were kept at low light for 18-24 h, after which the cover was removed and plants were transferred back to the greenhouse. These T0 plants were grown for a further 3-5 weeks until siliques were ripe for harvesting.

Selection of transformants

Primary transformants (T1) were selected by plating approximately 3000 seeds in ½xMS soft medium (same as ½xMS but with only 0.5% [w/v] agar; supplemented with 250 µg/ml cefotaxim, 250 µg/ml vancomycin and 15 µg/ml hygromycin) onto petri dishes containing

½xMS plates 1% (w/v) sucrose supplemented with the same antibiotics in the same concentrations as for the soft medium. Hygromycin ensures the selection of transformed plants, whereas vancomycin and cefotaxim are used as anti-microbials. Seeds were stratified for 3 days at 4°C in darkness, than were placed in a standard growth chamber (MLR-350, Sanyo Electric Co., Ltd.) at 25°C with a 12 h/12 h light/dark cycle (fluence rate = 16.1 µmole m^{-2} sec^{-1}) and were grown for 7-10 days. Resistant seedlings were transferred to soil and grown until harvesting.

Transgenic T1 lines segregating resistant to sensitive seedlings in 3 to 1 ratio in the next generation (T2) were classified as carrying single locus insertions. Homozygous lines were selected in the next generation (T3).

3.3.5. Generation of *tt4 cop1-4* double mutants

The *tt4* allele used was the T-DNA insertion line SALK_020583 in Col background (Alonso et al., 2003). Homozygous *tt4* mutants were selected according to their seed coat phenotype (transparent testa; yellow seed color) that is determined by the parental genotype. *cop1-4* mutant was crossed to *tt4* by pollinating emasculated *tt4* flowers with *cop1-4* pollens. The resulting F1 plants were self-pollinated. F2 siblings with the indicated genotypes were selected as followings: *tt4/tt4 cop1/cop1* according to F2 seedling phenotype (constitutively photomorphogenic) and F3 seed phenotype; *tt4/tt4* COP1/COP1 according to F3 seed and seedling phenotypes. TT4/TT4 *cop1/cop1* according to F2 seedling and F4 seed phenotypes; TT4/TT4 COP1/COP1 according to F3 seedling and F4 seed phenotypes. F3 plants were used in the experiment on Figure 4.3.2.D.

3.4. Cell-biological methods

3.4.1. Epifluorescent microscopy

For epifluorescence and light microscopy 1-3 seedlings were transferred to 76x26 mm glass slides (Roth) in a few droplets of water and were covered with 24x24 mm cover glass (Roth). These were analysed with an Axioscope II epifluorescence and differencial interference contrast (DIC) microscope setup (Zeiss) equipped with a HBO 100 mercury short-arc lamp as the excitation light source. For the excitation and detection of YFP a standard YFP-filter-set (excitation filter: HQ 500/20; dichroic mirror: Q 515 LP; emission filter: HQ 535/30; AHF Analysentechnik), and for the detection of background chlorophyll fluorescence a Rhodamin-filter-set was used (excitation filter: BP 546/12; dichroic mirror: FT 580; emission filter: LP 590). Seedlings were investigated with a 63x Plan-Apochromat oil immersion objective (N.A. = 1.4; Zeiss). For image acquisition an AxioCam digital camera (CCD pixel size = 6.7 µm; Zeiss) and the Axiovision software (version 2.0.6.1, Carl Zeiss

Vision GmbH) was used. Images were taken during the first two minutes of microscopic analysis. Image processing (scale bars, merges, crops) were done by ImageJ 1.37v (http://rsb.info.nih.gov/ij/).

4. RESULTS

An important entry point to analyse UV-B responses is to monitor global gene expression changes provoked by this stimulus. A previous study of our group reported a microarray analysis of UV-B treated seven-day-old white-light-grown *Arabidopsis* seedlings of the Wassilewskija (Ws) accession (Ulm et al., 2004). This comprehensive survey using the Affymetrix ATH1 full genome chip representing ~24000 *Arabidopsis* genes demonstrated that irradiation with 15 minutes of different UV-B spectra results in the altered regulation of a robust set of genes. 100 genes are at least two-fold induced, and another 7 are repressed specifically by low-level, longer wavelength UV-B irradiation. The transient up-regulation of the induced genes, as well as the overrepresentation of transcriptional regulators among these genes was apparent. On the basis of this study it was of interest to analyse further the low-level, non-damaging UV-B-induced responses in young *Arabidopsis* seedlings, with the additional goal of identifying new players in UV-B signalling.

4.1. Analysis of UV-B induced transcriptional changes

4.1.1. UV-B triggers early transcriptional responses

To confirm and characterize further the results from the microarray experiments, the UV-B treatments were repeated under the same conditions in Ws, and in two additional wild-type *Arabidopsis* accessions: Landsberg *erecta* (Ler) and Columbia (Col). Seedlings were grown for seven days in standard white light growth chamber (12 hours light/12 hours dark cycles) after which they were exposed to 15 minutes of polychromatic irradiation under the broadband UV-field at midday. Decreasing short wave cut-off filters were used to generate four different UV spectra: WG327 (i.e., half maximal transmitted wavelengths at 327nm; minus UV-B control), WG305 (305nm; low level UV-B), WG295 (295nm; stronger UV-B) and quartz (unfiltered irradiation; strong UV-B) (see Figure 3.1.). After irradiation, seedlings were transferred back to standard growth conditions, and samples were harvested after one hour from the onset of the UV-B treatments. In addition to the WG327 minus UV-B control, another control sample was generated by keeping the seedlings in the standard growth chamber until harvesting (non-treated control). The total RNA isolated from these samples were analysed by RNA gel blots using specific probes of selected genes that were shown to be inducible by low level UV-B according to the expression profiling. Figure 4.1.1.A and C shows that in the three wild-type accessions (Ws, Ler, Col) all the analysed genes exhibit the UV-B dependent transcriptional up-regulation on RNA gel blots as well. This indicates that the observed transcriptional gene induction is a general response in *Arabidopsis*. For all the

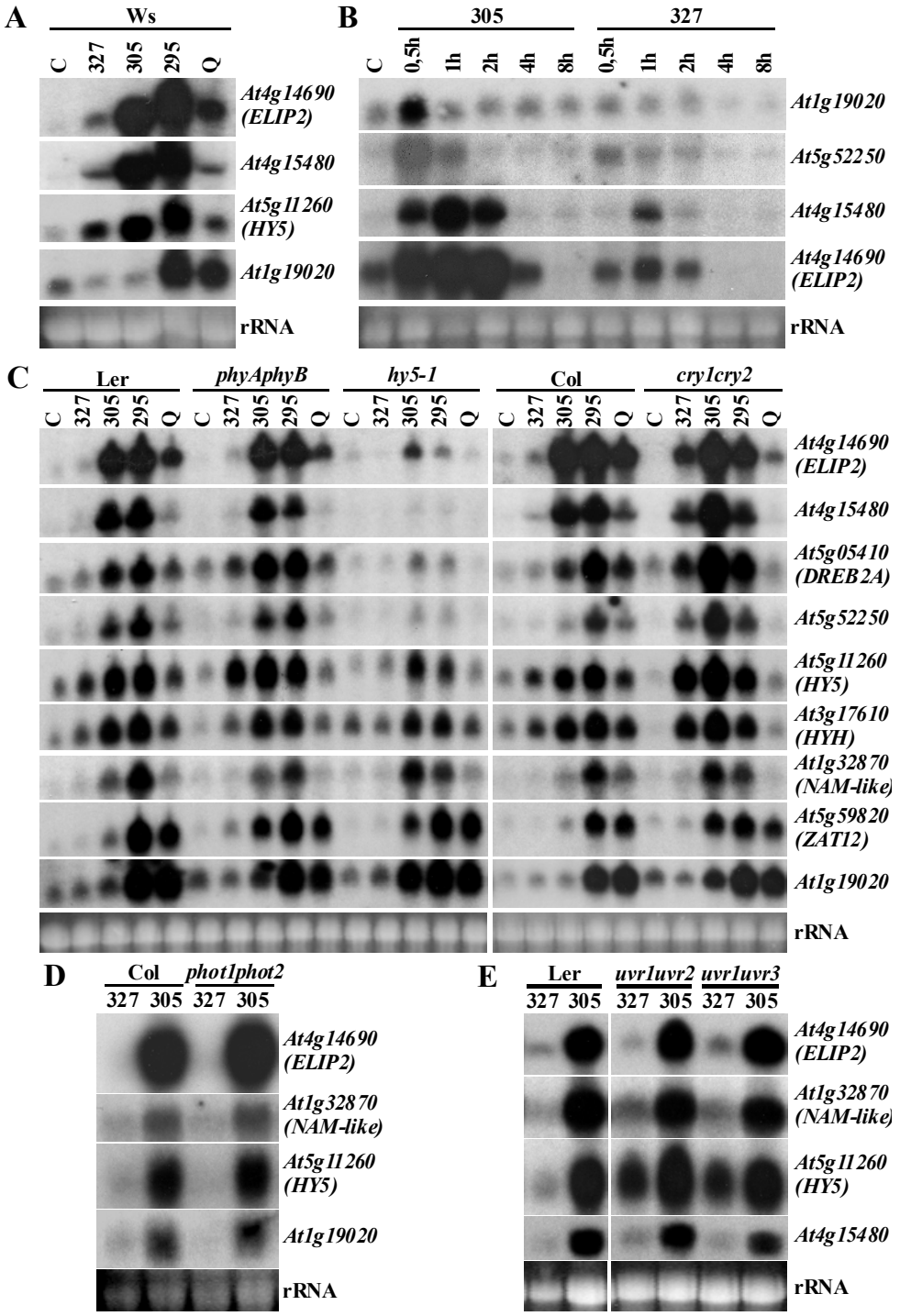

Figure 4.1.1. Non-damaging UV-B induced transient activation of UV-responsive genes is independent of known photoreceptors, but requires HY5

RNA gel blot analysis of 7-d-old white light-grown seedlings of the indicated genotypes that were irradiated under the broadband UV field for 15 min with different UV spectra (-UV-B under cut-off WG327; +UV-B under decreasing short wave cut-offs WG305, WG295 or quartz glass [Q]). After irradiation, seedlings were transferred back to standard conditions and for **(A), (C), (D)** and **(E)** seedlings were harvested after one hour from the onset of irradiation, for **(B)** after 30 min, 1-, 2-, 4- and 8 hours from the onset of irradiation, as indicated on the figure. Control samples were kept in the standard growth chamber until harvesting **(C)**. Blots were sequentially hybridised with specific probes for the indicated genes listed on each panel. Ethidium bromide-stained rRNA is shown as loading control. Experiments kindly contributed by Alexander Baumann.

genes tested, the transcript accumulation is higher in low-level UV-B (WG305) treated samples then in the minus UV-B controls (WG327) (Ws, Ler and Col on Figure 4.1.1.A and C). Exception is *At1g19020* where the mRNA accumulates to similar levels 1 hour after the

UV-B treatment under both WG327 and WG305. However, 30 minutes after the UV-B treatment the transcript accumulation of this gene is higher under WG305 than WG327 (see later and Figure 4.1.1.B), indicating that this gene is also responsive to low-level UV-B, but with faster induction kinetics.

A subset of the low-level UV-B induced genes showed the expected expression characteristics. This is represented by stronger gene induction in response to stronger UV-B treatment (i.e. shorter wavelength UV-B) as indicated by the higher transcript accumulation under WG295 and quartz compared to WG305 cut-off filters (e.g. *At5g59820*/*ZAT12*, *At1g19020* on Figure 4.1.1.A and C). Surprisingly though, another class of UV-B induced genes showed reduced transcript accumulation under the quartz filters compared to the weaker (i.e. longer wavelength) UV-B treatments under the WG305 cut-off filters (e.g. *At4g15480*, *At5g11260*/*HY5*). These findings are in good agreement with the microarray data, where a set of 62 genes were exclusively induced by longer wavelength UV-B, while another set of 38 genes were up-regulated by the short wavelength quartz treatment as well (Ulm et al., 2004). This is particularly interesting because the UV-B spectra for WG305 and quartz treatments are identical except for the extension to the shorter wavelength ranges in the later (see Figure 3.1.). Altogether these findings indicate that the shorter wavelength UV-B irradiation negatively interferes with the transcriptional up-regulation of a particular subset of low-level UV-B induced genes, suggesting the presence of at least two interacting UV-B perception and signalling pathways.

4.1.2. UV-B responsive genes are rapidly and transiently induced

Transient induction characteristics of the low-level UV-B induced genes were reported through the microarray analysis (Ulm et al., 2004 and Figure 5.1.A), where almost all of the genes that were up-regulated by low-level UV-B after 1 hour, were no longer induced after 6 hours. To investigate further the temporal expression pattern of a set of UV-B induced genes, we analysed the transcript levels of these marker genes over 8 hours by RNA gel blots as before. Seven-day-old Ws seedlings were subjected to 15 minutes UV-B irradiation as described above, using the WG327 and WG305 cut-off filters. Seedlings then were transferred back to standard growth conditions and samples were harvested after 30 minutes, 1-, 2-, 4- and 8 hours from the onset of irradiation. Control samples were kept in the standard growth chamber and harvested together with the 8 hours time-point samples. The genes investigated and depicted on Figure 4.1.1.B show transient mRNA induction upon UV-B treatment in agreement with the expression profiling data. The time resolution of the RNA gel blot analysis indicates that some genes are induced more rapidly and transiently (e.g.

At5g52250), while others show delayed induction with prolonged induction kinetics (e.g. *At5g15480*, *At4g14690*), but all are within a six hours timeframe. Note the extremely fast and transient response of *At1g19020*, where already 1 hour after the UV-B treatment the transcript falls back to background level. The different temporal distribution of the induction kinetics of the UV-B induced genes may indicate that these genes are components of a regulatory network triggered by low level UV-B irradiation. This notion is further supported by the overrepresentation of transcription factors among the early UV-B induced genes as indicated by microarray analysis (Ulm et al., 2004).

4.1.3. Transcriptional UV-B responses are independent of known photoreceptors

It is well known that complex regulatory networks are also operating during the photoreceptor mediated processes regulated by visible light. Therefore it was of interest to investigate further, whether the transcriptional UV-B responses described above are mediated by any of the known photoreceptors. To this end, compound mutants of the photoreceptors phytochromes A and B (*phyAphyB*), cryptochromes 1 and 2 (*cry1cry2*), and phototrophins 1 and 2 (*phot1phot2*) were analysed with regard to their low-level UV-B induced transcriptional responses compared to the corresponding wild-type backgrounds. Seven-day-old white light-grown seedlings were irradiated with 15 minutes of UV-B using WG327, WG305, WG295 and quartz filters under the broadband UV-field, and samples were harvested after one hour from the onset of irradiation, and analysed by RNA gel blots as described before.

Figure 4.1.1.C and D clearly shows that all three photoreceptor double mutants exhibit both qualitatively and quantitatively similar UV-B responses to their corresponding wild-types. For example, in *phyAphyB* double mutant the mRNA of *ELIP2* (*At4g14690*) is induced to similar level under WG305 cutoff compared to WG327 as in Ler. The level of this transcript is also similar in the two genotypes under WG295 and under quartz, indicating that not only the induction in response to low-level UV-B, but also the antagonistic effect of the short wavelength ranges are operating in the mutant (Figure 4.1.1.C). Consistent with these findings, all the other genes tested show transcriptional responses independent of the investigated photoreceptors (Figure 4.1.1.C and D). Similar results were also obtained in case of a *phyABDE* quadruple mutant (data not shown). It also should be noted here, that the Ws accession used for the expression profiling is PhyD deficient (Ulm et al., 2004). Taken together, the transcriptional responses to low level UV-B, at least in case of the analysed genes, are independent of the red/far-red and blue/UV-A sensing photoreceptors tested, strongly suggesting that the induction of these genes occur through the action of a specific UV-B photoreceptor(s).

It is of note though, that the induction properties of a set of genes show a slight shift towards the longer wavelength range in the *cry1cry2* double mutant (Figure 4.1.1.C), indicating hypersensitivity due to the loss of the two cryptochromes. This is marked by enhanced transcript accumulation in the mutant under WG327 and WG305, and reduced mRNA levels under WG295 and quartz, compared to the corresponding wild-type.

However it is interesting to note, that mRNAs of the genes that are not antagonised by shorter wavelength UV-B are not significantly altered in the double mutant under WG295 and quartz, whereas their levels are elevated under WG305 (*At5g59820*, *At1g19020* on Figure 4.1.1.C). This further supports the idea of the existence of at least two different but interacting perception and signalling pathways in UV-B that are differently affected by the cryptochrome pathway. It follows that the effect in the *cry1cry2* mutant indicates a possible interaction between the blue light and UV-A sensing cryptochrome pathways and the postulated UV-B pathway.

4.1.4. Low level UV-B induced processes are not related to DNA damage responses

When analyzing responses to low level UV-B irradiation, it is important to clarify that the processes under investigation are not general damage responses triggered by the UV treatment. We used *uvr1uvr2* and *uvr1uvr3* DNA repair double mutants to examine this possibility. Both of these mutants are deficient in DNA excision repair (*uvr1*) (Britt et al., 1993); in addition, the former being unable to perform the photorepair of CPDs (*uvr2*), the later that of the 6-4 photoproducts (*uvr3*) (Jiang et al., 1997). If the UV-B dependent gene inductions are related to stress responses due to the damaged DNA caused by the irradiation, these mutants are expected to show hyper-responsiveness. Seven-day-old seedlings of these double mutants together with the corresponding wild-type (Ler) were subjected to low level UV-B for 15 minutes under the broadband UV-field using the WG305 and WG327 cut-off filters, and were analysed by RNA gel blots. Figure 4.1.1.E shows, that in response to low-level UV-B the transcriptional induction of the genes tested are similar in wild-type and in the two double mutants. Furthermore, in the *uvr2* single mutant no enhancement of the antagonistic effect of short-wavelength-UV-B has been observed (Ulm et al., 2004). These results with the repair mutants indicate that the responses described above are not triggered by DNA damage caused by the UV-B treatment.

4.2. HY5 as a major regulator of UV-B signalling

4.2.1. The bZIP transcription factor HY5 is induced by UV-B

The analysis of RNA gel blots of UV-B treated *Arabidopsis* seedlings of three different accessions revealed that the well established light signal transduction regulator, the bZIP transcription factor *HY5* (*At5g11260*) is transcriptionally induced by low-level UV-B irradiation (Figure 4.1.1.A and C). This finding is further strengthened by the results of the microarray analysis, where *HY5* was also shown to be up-regulated under the same conditions (Ulm et al., 2004). It is also apparent, that *HY5* is one of the genes, the induction of which is antagonised by shorter wavelength ranges in UV-B (Figure 4.1.1.A and C). Furthermore it should be emphasized here, that while the light dependent transcriptional activation of *HY5* requires functional phytochromes (Tepperman et al., 2001; Quail, 2002a), the UV-B triggered *HY5* induction is independent of all the tested photoreceptors (Figure 4.1.1.A and C). This implies that HY5 is also a component of a pathway that is specific for the signal relay after perception of low level UV-B. Therefore it was of interest to test, whether the UV-B dependent up-regulation of *HY5* is also manifested on protein level, which would further suggest a role of this protein in UV-B signalling.

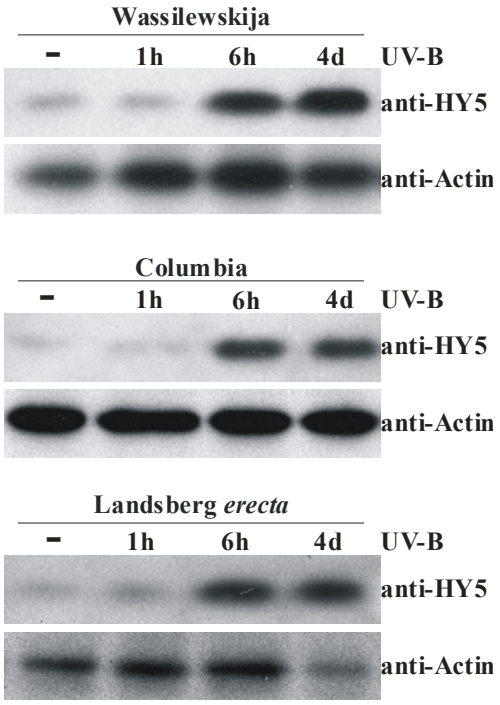

Figure 4.2.1. HY5 protein accumulates to high level in response to supplementary UV-B irradiation
Immunoblot analysis of Ws, Col and Ler seedlings grown for four days in the narrowband UV-field either in UV-B supplemented white light under WG305 cut-off (4d UV-B) or in white light only under WG327 cut-off (-UV-B). 1 and 6 h supplementary UV-B treated samples (1 h and 6 h UV-B) were generated by exchanging the WG327 cut-off to WG305 1 and 6 h before harvesting. Protein gel blots were sequentially probed with anti-HY5 and anti-Actin antibodies.

The endogenous HY5 protein levels in wild-type *Arabidopsis* seedlings (Col, Ler and Ws) subjected to 1h, 6h and 4 days of continuous supplementary UV-B (WG305 under the narrowband UV-field) were analysed by immunoblots using polyclonal anti-HY5 antibody. Figure 4.2.1. shows the HY5 protein accumulation after 6 hours in response to UV-B when compared to the non-UV-B treated controls (i.e. continuous white light). Furthermore, the high HY5 protein level is maintained in the four days UV-B treated samples, as indicated by the similar immunoreactivity of the HY5 bands in the 6 hours and four days UV-B samples.

To confirm these result, and to gain further information on the subcellular localisation of HY5, epifluorescent microscopy was used to analyse stable transgenic *Arabidopsis hy5-1* mutants expressing the HY5-YFP fusion protein under the control of the *HY5* promoter (*hy5-1/Pro$_{HY5}$:HY5-YFP*). The fusion construct is functional and is able to rescue the *hy5-1* mutation as it is shown by the complementation of the loss of flavonoid induction in the *hy5-1* mutant (see below and Figure 4.2.3.). Yet the complementation is only partial, as indicated by the 1.7 fold induction of flavonoid content in the transgenic line compared to the 2.3 fold induction in the wild-type (Figure 4.2.3.), which may be due to the disturbing effect of the relatively large (~27 kDa) YFP tag (note: HY5 is only 18 kDa). Nevertheless, the examination of the subcellular localisation of this construct revealed clear nuclear fluorescence in white light-grown seedlings (no UV-B in Appendix 2.1.), which is in good agreement with previous work (Oyama et al., 1997) and the well established function of HY5 as a transcription factor in light signalling. Furthermore, under the same conditions that were used for the Western blot experiments before (Figure 4.2.1.), strong nuclear enrichment of the HY5-YFP protein was detected in response to UV-B radiation. The induction is obvious after 6 hours, and remains high after four days (Appendix 2.1.) which correlates well with the Western blot results presented above.

We note here the modest variation observable in the HY5-YFP expression level in different samples after 1 hour of UV-B treatment (Appendix 2.1.). Part of these samples exhibit elevated nuclear fluorescence already after 1 hour, while others have still similar level to the minus UV-B control. It is yet important to emphasize that in case of the 6 hours and the 4 days continuously UV-B treated samples the fluorescence is homogenously high without significant variation.

4.2.2. HY5 is required for the UV-B induced transcriptional up-regulation of a set of UV-responsive genes

The UV-B mediated activation on both transcriptional and protein level suggests a possible role for HY5 in UV-B signalling. We assessed the importance of HY5 in these responses by analyzing *hy5-1* null mutants, first with regard to the transcriptional activation of UV-B induced genes. The transcriptional responses in the *hy5-1* mutants were assayed exactly under the same conditions as in case of the photoreceptor mutants in Figure 4.1.1.C. The RNA gel blot in Figure 4.1.1.C shows that the loss of HY5 leads to the deregulation of a set of UV-B induced genes (e.g. *At4g14690*, *At4g15480*, *At5g05410, At5g52250*), as indicated by the greatly reduced transcript accumulation in the UV-B treated (305) samples in *hy5-1* mutant compared to the corresponding wild-type (Ler). Thus HY5 is required for the UV-B induced transcriptional activation of a subset of UV induced genes. However, another set of

UV-B regulated genes was unaffected (e.g. *At3g17610*, *At1g32870*) by the *hy5-1* mutation, demonstrated by the similar level of UV-B induced mRNA production of these genes in the mutant and the wild-type.

In a subset of the HY5 dependent genes a residual UV-B dependent activation is still present in the *hy5-1* mutant (Figure 4.1.1.C, e.g. *At4g14690*, *At5g52250*), as indicated by a slight increase in the transcript levels of these genes upon UV-B treatment. This residual inducibility may be accounted for one or more additional factors regulating these genes together with HY5, yet to a smaller extent. It is interesting to note here, that the antagonistic effect of quartz irradiation still seems to be operating in the *hy5-1* mutant on these residually induced genes, indicating that HY5 is not involved in the pathway mediating the "quartz antagonism". It is also noteworthy, that according to the results from Figure 4.1.1.C, HY5 does not seem to regulate its own expression, as in the *hy5-1* mutant the *HY5* transcript accumulates in a similar manner in response to UV-B as it does in case of the wild-type. It has to be mentioned in this regard, that the *hy5-1* mutation is a point mutation resulting in a C to T exchange which changes the third codon of the *HY5* mRNA to a STOP codon (Oyama et al., 1997). These mutants produce no HY5 protein (see below), though the full length, but mutated transcript can still accumulate in the cells (verified by cloning and sequencing, data not shown).

The importance of HY5 in regulating the transcriptional UV-B responses is further supported by global gene expression profiling using the Affymetrix ATH1 oligonucleotide microarray performed in our group. 31.4% (127 out of 405 genes) of the early UV-B activated genes require HY5 for their UV-B induction, indicating the broad effect of the *hy5-1* mutation on the transcriptional activation of the UV-B responsive genes (Oravecz et al., 2006). Interestingly, the list of such HY5 dependent genes includes components that can be linked to UV tolerance, such as the photolyases of CPDs (*PHR1*/*UVR2*) and 6-4 photoproducts (*UVR3*) (Britt, 2004), enzymes of the phenylpropanoid metabolism pathways like *4CL1*, *4CL3* (Ehlting et al., 1999), *CHI*, *CHS*, *C4H*, *PAL1*, *PAL2* and *FLS1* (Winkel-Shirley, 2002), and their transcriptional regulators like *MYB12*, *MYB111* and *MYB32* (Mehrtens et al., 2005; Stracke et al., 2007) (Table 4.2.2.).

	Ler		*hy5-1*		Col		*cop1-4*			
Gene	expr	**fold**	expr	**fold**	expr	**fold**	expr	**fold**	*Genename*	
At1g12370	97	**3,0**	36	**1,4**	87	**3,0**	53	**-1,1**	*PHR1*	*PHR1* (type II CPD photolyase)
At3g15620	43	**2,0**	36	**-1,2**	47	**2,4**	52	**1,0**	*UVR3*	*UV Resistance 3* (6-4 photolyase)
At1g51680	1205	**1,9**	1235	**-1,1**	1384	**2,5**	581	**1,0**	*4CL1*	*4-coumarate-CoA ligase 1*
At1g65060	308	**2,7**	125	**-1,3**	659	**2,4**	427	**-1,3**	*4CL3*	*4-coumarate--CoA ligase 3*
At5g13930	2562	**2,4**	711	**1,0**	3105	**2,2**	3772	**1,0**	*CHS*	*chalcone synthase*
At3g55120	738	**2,6**	375	**1,0**	902	**2,5**	852	**1,0**	*CHI*	*chalcone isomerase*
At5g05270	215	**2,8**	134	**1,1**	380	**3,0**	330	**-1,1**	*CHI-like*	*chalcone isomerase family protein*
At2g30490	1514	**2,2**	1355	**1,2**	1819	**2,2**	1385	**1,0**	*C4H*	*cinnamic acid 4-hydroxylase*
At2g37040	1532	**2,5**	1240	**1,0**	2074	**2,4**	1250	**1,0**	*PAL1*	*phenylalanine ammonia-lyase 1*
At3g53260	923	**2,5**	927	**1,4**	1971	**2,9**	1379	**1,1**	*PAL2*	*phenylalanine ammonia-lyase 2*
At3g51240	1158	**4,3**	145	**3,0**	1906	**3,4**	773	**-1,1**	*F3H*	*flavanone 3-hydroxylase*
At5g08640	868	**3,2**	139	**1,1**	1453	**2,5**	1476	**-1,2**	*FLS1*	*flavonol synthase 1*
At2g47460	109	**5,2**	21	**1,2**	141	**4,7**	73	**-1,1**	*MYB12*	*myb family transcription factor*
At5g49330	70	**3,9**	74	**-1,9**	94	**4,1**	192	**-1,9**	*MYB111*	*myb family transcription factor*
At4g34990	83	**1,5**	57	**-1,1**	97	**2,3**	74	**-1,4**	*MYB32*	*myb family transcription factor*

Table 4.2.2. A subset of UV-B-induced genes that are blocked in *hy5-1* and/or *cop1-4* mutants according to comparative microarray analysis

Microarray data from two biological replicates of seven-day-old white light-grown seedlings of Ler, *hy5-1*, Col and *cop1-4* exposed to 15 min irradiation using WG327 (-UV-B) and WG305 (+UV-B) cut-off filters under the broadband UV-field. Analysis was restricted to the genes that are commonly UV-B induced in Ler and Col. Control expression values (expr) indicate expression levels of each genes under -UV-B conditions. Fold induction (fold) depicts the ratio of expression values of +UV-B/-UV-B samples. Values are averages of two array hybridisations (after Oravecz et al., 2006).

4.2.3. HY5 is essential for the establishment of UV tolerance

The induction of phenylpropanoid biosynthetic pathway components is a well established UV-B effect proposed to be mediated by the postulated UV-B photoreceptor, ultimately leading to the accumulation of sunscreen flavonoids (Beggs and Wellmann, 1994; Kucera et al., 2003). The deregulation of these components in the *hy5-1* mutant suggests that this mutant accumulates a reduced amount of protective phenolic compounds (such as flavonoids) under UV-B conditions. To test this, we measured the flavonoid content of four-day-old wild-type (Ler) and *hy5-1* mutant seedlings grown in UV-B supplemented white light (WG305 cut-off) or only in white-light (WG327 cut-off) under the narrowband UV-field. Figure 4.2.3. shows a 2.3 fold increase in the flavonoid content of Ler seedlings in UV-B relative to white-light, confirming that UV-B leads to the elevated production of UV protective compounds. However, this response is strongly impaired in the *hy5-1* mutant, with a remaining marginal 1.2 fold increase under supplementary UV-B, which is in good agreement with the reduced transcriptional regulation of genes encoding proteins involved in the phenylpropanoid biosynthesis pathway.

Consistently *hy5-1* mutants were found to be less tolerant to sustained UV-B irradiation resulting in increased mortality (Figure 5.4.1.A). Taken together, these results indicate that HY5 regulates gene expression required for the protective responses against UV-B and that HY5 is required for the survival of plants in the presence of UV-B radiation.

4.2.4. HYH has a minor role in regulating UV-B induced genes

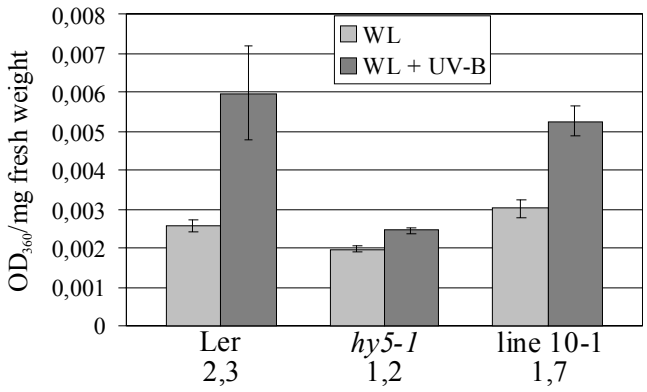

Figure 4.2.3. *hy5-1* is deficient in UV-B-responsive flavonoid induction
Wild-type (Ler), *hy5-1* mutant and *hy5-1/Pro$_{HY5}$:HY5-YFP* line 10 were grown for four days under the narrowband UV-field in continuous white light either in the presence (under WG305 cut-off [WL+UV-B]) or in the absence (under WG327 cut-off [WL]) of supplementary UV-B. The flavonoid content of 50 seedlings of each treatment was measured as described in Materials and Methods. Bars show SE of the mean (n=4).

The residual UV-B activation of a set of genes in *hy5-1* mutant (Figure 4.1.1.C) indicates that some other components beside HY5 may regulate these genes under UV-B. An obvious candidate would be HYH, the closest homologue of HY5 in *Arabidopsis* that is also induced transcriptionally by UV-B irradiation (Figure 4.1.1.C). It has been shown that HYH has a predominant role in blue light and that HY5 and HYH have overlapping functions in light signalling. Furthermore, it has also been demonstrated that the two proteins can form heterodimers (Holm et al., 2002). Therefore it was of interest to test if HYH also has a role in UV responses. To test this, *hyh hy5* double, and *hy5* and *hyh* single mutants along with their corresponding wild-type (Ws) were analysed with regard to the transcriptional induction of UV-B responsive genes both using the 15 minutes irradiation under the broadband UV-field (Figure 4.2.4.A.) and the continuous supplementary UV-B treatment under the narrowband UV-field (Figure 4.2.4.B.). In case of *At4g14690, At5g52250* and *CHS* genes the residual transcript induction in the *hy5* allele is apparent under both conditions. Figures 4.2.4.A and B also show that the mRNA of all genes tested induced to the wild-type level in the *hyh-1* mutant (except, of course, for the *HYH* mRNA).The residual *At4g14690* activation observable in the *hy5-ks50* mutant is lost in the *hyh hy5* double mutant under the broadband UV-field, and is greatly reduced under continuous UV-B (Figure 4.2.4.A and B). Similarly, the retained transcript accumulation of *At5g52250* is lost in the double mutant, compared to the *hy5* single mutant under supplementary UV-B (Figure 4.2.4.B). However, other genes that are still weakly up-regulated in *hy5* mutants still retain this residual inducibility in the *hyh hy5* double mutant, such as *CHS* (Figure 4.2.4.B). An example for a gene that has no significant residual activation in *hy5* mutants (*At4g15480*) is also shown (Figure 4.2.4.B). The *HYH* and *HY5* transcripts are demonstrated to verify the mutants (Figure 4.2.4.B).

Beside the transcriptional responses, we also sought to test the possible effects of HYH at the physiological level. To this end the flavonoid accumulation in response to UV-B was

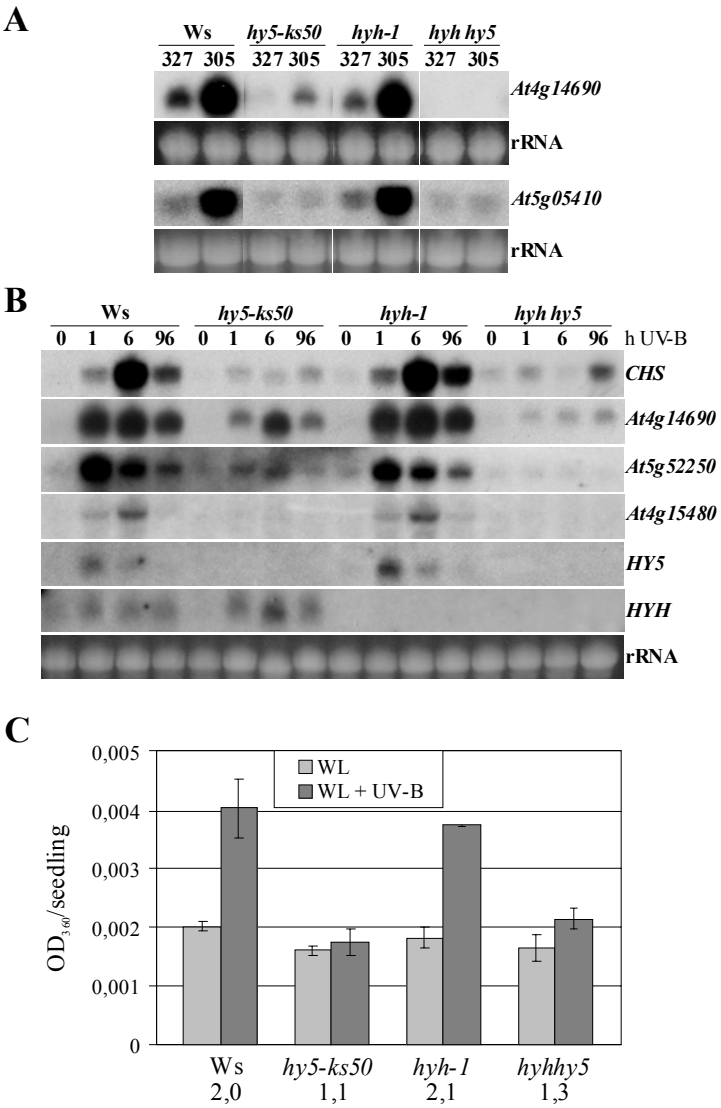

Figure 4.2.4. HYH contributes to the regulation of a subset of HY5 dependent genes, but does not play significant role in flavonoid induction in UV-B

(A) RNA gel blot analysis of seven-day-old white light-grown seedlings exposed to 15 min irradiation using WG327 (-UV-B) and WG305 (+UV-B) cut-off filters under the broadband UV-field.

(B) RNA gel blot analysis of seedlings grown for four days in the narrowband UV-field either in UV-B supplemented white light under WG305 cut-off (96 h UV-B) or in white light only under WG327 cut-off (0 h UV-B). 1 and 6 h supplementary UV-B treated samples (1 h and 6 h UV-B) were generated by exchanging the WG327 cut-off to WG305 1 and 6 h before harvesting.

(A) and **(B)** Blots were sequentially hybridised with specific probes for the indicated genes. Ethidium bromide-stained rRNA is shown as loading control.

(C) Flavonoid measurement of 50 seedlings of each genotype grown for 4 d in the narrowband UV-field in continuous white light either with (WG305 [WL+UV-B]) or without (WG327 [WL]) supplementary UV-B. Numbers below the genotypes show WG305/WG327 values indicating fold inductions. Bars show SE of the mean (n=3).

analysed. Seedlings were grown for four days under the narrowband UV-field in continuous white light either with (WG305) or without (WG327) supplementary UV-B, after which the flavonoid content of the seedlings were measured. Figure 4.2.4.C shows that UV-B irradiation results in a 2- fold increase in the flavonoid content of wild-type Ws seedlings, which correlates well with previous findings in the Ler accession (Figure 4.2.4.A). Furthermore, also in line with results presented before (Figure 4.2.3.), this induction is abolished in *hy5-ks50* mutant, as demonstrated by the marginal 1.1- fold increase (Figure 4.2.4.C). However it is also clear that the flavonoid induction in the *hyh-1* mutant in not significantly different from the wild-type (2.1 fold induction, Figure 4.2.4.C). Furthermore, the marginal induction in *hy5-ks50* is not reduced in the *hyh hy5* double mutant, rather it shows a slight increase, which correlates with the RNA gel blot result showing slightly elevated *CHS* expression in the double mutant compared to the *hy5-ks50* under sustained UV-B (Figure 4.2.4.B).

Taken together, these data indicate that HYH is responsible for the residual induction of some of the

UV-B-responsive genes in *hy5* mutants, suggesting a partly overlapping role with HY5. However, as the *hyh* mutant shows the same phenotype as wild-type on both molecular and physiological level, and in the *hyh hy5* double mutant the loss of HYH does not confer additional defects to the *hy5* mutant, we conclude that the role of HYH is only minor under the conditions tested. Moreover, the persisting weak activation of, for example, *At4g14690* in the *hyh hy5* double mutant (Figure 4.2.4.B) suggests that in addition to HYH other minor regulators also may act besides HY5.

4.2.5. HY5 dependent gene regulation temporally correlates with *HY5* gene induction and protein accumulation

It was previously shown that HY5 binds to *CHS* promoter elements *in vitro* and *in vivo*, and regulates light induced transcriptional activation of *CHS* (Ang et al., 1998; Lee et al., 2007). Consistently, *CHS* was found to be transcriptionally induced by UV-B in a HY5-dependent manner (Figure 4.2.4.B). Therefore, we sought to follow the temporal relationship of *HY5* induction and the activation of HY5 dependent genes, including *CHS*, in UV-B. To this end a timecourse assay on the wild-type accession Col was performed. Plants were grown for four days under the narrowband UV-field, and seedlings were treated with UV-B for 30 minutes, 1-, 2-, 3-, 4-, 6-, 8-, 12-, 24-, 48- and 96 hours (see Figure 4.2.5. legend for

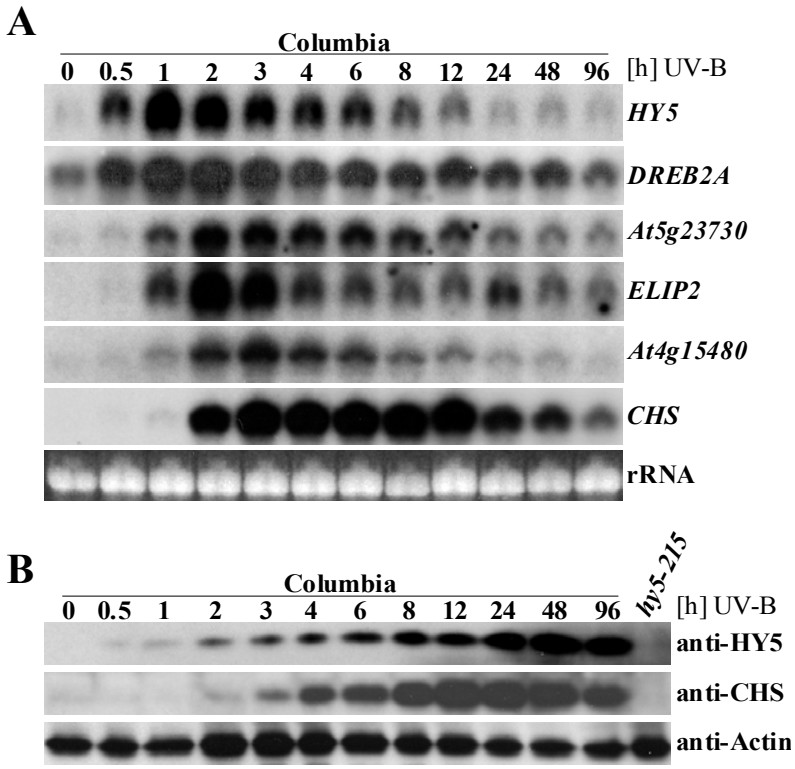

Figure 4.2.5. UV-B induced *HY5* activation and protein accumulation is in temporal correlation with HY5-dependent gene induction

Wild type Col seedlings were grown for four days in the narrowband UV-field either in white light only under WG327 cut-off (0 h UV-B) or in UV-B supplemented white light under WG305 cut-off (96 h UV-B). UV-B treatments of different durations were performed by exchanging WG327 cut-offs to WG305 for the indicated times before harvesting (i.e. 8 h UV-B means 88 h WG327 followed by 8 h WG305). Samples were divided for RNA and for protein extraction.

(A) RNA gel blot analysis of the above samples. Blots were sequentially hybridised with specific probes for the indicated genes. Ethidium bromide-stained rRNA is shown as loading control.

(B) Protein gel blot analysis of the above samples. 96h UV-B-treated *hy5-215* is shown as control for antibody specificity. Blots were sequentially probed with anti-HY5, anti-Actin and anti-CHS antibodies.

details).

The rapid accumulation of *HY5* mRNA is detectable already after 30 minutes of UV-B treatment, and peaks at about 1 hour (Figure 4.2.5.A). The transcriptional induction of *HY5* is followed by the HY5 protein accumulation that is very weakly detectable already after 30 minutes, increases further during the next hours, and remains on high levels during the UV-B treatment. *CHS* gene induction follows the accumulation of HY5 protein: it is strongly induced 1 to 2 hours after the UV-B treatment, and remains on high levels during 12 hours of irradiation (Figure 4.2.5.A). The CHS protein is weakly detectable after 2 hours, and accumulates to high level after 3 to 4 hours. It reaches its maximum at around 12 hours, and remains on elevated levels during the UV-B treatment (Figure 4.2.5.B). The induction of other HY5 dependent genes is also delayed compared to *HY5*. Increase in *DREB2A* transcript level is detectable after 30 minutes, and it peaks at about 1 to 2 hours. *At5g23730* and *ELIP2* are induced around 1hour, and they reach their maximum at about 1 to 2 hours. *At4g15480* is the latest, with weak increase around 1 hour, and a peak at about 3 hours. These results demonstrate that *HY5* gene induction and subsequent HY5 protein accumulation is in temporal correlation with the transcriptional up-regulation of the HY5-dependent genes.

In case of the genes tested, including *HY5*, transcript accumulation is transient even in the continuous presence of the UV-B stimulus. This is particularly interesting in case of HY5, as under continuous UV-B the protein remains at high levels even when the transcript amount is already back to background levels (24 to 96 h, Figure 4.2.5.A and B). Similar observation can also be made for CHS, though the decrease in its transcript level is less pronounced under these conditions, as it stays considerably higher than background after four days of UV-B (Figure 4.2.5.A). These findings indicate that in addition to transcriptional induction, UV-B may regulate HY5 and CHS levels by means of protein stability.

4.2.6. HY5 overexpression complements the *hy5* phenotype, but does not lead to hyper-responsiveness to UV-B

The data thus far supports the crucial role of HY5 in UV-B responses indicated by the severe defects in *hy5* mutant on both molecular and phenotypic level. HY5 function was further investigated by analysing its overexpression under UV-B conditions. For this purpose, stable transgenic lines expressing *HY5* under the regulation of the strong constitutive cauliflower mosaic virus 35S promoter in a *hy5-215* mutant background (*hy5-215/Pro$_{35S}$:HY5*) were generated. Protein gel blot of extracts from four-day-old weak white light-grown (under WG327 in the narrowband UV-field) seedlings of three independent transgenic lines together with the corresponding wild-type (Col) and mutant (*hy5-215*) is shown in Figure 4.2.6.A. The blot was analysed with polyclonal anti-HY5 antibody. The

strong immunoreactivity of the HY5 band in the three transgenic lines compared to the Col demonstrate much higher level of HY5 protein expression in these lines than in the wild-type.

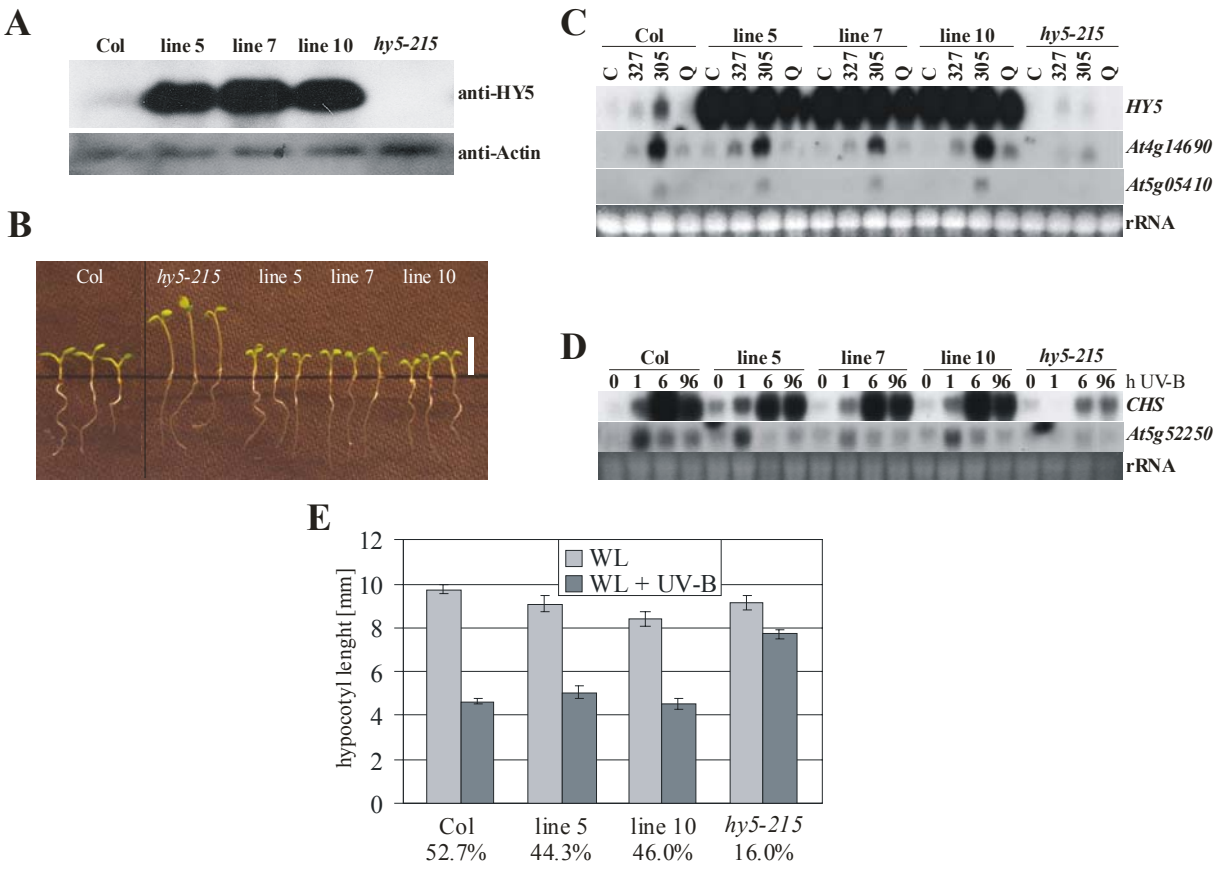

Figure 4.2.6. HY5 overexpression complements *hy5* phenotype, but does not result in UV-B hyper-responsiveness
hy5-215/Pro$_{35S}$:HY5 lines 5, -7 and -10 were compared to Col wild type and *hy5-215* mutant.
(A) Protein gel blot analysis of four-day-old seedlings grown in the narrowband UV-field in continuous white light under the WG327 cut-off filter. Blots were sequentially probed with anti-HY5 and anti-Actin antibodies.
(B) Photograph of four-day-old, continuous white light-grown seedlings. Bar = 5 mm.
(C) RNA gel blot analysis of seven-day-old white light-grown seedlings of the indicated genotypes exposed to 15 min irradiation in the broadband UV-field using different UV-B spectra under WG327, WG305 and quartz (Q) cut-off filters. Control (C) samples were kept in the standard growth chamber.
(D) RNA gel blot analysis of seedlings of the indicated genotypes grown for four days in the narrowband UV-field either in UV-B supplemented white light under WG305 cut-off (96 h UV-B) or in white light only under WG327 cut-off (0 h UV-B). 1 and 6 h supplementary UV-B treated samples (1 h and 6 h UV-B) were generated by exchanging the WG327 cut-off to WG305 1 and 6 h before harvesting.
(C) and **(D)** Blots were sequentially hybridised with specific probes for the indicated genes. Ethidium bromide-stained rRNA is shown as loading control.
(E) Hypocotyl measurement of seedlings grown for 4 d in the narrowband UV-field in continuous white light either with (WG305 [WL+UV-B]) or without (WG327 [WL]) supplementary UV-B. Numbers below the genotypes show the relative hypocotyl growth inhibition by UV-B as percentage [(1-(WG305/WG327))x100]. Bars show SE of the mean (n=30).

To assess the functionality of this overexpressed construct, we examined whether it is able to complement the long hypocotyl phenotype of *hy5-215* in white light conditions. Seedlings of the transgenic lines, wild-type and mutant were grown for four days in continuous strong white light, after which a picture of representative seedlings was taken. The three transgenic lines showed a complete rescue of the long hypocotyl phenotype of *hy5-215* (Figure 4.2.6.B),

demonstrating the functionality of the cloned *HY5* gene in light. It is of note that no hypersensitivity to light was observed in the three transgenic lines (i.e. their hypocotyls are not shorter than wild-type), indicating the lack of an overexpression phenotype in white light conditions, as already has been reported for HY5 overexpression before (Ang et al., 1998).

It was of interest to test the HY5 overexpression lines in UV-B as well. First the UV-triggered transcriptional responses were analysed in the three *hy5-215/Pro$_{35S}$:HY5* lines together with wild-type and mutant under both the broadband UV-field using the 15 minutes irradiations and under the narrowband field using continuous supplementary UV-B treatments. The high level of *HY5* mRNA accumulation in the transgenic lines is apparent under all conditions on Figure 4.2.6.C. The overexpression of HY5 rescues the transcriptional UV phenotypes of *hy5-215* in case of both the 15 minutes broadband (Figure 4.2.6.C) and the supplementary narrowband UV-B treatments (Figure 4.2.6.D), as demonstrated by the similar transcript accumulation of the UV-B induced genes in the transgenic lines compared to wild-types. However, the similar levels of these transcripts in HY5 overexpressors and wild-types under conditions devoid of UV-B (C and 327 on Figure 4.2.6.C and 0 h UV-B on Figure 4.2.6.D) suggest that HY5 in itself is not sufficient to induce its UV-regulated target genes. Moreover, in line with this observation, the UV-B induced transcript accumulation in the transgenic lines is not significantly higher than in the wild-type (Figure 4.2.6.C and D), which again shows the lack of hyper-responsiveness in the HY5 overexpressing lines, similar to the situation in white light (Figure 4.2.6.B).

Besides the transcriptional regulation, it was also of interest to study a physiological response to UV-B with regard to HY5 overexpression. A well established photomorphogenic, non-stress-mediated effect of physiological UV-B is the hypocotyl growth inhibition response of *Arabidopsis* seedlings (Kim et al., 1998). To analyse this phenotype we grew seedlings of two transgenic lines (lines 5 and 10), a wild-type and a mutant for four days under the narrowband UV-field in continuous weak white light either in the presence or in the absence of supplementary UV-B (WG305 or WG327, respectively), and measured the hypocotyl length of 30 seedlings from each treatment. Figure 4.2.6.E shows that supplementary UV-B treatment results in 52.7% inhibition of hypocotyl growth in wild-type, compared to the much lower 16% inhibition in *hy5-215* mutant. Furthermore, the wild-type level inhibition is almost completely restored in the two HY5 overexpressing lines 5 and 10 with 44.3% and 46.0% inhibition rate, respectively. We note here that the hypocotyl length of the wild-type seedlings are as long as that of the *hy5* mutant under these conditions, which is most probably due to low levels of white light used in this experiment. It is important to note though, that the similar hypocotyl phenotypes of wild-type and *hy5-215* mutants in white light is specific for

this experiment in Figure 4.2.6.E, and was not the case for other experiments using this light field (see also Figure 4.2.7.). However, this data demonstrates that low-level UV-B induces hypocotyl growth inhibition even under conditions, when the white light background is too weak to trigger the same physiological response through the light signalling pathways. Furthermore, these data suggest, that HY5 is required for this hypocotyl growth inhibition response of Col accession. Moreover, the greatly reduced responsiveness of *hy5-215* mutant in UV-B is complemented in the HY5 overexpressing transgenic lines, further supporting their functionality, but again, HY5 overproduction does not result in overexpression phenotype in UV-B (Figure 4.2.6.E).

4.2.7. UV-B induced hypocotyl growth inhibition is HY5 dependent

It was of interest to confirm the UV-B induced hypocotyl growth inhibition phenotype in another *Arabidopsis* accession as well. Seedlings were grown for four days in continuous white light under the narrowband UV-field with (WG305 cut-off) or without (WG327 cut-off) supplementary UV-B. Hypocotyl length of *hy5-1*, *cop1-4*, *cop1*^{eid6} and of the corresponding wild-types (Ler and Col) was measured. Hypocotyl length of the *phyA-201 phyB-5* double mutant was also determined as control. Figure 4.2.7. shows that continuous UV-B irradiation results in hypocotyl growth inhibition in wild-type, 42% and 44% in Ler and Col respectively. The response is reduced in *hy5-1* to 9%, while in the *phyAB* double mutant (36% inhibition) that has similarly long hypocotyl in light as *hy5-1*, this UV-B response is comparable to the corresponding Ler wild-type. No significant hypocotyl growth inhibition response is detectable in *cop1-4*. Albeit this finding is less conclusive under these conditions, as this mutant has already very short hypocotyl in white light without UV-B, is consistent with a previous report (Kim et al., 1998). The same is true for *cop1*^{eid6} that, in contrast, do exhibit an apparent hypocotyl growth inhibition response to UV-B.

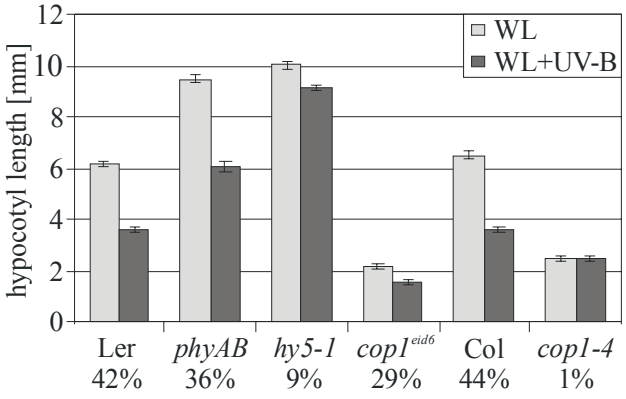

Figure 4.2.7. HY5 is required for the UV-B induced hypocotyl growth inhibition response
phyAphyB double mutant, *hy5-1*, Ler, *cop1-4*, *cop1*^{eid6} and Col were grown for 4 d in the narrowband UV-field in continuous white light either with (WG305 [WL+UV-B]) or without (WG327 [WL]) supplementary UV-B. Hypocotyl lengths of the seedlings were measured. Numbers below the genotypes show the relative hypocotyl growth inhibition by UV-B as percentage [(1-(WG305/WG327))x100]. Bars show SE of the mean (n=120).

4.3. COP1 is a crucial positive regulator of UV-B responses

4.3.1. COP1 is an essential positive regulator of UV-B triggered gene induction, including *HY5* activation

In dark, HY5 protein is ubiquitylated by the E3 ubiquitin ligase COP1 in the nucleus, leading to its subsequent degradation by the 26S proteasome. Light perception triggers the nuclear exclusion of COP1, and therefore stabilizes its target protein HY5, hence allowing light dependent transcription to occur. Thus COP1 functions as a major repressor of light signalling in plants (Yi and Deng, 2005). This well characterised connection between HY5 and COP1, and the utilisation of shared signalling components in the light and UV-B signalling pathways, exemplified by the crucial role of HY5 in both, led us to investigate the possible contribution of COP1 to UV-B signal transduction. As a first step, we analysed the UV-B induced transcriptional changes in *cop1 Arabidopsis* mutants. Since null mutants of COP1 are seedling lethal, we investigated the widely used, viable *cop1-4*, which is considered as a weak allele with regard to its phenotype in light. This allele contains a premature stop codon resulting in a truncated protein that contains only the N-terminal 282 amino acids and thus lacks the C-terminal WD40 repeats domain (McNellis et al., 1994b). In addition, it was shown that this truncated protein is present at highly reduced levels only (McNellis et al., 1994b).

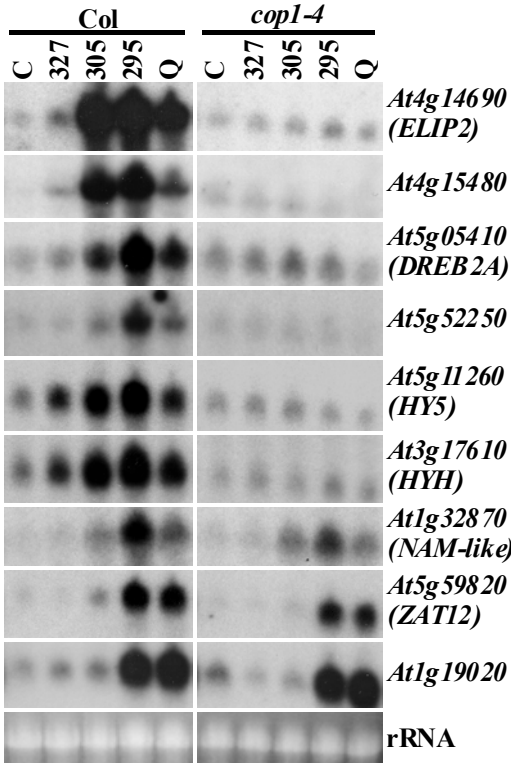

Figure 4.3.1. COP1 is required for the activation of a set of UV-B induced genes, including *HY5*
RNA gel blot analysis of seven-day-old white light-grown seedlings exposed to 15 min irradiation in the broadband UV-field using different UV-B spectra under WG327, WG305, WG295 and quartz cut-off filters. Control (C) samples were kept in the standard growth chamber. Blots were sequentially hybridised with specific probes for the indicated genes. Ethidium bromide-stained rRNA is shown as loading control.

Seven-day-old *cop1-4* mutant seedlings were irradiated for 15 minutes under the broadband UV-field exactly as described before in Section 4.1.1. Surprisingly, the induction of a set of UV-B regulated genes was found to be blocked in *cop1-4* (Figure 4.3.1.), as indicated by the lack of elevated transcript accumulation in the mutant in response to UV-B. In contrast to *hy5-1*, the block of induction in *cop1-4* seems to be complete in case of these genes (compare e.g. *At4g14690*, *At5g52250* in Figure 4.1.1.C and Figure 4.3.1.). It is also evident from Figure 4.3.1., that *HY5* itself is among the

COP1 dependent genes in UV-B. Thus, in sharp contrast to their antagonistic role in the dark-to-light transition, both COP1 and HY5 seem to act positively in UV-B-mediated gene regulation.

The COP1 dependence of select genes that are not antagonised by shorter wavelength UV-B was also tested (e.g., *At5g59820*, *At1g19020*). The similar transcript accumulation in wild-type and *cop1-4* under WG295 and quartz (Figure 4.3.1.) indicates that these genes do not require COP1 similar to the case of HY5 (Figure 4.1.1.C). These data suggest that COP1 is acting in the postulated UV-B photoreceptor pathway, rather than in the more general UV-B stress pathway, tentatively characterised by these marker genes.

Further evidence pointing to the crucial role of COP1 in UV-B signalling comes from an independent approach using a luciferase reporter gene-based genetic screen. This approach aimes at the identification of components in the UV-B pathway through EMS mutants deficient in the UV-B-dependent activation of a *HY5* promoter-driven luciferase reporter (Ulm et al., 2004). A mutant that has a C-T transition changing the Gln-283 codon to a stop codon in the *COP1* gene was found by this screen (data from A. Brzezinska, see Oravecz et al., 2006). This mutation in Ws is in fact identical to the *cop1-4* allele in Col. In addition to these, the strong *cop1-1* allele was also found to be deficient in the UV-B dependent up-regulation of a set of UV-B responsive genes (see below and Figure 4.3.8.).

Taken together, we conclude that COP1 and HY5 are major positive regulators of UV-B triggered gene induction, and COP1 is required for the activation of *HY5*.

4.3.2. Sequential action of COP1 and HY5 regulates the establishment of UV tolerance

All the HY5 dependent genes tested by RNA gel blots were found to be COP1 dependent as well (*At4g14690*, *At4g15480*, *At5g05410*, *At5g52250* Figure 4.3.1. and Figure 4.1.1.C), while other COP1-dependent genes do not require HY5 under UV-B (e.g. *At3g17610*). This points to the broader effect of the *cop1* mutation on UV-B signalling than that of *hy5* and suggests a sequential COP1-HY5 action on these genes. In fact, a whole transcriptome profiling of seven-day-old white light-grown and 15 minutes UV-B treated *Arabidopsis* seedlings using the ATH1 oligonucleotide microarrays confirmed the broad impact of the *cop1-4* mutation on UV-B triggered gene induction: 75.3% (272 out of 361) of the UV-B induced genes (including *HY5*) in Col require functional COP1 for their ability to respond to UV-B (Oravecz et al., 2006). In addition, comparative analysis of *cop1-4* and *hy5-1* microarrays confined to the genes commonly induced by UV-B in both respective wild-type accessions indicates that close to half of the COP1-dependent genes (43%; 103 out of 240) require HY5 as well, and almost all of the HY5-dependent genes (96%; 103 out of 107) are

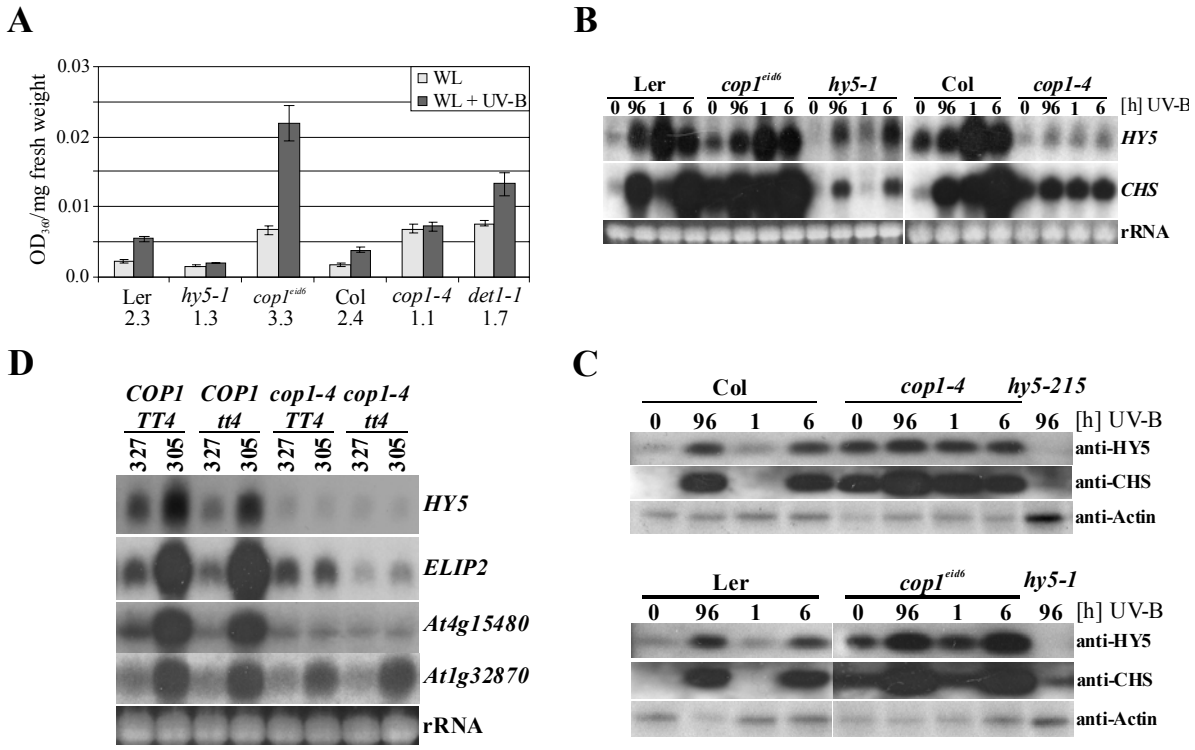

Figure 4.3.2. Functional COP1 and HY5 are required for the induction of UV-B-responsive flavonoid biosynthesis

(A) to **(C)** Seedlings of the indicated genotypes were grown for four days in the narrowband UV-field either in UV-B supplemented white light under WG305 cut-off (96 h UV-B or WL+UV-B) or in white light only under WG327 cut-off (0 h UV-B or WL). Where indicated, 1 and 6 h supplementary UV-B treated samples (1 h and 6 h UV-B) were generated by exchanging the WG327 cut-off to WG305 1 and 6 h before harvesting.

(A) *hy5-1, cop1-4 but not cop1^{eid6} and det1-1 mutants are deficient in UV-B induced flavonoid accumulation.* Flavonoid measurement of Ler, *hy5-1, cop1^{eid6}*, Col, *cop1-4* and *det1-1* seedlings. Numbers below the genotypes show WG305/WG327 values indicating fold inductions. Bars show SE of the mean (n=9).

(B) COP1 *dependent HY5 and CHS gene induction in response to UV-B is impaired in hy5-1 and cop1-4 mutants, but not in cop1^{eid6}*. RNA gel blot analysis of Ler, *hy5-1, cop1^{eid6}*, Col and *cop1-4* seedlings.

(C) *UV-B induced* HY5 *and* CHS *protein accumulation is impeded in cop1-4 but not in cop1^{eid6} mutants.* Immunoblot analysis of Col, *cop1-4*, Ler and *cop1^{eid6}* seedlings. *hy5-1* and *hy5-215* samples are shown as controls for antibody specificity. Blots were sequentially probed with anti-HY5 anti-Actin and anti-CHS antibodies.

(D) *Absence of* CHS (TT4) *does not restore wild-type molecular UV-B responses in cop1-4.* RNA gel blot analysis of seven-day-old white light-grown *cop1-4 tt4* double, *cop1-4* and *tt4* single mutants and wild type. Seedlings were exposed to 15 min irradiation using WG327 (-UV-B) and WG305 (+UV-B) cut-off filters under the broadband UV-field.

(B) and **(D)** Blots were sequentially hybridised with specific probes for the indicated genes. Ethidium bromide-stained rRNA is shown as loading control.

COP1-dependent (Oravecz et al., 2006). These results are consistent with a sequential action of COP1 and HY5 on this subset of genes, and the 103 COP1- and HY5-dependent genes may delineate the output of the UV-B-activated COP1-HY5 pathway.

As described before the HY5-dependent genes under UV-B include enzymes performing DNA damage repair, and members and regulators of the biosynthetic pathways that generate UV-protective pigments (see above and Table 4.2.2.). It is apparent from the comparative microarray analysis that the same genes are also among the COP1-dependent genes (Table 4.2.2.) indicating that all the analysed UV-tolerance components that require HY5 for their

transcriptional activation in response to UV-B also require COP1. However, the induction of, for example, a flavanone 3-hydroxylase (F3H, *At3g51240*) is blocked in *cop1-4*, but is not affected in *hy5-1*. In line with these results, similar to *hy5*, *cop1-4* was also found to exhibit reduced UV-B tolerance in supplementary UV-B, although to a lesser (Figure 5.4.1.B).

Since the UV-B-induced genes of the phenylpropanoid pathway are regulated by the sequential action of COP1 and HY5, and as in the two corresponding mutants the deregulation leads to decreased UV-B tolerance (Figure 5.4.1.), we were interested whether the accumulation of flavonoids in response to UV-B correlated with gene expression and tolerance in *cop1-4*, as it did in the case of *hy5-1*. Seedlings were grown for four days in continuous white light under the narrowband UV-field with (WG305 cut-off) or without (WG327 cut-off) supplementary UV-B and the flavonoid content of the samples was measured. Figure 4.3.2.A. shows the increased flavonoid accumulation in wild-types Col (2.4 fold) and Ler (2.3 fold) in response to UV-B, while in both *cop1-4* and *hy5-1* mutants this response is severely reduced to a 1.1 and 1.3 fold increase, respectively. Interestingly, *det1-1*, another constitutively photomorphogenic mutant, that has similar phenotype to *cop1-4* in light, is able to increase its flavonoid content by 1.7 fold in response to UV-B treatment, in agreement with its retained UV-B tolerance demonstrated in Figure 5.4.1.B.

4.3.3. The nonconstitutive photomorphogenic *cop1^{eid6}* allele is not impaired in UV-B responses

cop1^{eid6} is a nonconstitutive photomorphogenic *cop1* allele that has a mutation changing the conserved His-69 residue of the RING finger motif to a Tyr. These seedlings exhibit normal etiolated growth in the dark but are strongly hypersensitive to visible light (Dieterle et al., 2003), resulting in a dwarf growth phenotype comparable to *cop1-4*. In sharp contrast to *cop1-4* however, *cop1^{eid6}* is proficient in the activation of its flavonoid biosynthesis in response to UV-B similar to wild-type, as demonstrated by the 3.3 fold increase in the flavonoid content of the UV-B-irradiated *cop1^{eid6}* mutants (Figure 4.3.2.A).

To further understand the molecular events leading to the differential UV-B induced flavonoid accumulation in *hy5-1*, *cop1-4* and *cop1^{eid6}*, *CHS* and *HY5* gene expression and protein accumulation were analysed in seedlings subjected to 1- and 6 hours and 4 days of supplementary UV-B under the narrowband UV-field. The results show in both wild-types (Col and Ler) the clear induction of the *HY5* transcript one hour after the UV-B treatment, and it recedes to near background level after four days in continuous UV-B (Figure 4.3.2.B). Transcriptional activation is followed by HY5 protein accumulation as demonstrated by the enhanced immunoreactivity of HY5 detectable 6 hours after the UV-B treatment that is retained at high level after 4 days of UV-B (Figure 4.3.2.C). The HY5 protein accumulation is

followed by the *CHS* gene induction with the highest transcript level at 6 hours (Figure 4.3.2.B), and the subsequent production of CHS protein indicated by the elevated immunoreactivity of CHS in wild-types (Figure 4.3.2.C). In accordance with CHS induction, these wild-type seedlings accumulate elevated levels of flavonoids (Figure 4.3.2.A) in response to UV-B. These findings correlate well with previous results shown in Figures 4.2.1., 4.2.5., 5.4.1.B and Appendix 2.1.

However, in *cop1-4* mutant neither *HY5* nor *CHS* transcript is induced upon UV-B stimulus (Figure 4.3.2.B), and accordingly no considerable increase in HY5 and CHS protein level is apparent (Figure 4.3.2.C top). Likewise, in the *hy5-1* mutant *HY5* and *CHS* mRNA accumulation in response to the UV-B treatment is severely reduced (Figure 4.3.2.B). In contrary though, the two genes are clearly induced in $cop1^{eid6}$ on both transcript (Figure 4.3.2.B) and protein level upon UV-B irradiation (Figure 4.3.2.C bottom). Thus, in agreement with the flavonoid accumulation in Figure 4.3.2.A, the UV-B inducibility of *HY5* and *CHS* mRNA and that of the corresponding proteins is maintained in $cop1^{eid6}$, but is severely impaired in *cop1-4* and *hy5-1* mutants. This indicates that while *cop1-4* and *hy5-1* are deficient in their UV-B responses, $cop1^{eid6}$ retains its ability to react upon UV-B. We note the elevated basal level of HY5 and CHS proteins and *CHS* mRNA coupled with higher basal flavonoid accumulation in *cop1-4* compared to Col in white light. Similarly, $cop1^{eid6}$ exhibits an already elevated gene and protein expression of CHS and HY5, and also increased flavonoid accumulation compared to Ler (Figure 4.3.2.A, B and C). However, in $cop1^{eid6}$ these transcripts, proteins and flavonoids can be further induced by UV-B, in *cop1-4* this inducibility is abolished.

Figure 4.3.2.B shows the reduced *HY5* mRNA accumulation in *hy5-1* mutant after one hour of supplementary UV-B treatment compared to wild-type. This contrasts to our previous results presented in Figure 4.1.1.C, where upon 15 minutes pulse of UV-B treatment *HY5* transcript accumulated to wild-type level in *hy5-1*. However, *HY5* transcript accumulation after 6 hours and 4 days of UV-B is rather similar to the wild-type in the *hy5-1* mutant. This may indicate slower induction kinetics and perhaps a lower level of activation of *HY5*, rather than a complete loss of induction in this mutant. We also note the residual *CHS* gene activation and flavonoid accumulation in the *hy5-1* (but not in *cop1-4*) mutant in response to supplementary UV-B treatment (Figure 4.3.2.A and B) that contrasts with the apparent lack of such residual gene induction in the microarray analysis (Table 4.2.2.) of 15 min UV-B pulse treated seedlings. Nevertheless, this remaining up-regulation of *CHS* and flavonoid production in *hy5-1* may represent a HY5 independent branch of the pathway downstream of COP1 (no residual effect is detectable in *cop1-4*).

Flavonoids are potential sunscreen metabolites that may interfere with the UV-B response (Li et al., 1993). Thus it follows, that the reported elevated flavonoid content of *cop1-4* (see above) may be the cause of reduced UV-B responses in this mutant. Both *det1-1* and *cop1*^{eid6} share the increased sunscreen phenotype of *cop1-4*. These two former mutants though exhibit UV-B responses comparable to wild-type, while *cop1-4* is broadly impaired in it. This indicates that the elevated flavonoid content is not the cause of the strong decrease of UV-B responses in *cop1-4*. To investigate this notion further, the UV-B triggered gene induction was analysed in a double mutant of *cop1-4* and *CHS* knockout (*cop1-4 tt4*; TT4=CHS). Obviously, *tt4* does not have the key enzyme of flavonoid biosynthesis, CHALCONE SYNTHASE. Seven-day-old white light-grown seedlings of wild-type, *tt4* and *cop1-4* single mutants and *cop1-4 tt4* double mutants were exposed to 15 minutes irradiation under the broadband UV-field using WG327 and WG305 cut-off filters as described before. The RNA gel blot analysis shows that *cop1-4 tt4* double mutants are similarly defective in inducing UV-B responsive genes than their *cop1-4* TT4 siblings that have functional CHS (Figure 4.3.2.D), further suggesting that elevated flavonoid content is not the cause of the diminished UV-B responses in *cop1-4*.

4.3.4. The YFP-COP1 fusion protein complements the *cop1-4* phenotypes

Nucleocytoplasmic partitioning of COP1 is thought to be an important regulatory mechanism to modulate its function in the dark-to-light transition (von Arnim and Deng, 1994; Subramanian et al., 2004). Therefore it was of interest to investigate COP1 protein accumulation and subcellular localisation in response to UV-B. To this end, stable transgenic lines expressing the YFP-COP1 fusion protein under the control of the strong constitutive 35S promoter of cauliflower mosaic virus (in the *cop1-4* mutant background *cop1-4/Pro*$_{35S}$*:YFP-COP1*) were used.

Initially, a set of experiments were performed to verify the functionality of YFP-COP1 fusion protein in both light and UV-B responses. First, we examined whether the YFP-COP1 fusion protein is able to rescue the *cop1-4* constitutive photomorphogenic phenotype in darkness and continuous light. Seedlings of three independent transgenic lines (lines 1, 7 and 21) together with the corresponding *cop1-4* mutant and wild-type (Col) were grown for four days either in continuous darkness or white light. The etiolated phenotype of the dark grown Col (Figure 4.3.4.A, left) is completely restored in all three transgenic lines as indicated by the long hypocotyls and closed cotyledons in contrast to the short hypocotyls and open cotyledons of the *cop1-4* mutant. Similarly, in continuous white light-grown seedlings the short hypocotyl phenotype of *cop1-4* is complemented in the three transgenic lines (Figure

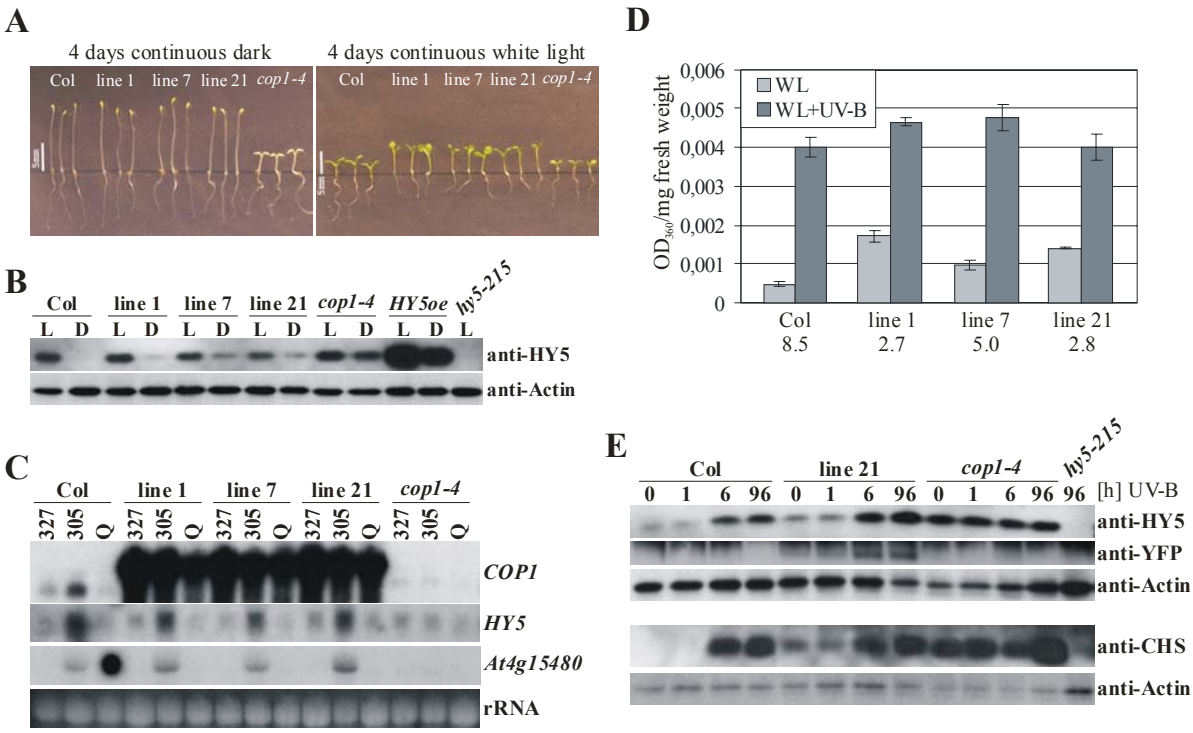

Figure 4.3.4. HY5 protein accumulates to high levels in UV-B even in the overexpressed presence of functional YFP-COP1

cop1-4/Pro$_{35S}$:YFP-COP1 lines 1, -7 and -21 were compared to Col wild type and *cop1-4* mutant.

(A) YFP-COP1 *complements both the dark and light phenotypes of cop1-4.* Photographs of representative seedlings of the indicated genotypes grown for four days in continuous dark (left) or white light (right). Bar = 5 mm

(B) YFP-COP1 *is proficient in degrading HY5 in darkness.* Protein gel blot analysis of seedlings of the indicated genotypes grown for 4 d in white light in the standard growth chamber and were either left there (L) or transferred to darkness (D) for an additional day. The HY5 overexpressor *hy5-215/Pro$_{35S}$:HY5* line 10 is shown as comparison and *hy5-215* mutant as antibody control. The blot was sequentially probed with anti-HY5 and anti-Actin antibodies.

(C) YFP-COP1 *overexpression complements transcriptional UV-B responses.* RNA gel blot analysis of seven-day-old white light-grown seedlings of the indicated genotypes exposed to 15 min irradiation in the broadband UV-field using different UV-B spectra under WG327, WG305 and quartz (Q) cut-off filters. Blots were sequentially hybridised with specific probes for the indicated genes. Ethidium bromide-stained rRNA is shown as loading control.

(D) and **(E)** Seedlings were grown for four days in the narrowband UV-field either in UV-B supplemented white light under WG305 cut-off (96 h UV-B or WL+UV-B) or in white light only under WG327 cut-off (0 h UV-B or WL). Where indicated, 1 and 6 h supplementary UV-B treated samples (1 h and 6 h UV-B) were generated by exchanging the WG327 cut-off to WG305 1 and 6 h before harvesting.

(D) YFP-COP1 *complements the UV-B induced flavonoid phenotype of cop1-4.* Flavonoid measurement of Col, *cop1-4/Pro$_{35S}$:YFP-COP1* lines 1, -7 and 21. Numbers below the genotypes show WG305/WG327 values indicating fold inductions. Bars show SE of the mean (n=3).

(E) *High level of endogenous* HY5 *accumulation in the presence of* YFP-COP1. Immunoblot analysis of seedlings of the indicated genotypes. Top: 10% SDS-PAGE blot sequentially probed with anti-YFP, anti-HY5 and anti-Actin antibodies. Bottom: 12% SDS-PAGE blot sequentially probed with anti-CHS and anti-Actin antibodies.

4.3.4.A, right). Moreover, the three transgenic lines also manifest a clear hyposensitivity to light, as indicated by the even longer hypocotyls than in case of the wild-type, which has previously been shown to be an effect of COP1 overexpression (McNellis et al., 1994a).

To test YFP-COP1 functionality in white light at the molecular level as well, we investigated its ability to promote HY5 degradation under dark conditions. For this purpose, the same set of lines were grown for four days in white light (12 h light/12 h dark cycles),

after which half of each sample was transferred to darkness for one additional day, while the other half was left under unchanged conditions. On the fifth day all samples were harvested, and HY5 protein levels were compared in the protein extracts by protein gel blot using anti-HY5 antibody. On Figure 4.3.4.B the loss or a strong reduction of HY5 protein level is evident for the wild-type and the three transgenic lines after dark treatment. On the contrary, in *cop1-4* the HY5 abundance is comparable in both light and after dark treatment. This indicates that YFP-COP1 rescues the deficiency of *cop1-4* to promote HY5 degradation in dark. However, the complementation seems to be partial, as indicated by the remaining, weakly detectable level of HY5 protein in the dark treated transgenic lines. This may be due to the relatively big YFP tag that may disturb the COP1 function, by reducing its ubiquitin ligase efficiency. Still, these results indicate that the YFP-COP1 fusion protein is functional in light responses, and is able to complement light phenotypes of *cop1-4* on both molecular and phenotypic level.

Beside the white light responses, we also sought to inspect if YFP-COP1 is working properly under UV-B conditions. To this end we exposed seven-day-old white light grown seedlings of Col, *cop1-4* and the three transgenics to 15 minutes irradiation in the broadband UV-field under WG327 (minus UV-B), WG305 and quartz (plus UV-B) filters, as before. RNA gel blot analysis shows the wild-type-level accumulation of *HY5* and *At4g15480* transcripts in response to low level UV-B irradiation (WG305) in the three transgenic lines, in contrast to the *cop1-4* mutant (Figure 4.3.4.C). The highly overexpressed *YFP-COP1* mRNA is also shown in the three transgenic lines. These results demonstrate that overexpression of *YFP-COP1* complements the *cop1-4* phenotype in UV-B on the molecular level. However, no overexpression phenotype, such as hyper-induction of UV-responsive genes is apparent in the transgenic lines, as the transcripts of the two analysed genes accumulate only to wild-type-level, and not higher, in response to UV-B (Figure 4.3.4.C).

On the phenotypic level, we assayed the UV-B induced flavonoid accumulation in the *cop1-4/Pro$_{35S}$:YFP-COP1* lines to further validate YFP-COP1 functionality. Seedlings were grown for four days under the narrowband UV-field in continuous white light either in the presence (WG305) or in the absence (WG327) of supplementary UV-B. Flavonoid content of wild-type and the three transgenic lines were measured. Figure 4.3.4.D shows that the flavonoid induction in the YFP-COP1 overexpressors is restored to some extent, as indicated by the 2.7, 5.0 and 2.8 fold increase in line 1, 7 and 21, respectively (see also Figure 4.3.2.A). However, this complementation seems to only be partial, when compared to the 8.5 fold increase in the wild-type. It is interesting to note though, that in the three transgenic lines the basal level of flavonoid production in white-light is significantly higher than in Col, and

accordingly one of these lines exhibit increased background CHS and HY5 protein expression under white-light conditions when compared to wild-type (Figure 4.3.4.E). On the other hand, in response to UV-B both flavonoid production, CHS and HY5 protein expression is induced to as high, or even higher level, than in the wild-type (Figure 4.3.4.D and E). These results suggest that rather it is the white-light-phenotypes of *cop1-4* that are partially complemented by the overexpressed YFP-COP1, as the flavonoids, HY5 and CHS proteins accumulate to lower level in the transgenic lines than in the *cop1-4* mutant, but still not as low as in the wild-type under white-light conditions. Notwithstanding this, UV-B induces the accumulation of these molecules to as high levels as in the wild-type, indicating full complementation with regard to the UV-B response. It is also noteworthy that again, no overexpression phenotype is attributable to COP1 overexpression, as no significant over-accumulation of either flavonoids or HY5 or CHS is detectable in the transgenic lines.

4.3.5. UV-B induces the nuclear accumulation of COP1 and HY5

In order to investigate the subcellular localisation of COP1 under UV-B conditions in living *Arabidopsis* seedlings, we took advantage of the three independent *cop1-4/Pro$_{35S}$:YFP-COP1* lines (lines 1,7 and 21). *hy5-1/Pro$_{HY5}$:HY5-YFP* line 10 was also included as comparison. Seedlings were grown for four days under the narrowband UV-field either only in continuous white-light (WG327) or in UV-B supplemented continuous white light (WG305). Seedlings were analysed with an epifluorescent microscope. An YFP filter set was applied to reveal YFP fluorescence, and thus YFP-COP1 and HY5-YFP signals. A rhodamine filter set was used for the detection of fluorescence from chlorophyll to be able to differentiate between YFP and background chlorophyll fluorescence in the YFP channel. The result is shown in Appendix 2.2. Interestingly, clear nuclear enrichment of YFP-COP1 was found under supplementary UV-B in all the three transgenic lines, as indicated by the markedly increased nuclear fluorescence in the +UV-B samples (Appendix 2.2.A). Similarly, in agreement with our previous results, HY5-YFP expressing seedlings also show greatly enhanced nuclear fluorescence in response to UV-B (Appendices 1 and 2B). By the microscopic analysis we also detected cytoplasmic aggregates of YFP-COP1, discernible, for example, in the line 21 +UV-B sample in Appendix 2.2.A. Such cytoplasmic inclusion bodies have already been demonstrated for overexpressed COP1 fusion proteins in *Arabidopsis* (see, for example, von Arnim and Deng, 1994). However, we note here that there were no sound changes detectable either in the size or in the amount of these inclusion bodies in UV-B treated versus non-treated samples. Taken the microscopic data together, we conclude that

both COP1 and HY5 accumulate to high level in the nuclei of UV-B treated *Arabidopsis* seedlings.

To investigate the kinetics of this nuclear accumulation of YFP-COP1 in response to UV-B, and to test whether removal of the UV-B stimulus reverses this effect, time-course experiments were performed using one of the YFP-COP1 overexpressing lines (line 21). Two sets of samples were grown for four days under the narrowband UV-field in continuous white light either in the presence (WG305) or in the absence (WG327) of supplementary UV-B. On the fourth day half of the white light-grown samples were left in white light, while on the other half the WG327 cut-offs were exchanged by WG305 (UV-B treatment). Similarly, half of the UV-B-grown samples were left in UV-B, while on the other half the WG305 cut-offs were exchanged by WG327 (removal of UV-B). 30 minutes, 1, 3, 6, 12, 18 hours, 1 and 2 days later seedlings from each of the 4 sets of treatments were analysed by epifluorescent microscopy. Additionally, two days after the filter switch, the previously changed cut-off filters were exchanged back to the original ones for one additional day (i.e.; for example 4d WG327 + 2d WG305 + 1d WG327). The continuous UV-B and white light controls were left unchanged for this third day as well. These samples were also analysed with the epifluorescent microscope. The results are shown in Appendix 2.3.

In all analysed time-points the continuous UV-B treatment results in enhanced nuclear accumulation of YFP-COP1 when compared to the controls without supplementary UV-B, as demonstrated by the stronger YFP fluorescent signals (compare 28-36 to 1-9 in Appendix 2.3.) - in good agreement with our previous findings (Appendix 2.2.A). For the first six hours of UV-B treatment of previously white light-grown seedlings no significant difference in YFP fluorescence is detectable compared to the white light-grown controls (compare 10-13 and 1-6 in Appendix 2.3.). However, from 12 hours on, when compared to the white light controls and also to the previous time-points, a strong increase of nuclear fluorescence is present in the UV-B treated samples (12, 18 hours, 1 and 2 days) that is comparable to continuously UV-B treated controls (compare 14-17 to 5-8, 10-13 and 32-35 in Appendix 2.3.). When from these samples the supplementary UV-B was removed for one additional day, the nuclear YFP fluorescence was restored to the level of the white-light-control (compare 18 and 9 in Appendix 2.3.). In parallel to these, in the first six hours after the removal of supplementary UV-B no significant change in the strong YFP signals was apparent when compared to the continuously UV-B treated samples (compare 19-22 and 28-31 in Appendix 2.3.). From 12 hours on the YFP fluorescence is markedly weaker compared to the previous time-points of the same treatment and to the continuously UV-B treated controls, and this lowered fluorescence is comparable to the white light-grown controls (compare 23-26 to 32-35, 19-22

and 5-8). Furthermore, when these UV-B deprived samples were exposed again to supplementary UV-B for one additional day, the YFP fluorescence was strongly re-induced to the level of the continuous UV-B control (compare 27 and 36 in Appendix 2.3.).

These results show that enhanced YFP-COP1 accumulates in the nuclei of UV-B treated seedlings occurs between 6 and 12 hours from the onset of the irradiation. Likewise, upon the removal of the UV-B stimulus the drop of nuclear YFP-COP1 level is observable in a similar timeframe (Appendix 2.3.). This UV-B-dependent nuclear accumulation and depletion of YFP-COP1 occurs on a similar time scale as the nuclear exclusion of COP1 in light signalling (~24 h) (von Arnim and Deng, 1994). Furthermore, the actual nuclear YFP-COP1 level follows the presence or absence of UV-B stimulus: when exposed to UV-B again, re-accumulation of YFP-COP1 can be triggered in UV-B deprived seedlings that have already reduced levels of the fusion protein. And conversely, the highly elevated nuclear YFP-COP1 level can be reverted to the white light stage by the removal of the UV-B stimulus.

4.3.6. COP1 promoted HY5 degradation is hindered by supplementary UV-B

Our microscopic data indicates that both COP1 and HY5 accumulate to high levels in the nuclei of UV-B treated seedlings (Appendix 2.2.). In the dark, HY5 is a major target of COP1-mediated ubiquitylation in the nucleus, which ultimately leads to HY5 degradation by the 26S proteasome (Figure 4.3.4.B, see also Osterlund et al., 2000). The parallel and highly abundant nuclear accumulation of both proteins under UV-B is in stark contrast to this, and thus prompted us to investigate HY5 protein levels under conditions when overexpressed YFP-COP1 is present in the nucleus. To this end the YFP-COP1 overexpressor (line 21) together with the corresponding wild-type and mutant were subjected to 1- and 6 hours and 4 days of supplementary UV-B (WG305 cut-off) and were compared to seedlings grown only in white light for 4 days (WG327 cut-off) as described in Materials and Methods. The protein extracts of these samples were analysed by protein gel blots using polyclonal anti-HY5 and anti-CHS, and monoclonal anti-GFP antibodies.

The similar immunoreactivity of HY5 after six hours and four days of supplementary UV-B in the YFP-COP1 overexpressor and wild-type (Col) is apparent (Figure 4.3.4.E). In the same extracts of the transgenic line high level of YFP-COP1 protein is detectable. This indicates that endogenous HY5 protein can accumulate to wild-type, or higher level even in the abundant presence of YFP-COP1. The same is true for the HY5-dependent CHS. We note the stronger YFP-COP1 signals after six hours and four days of UV-B compared to the 0 and one hour as revealed by the GFP antibody in the transgenic line. This may indicate an additional mechanism stabilizing COP1 under UV-B. On the basis of these results and the

microscopy data, we conclude that COP1-mediated HY5 degradation is hindered under UV-B conditions.

4.3.7. HY5 protein is stabilized in response to UV-B

Transient *HY5* gene induction in response to continuous UV-B is followed by the high level of protein accumulation. Under conditions, however, when the *HY5* transcript level is already declined to background stages, the protein is still retained at high abundance (Figures 4.2.5.A and B, 4.3.2.B and C). This suggests that beside its transcriptional activation, HY5 is a rather stable protein, potentially additionally stabilized under UV-B radiation. In light signalling, regulation of HY5 protein stability through COP1 is known to be a crucial mechanism of controlling HY5 function (see, for example, Yi and Deng, 2005). Therefore we were interested to investigate HY5 protein stability and the potential regulatory role of COP1 under UV-B. To this end, we first analysed wild-type (Col) and *cop1-4* mutant seedlings grown for four days in supplementary UV-B under the narrowband UV-field (WG305 cut-off). On the fourth day, on half of the samples the WG305 cut-offs were exchanged by WG327 (removal of UV-B), while the other half was left unchanged. From both sets, seedlings were harvested 5, 10, 15 and 25 hours after the filter switch. Additionally, one sample before the filter switch from both lines was also harvested. Total proteins extracted from these samples were analysed by protein gel blots with anti-HY5 antibody. The results are shown in Figure 4.3.7.A. In the wild type a decrease in the immunoreactivity of HY5 after the removal of UV-B is evident, while there is no significant change in signals in case of the samples left under UV-B. The reduced HY5 level is already detectable 5 hours after the removal of UV-B, and it becomes more apparent after 10 and 15 hours. On the other hand, in the *cop1-4* mutant the cease of UV-B irradiation does not result in any observable change in HY5 protein levels. These data indicate further that UV-B acts to stabilize HY5 protein, and the removal of supplementary radiation results in HY5 destabilisation. Furthermore, this destabilisation of HY5 under white light conditions deprived of UV-B seems to require COP1.

To test further, and to compare the HY5 degradation in light and darkness after UV-B treatment, we repeated the experiment with Col. In order to exclude variation in *HY5* transcription we also used a *hy5-215/Pro$_{35S}$:HY5* line that constitutively overexpresses *HY5* mRNA (see Figure 4.2.6.C). Furthermore we included a *cop1-4*/*Pro$_{35S}$:YFP-COP1* line to study the effects of COP1 overexpression on HY5 stability in UV-B. These three genotypes were grown for four days under the narrowband UV-field in supplementary UV-B (WG305), after which one part of the samples were left under WG305, for another part the WG305 cut-

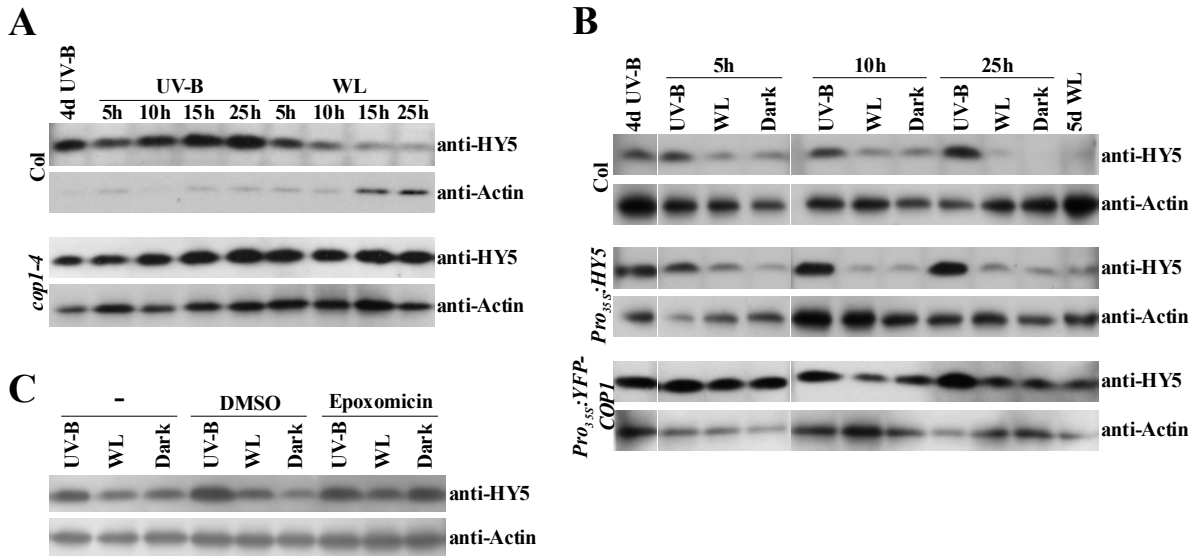

Figure 4.3.7. UV-B regulates HY5 stability through COP1 and the proteasome pathway
(A) and **(B)** Seedlings of the indicated genotypes were grown for 4 d in UV-B supplemented white light under WG305 cut-off in the narrowband-field (4d UV-B) and than were either left under WG305 (UV-B) or replaced under WG327 cut-offs (WL) or, when indicated, transferred to darkness (D) for the times depicted.
(A) (B) and **(C)** Protein gel blots were sequentially probed with anti-HY5 and anti-Actin antibodies.
(A) *Removal of supplementary UV-B leads to COP1 dependent HY5 destabilisation.* Immunoblot analysis of Col (top) and *cop1-4* (bottom) seedlings.
(B) *UV-B deprivation induced HY5 destabilisation occurs in HY5 and YFP-COP1 overexpressors.* Immunoblot analysis of Col (top), *hy5-215/Pro$_{35S}$:HY5* line 7 (middle) and *cop1-4/Pro$_{35S}$:YFP-COP1* line 21 (bottom) seedlings. 5d WL samples were grown for 5 d in white light under WG327 cut-off in the narrowband-field.
(C) *HY5 destabilisation in white light and dark following UV-B is blocked by a proteasome inhibitor.* Immunoblot analysis of seedlings of the *hy5-215/Pro$_{35S}$:HY5* line 7 grown for 4 d in UV-B supplemented white light under WG305 cut-off in the narrowband-field (3 d on solid-, 1d in liquid-media). Seedlings then were either left untreated, or mock (DMSO) or 10 μM epoxomicin treated for 45 min following which from each treatment samples were either left under WG305 cut-off (UV-B), or replaced under WG327 cut-off (WL) or transferred to darkness (D) for 10 h. Blots were sequentially probed with anti-HY5 and anti-Actin antibodies.

off was exchanged by WG327 while a third part was transferred to darkness. 5, 10 and 25 hours later samples were harvested from each treatment. In addition, seedlings of each genotype were grown for five days in continuous white light (WG327), to demonstrate background level of HY5 protein expression; these seedlings were harvested together with the 25 hours time-point samples. Four-day-old seedlings grown in supplementary UV-B (WG305) were also harvested as controls. Total protein extracts from these samples were analysed by protein gel blots using anti-HY5 antibody. The results are shown on Figure 4.3.7.B. In Col, reduced immunoreactivity of HY5 is detectable as early as 5 hours after change to both white light and darkness, and after 25 hours it is decreased to the background level in white light, and to under the detection limit in darkness. The same is true for the HY5 overexpressor line (Figure 4.3.7.B, middle), except that after 25 hours of darkness following the UV-B treatment the HY5 protein level is reduced only to the background stage of five-day-old white light-grown samples. This incomplete HY5 degradation in dark after UV-B treatment in the HY5 overexpressor, which is similar to the case in light-to-dark transfer

(Figure 4.3.7.B), may be due to the high HY5 protein level (see Figure 4.2.6.A). In the YFP-COP1 overexpressing line reduction of HY5 immunoreactivity to the background level in both white light and darkness is also apparent after 10 and 25 hours (Figure 4.3.7.B, bottom). This result further supports the functionality of the YFP-COP1 construct (see also Figure 4.3.4.A to D). However, the incomplete HY5 degradation after 25 hours of darkness following the UV-B treatment may indicate reduced functionality of the fusion construct that may result from the possible disturbance of the relatively big YFP tag, as already has been proposed. Alternatively, heterodimerisation between the truncated COP1-4 and YFP-COP1 may impede the proper COP1 function. The somewhat slower kinetics of HY5 degradation in both white light and darkness (compare 5 h time-points of *Pro$_{35S}$:YFP-COP1*, Col and *Pro$_{35S}$:HY5* on Figure 4.3.7.B) is in line with these assumptions. It is also clear that upon transfer to white light from UV-B the HY5 protein abundance is only reduced to the background level of white light-grown seedlings, and not further (Figure 4.3.7.B bottom), indicating again the lack of YFP-COP1 overexpression effect on molecular level (see also Figure 4.3.4.B and C).

Taken all these together, our data suggests that beside the transcriptional induction, UV-B acts to stabilize HY5 protein, and accordingly, the removal of UV results in HY5 degradation both in white light and darkness. To study whether this degradation upon UV-B removal is mediated by the proteasome pathway, as it is in the dark, we used the potent proteasome inhibitor epoxomicin. Three sets of *hy5-215/Pro$_{35S}$:HY5* seedlings were grown under the narrowband UV-field in supplementary UV-B (WG305) for four days and then were treated with either epoxomicin or mock treated with DMSO or were left untreated as described in Materials and Methods. One part of each of the three different treatments was left under UV-B, on the second part the WG305 cut-off was exchanged by WG327, and the third part was transferred to darkness. All of the samples were harvested 10 hours later, and the total protein extracts were analysed by protein gel blot using anti-HY5 antibody. Figure 4.3.7.C shows that in the non-chemical-treated samples HY5 levels are significantly decreased 10 hours after the removal of UV-B in both white light and darkness, when compared to the sample that was continuously subjected to UV-B. Similarly, in the mock treated (DMSO) samples the reduction of HY5 protein levels under both conditions is apparent when compared to the corresponding UV-B control. These results correlate well with our previous findings (Figure 4.3.7.A and B). However, in the presence of epoxomicin, the immunoreactivity of HY5 in the white light and dark treated samples are not significantly different from the continuously UV-B exposed control. This indicates that the destabilisation of HY5 upon removal of UV-B is

blocked by the proteasome inhibitor, suggesting that HY5 degradation in response to these stimuli is carried out through the proteasome pathway.

On the basis of these results we postulate that supplementary UV-B stabilizes HY5 protein, and in the absence of UV-B radiation HY5 is degraded by the proteasome in a COP1-dependent manner, both in dark and visible light.

4.3.8. Transcriptional UV-B responses function independently of SPA1 to SPA4 proteins, the COP9 signalosome and DET1

HY5 degradation through the ubiquitin/proteasome system in light responses requires other members of the COP/DET/FUS proteins (Osterlund et al., 2000). Our previous results demonstrate that the light and UV-B signalling pathways use shared component, like HY5 and COP1 however there are strong evidences that the two responses may be mechanistically different (see also Discussion).

To investigate whether other light-signalling components are also involved in UV-B responses, UV-B-induced transcriptional changes in *spa1234* quadruple, *csn5a* and *det1-1* single mutants were compared to the corresponding wild type (Col). Additionally, another *cop1* allele, *cop1-1* was also included in the analysis. Seven-day-old white light-grown seedlings were exposed to 15 minutes irradiation in the broadband UV-field using the WG327, WG305 and quartz filters as described before. The results of the corresponding RNA gel blot analysis are shown in Figure 4.3.8. Similar to *cop1-4* (Figure 4.3.1.), the *cop1-1* mutant is also deficient in the UV-B induced activation of UV-responsive genes, including *HY5*, as indicated by the stable transcript levels of all tested genes irrespective of the UV-B treatment. On the contrary, in the *spa1234* quadruple and the *csn5a* single mutants the

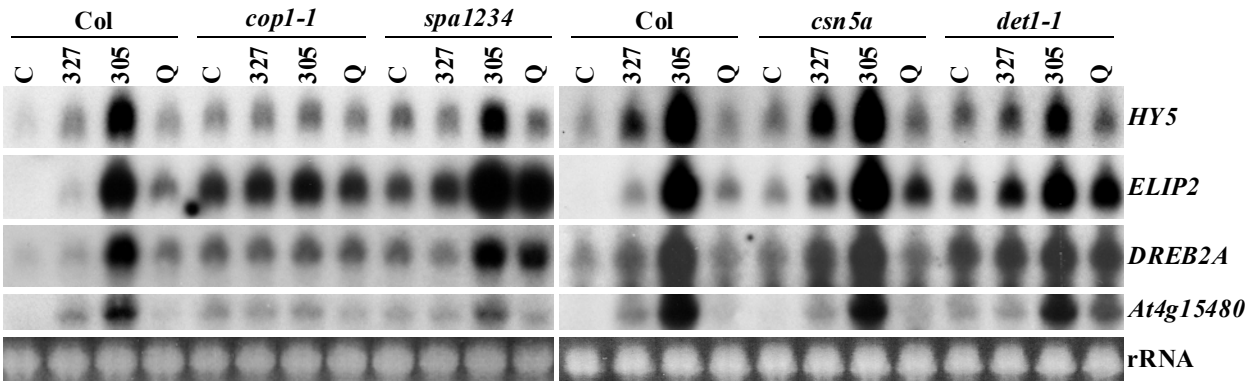

Figure 4.3.8. COP1 dependent transcriptional UV-B responses do not require SPA1 to SPA4 proteins, CSN5 and DET1-1
RNA gel blot analysis of seven-day-old white light-grown Col, *det1-1, csn5a-1, cop1-1* and *spa1-3 spa2-1 spa3-1 spa4-1* seedlings exposed to 15 min irradiation in the broadband UV-field using different UV-B spectra under WG327, WG305 and quartz cut-off filters. Control (C) samples were kept in the standard growth chamber. Blots were sequentially hybridised with specific probes for the indicated genes. Ethidium bromide-stained rRNA is shown as loading control.

transcript accumulation of all tested genes is similar to wild-type in response to UV-B. Similarly, in *det1-1* mutant the mRNA of all tested genes accumulates to as high level as they do in the wild-type upon UV-B irradiation, however in this mutant these genes has an already elevated background transcription under conditions devoid of UV-B. These results indicate, that the SPA proteins are not required for the UV-B induced gene activation, and also that in sharp contrast to the case in light signalling, COP1 can function properly without the four SPA proteins in UV-B responses. Likewise, DET1 and CSN5 (and therefore potentially the COP9 signalosome as well) seem not to be essential for the transcriptional UV-B responses, in contrast to their crucial role in light signalling. Therefore we conclude that the physiological UV-B response is mechanistically separate from the light responses.

4.4. Novel scientific results

Within the frame of the work presented in this study, the following novel results were obtained.

1. A set of RNA gel blot analyses revealed that low level UV-B irradiation triggers early and transient transcriptional changes in *Arabidopsis thaliana*. A substantial portion of the UV-B-induced genes were found to encode for known transcriptional regulators.

2. Further RNA gel blot analyses of *phyAphyB*, *cry1cry2*, *phot1phot2*, *uvr1uvrr2* and *uvr1uvr3* mutants demonstrated the independence of the transcriptional UV-B responses from the known photoreceptors and the general DNA damage pathway. Two subgroups of the low level UV-B induced genes were shown to be differentially regulated by shorter wavelength UV-B ranges.

3. The bZIP transcription factor *HY5* was found to be transcriptionally induced by UV-B irradiation. Western blot analysis of endogenous HY5 protein in wild type plants and epifluorescent microscopic examination of functional HY5-YFP fusion protein in stable transgenic lines proved the elevated HY5 protein production and nuclear accumulation in response to supplementary UV-B treatments.

4. RNA gel blot analyses and flavonoid measurements of *hy5* null mutants substantiated that HY5 is required for the transcriptional activation of a subset of UV-B-induced genes and for the elevated production of “sunscreen” flavonoid pigments in response to UV-B. Consistently, regulators and components (such as CHS) of the flavonoid biosynthetic pathway were found to be among the HY5-dependent UV-B induced genes, and *hy5* mutants exhibited reduced UV-tolerance.

5. RNA gel blot and Western blot analyses indicated that the UV-B induction of HY5-dependent genes (including *CHS* gene induction and protein accumulation) is in temporal

correlation with *HY5* gene activation and protein accumulation. The same experiments showed that HY5 protein accumulation remains on high levels in supplementary UV-B under conditions when *HY5* transcription is already decreased to background levels.

6. Hypocotyl growth inhibition assays and RNA gel blot analyses of stable transgenic lines that ectopically overexpress HY5 under the regulation of the CaMV 35S promoter demonstrated that HY5 overexpression complements the *hy5* mutant phenotypes, but is insufficient to provoke UV-B hyper-responsiveness.

7. HYH, the closest homolog of HY5 was also shown to be transcriptionally induced by low level UV-B irradiation as. Transcriptional assays and flavonoid measurements of *hyh* and *hyhhy5* mutants revealed a minor role of HYH in the transcriptional regulation of UV-B induced genes.

8. RNA gel blot analyses of *cop1* mutants proved that the COP1 E3 ubiquitin ligase is required for the UV-B induced transcriptional up-regulation of a broad range of UV-B responsive genes, including *HY5*. Consistently, COP1 is essential for the activation of *CHS* gene transcription and protein production, flavonoid accumulation and for UV-B tolerance, as demonstrated be RNA gel and Western blots and flavonoid measurements.

9. The same set of experiments on the non-constitutive photomorphogenic $cop1^{eid6}$ allele demonstrated that this *cop1* mutant is proficient in its UV-B responses.

10. Epifluorescent microscopic analyses demonstrate the nuclear enrichment of a functional, overexpressed YFP-COP1 fusion protein in stable transgenic seedlings in response to supplementary UV-B in a reversible manner. Such examinations together with Western blot analysis demonstrate that both HY5-YFP and YFP-COP1 is enriched in the nuclei of UV-B treated seedlings, and that endogenous HY5 protein accumulates to high levels in the abundant presence of functional YFP-COP1.

11. RNA gel blot analyses of *spa1234*, *csn5a* and *det1-1* mutants demonstrated that these mutants exhibit transcriptional UV-B responses similar to the wild type.

5. DISCUSSION

5.1. Low-level UV-B induced transcriptional changes define interacting regulatory responses

An initial step to analyse the impact of low-level UV-B irradiation was the assessment of the transcriptional changes it triggers. *Arabidopsis* seedlings exposed to weak UV-B radiation using two different UV-fields induced gene activation in all accessions analysed by RNA gel blots: both the 15 minutes irradiation using the broadband UV-field and the continuous treatment using the narrowband UV-field resulted in the rapid and transient induction of a set of UVB-responsive genes in Columbia, Wassilewskija and Landsberg *erecta* ecotypes (Figures 4.1.1.A, B and C, 4.2.4.A and B, 4.2.5.A). This result is in good agreement with microarray data from 15 minutes broadband UV-B treated Ws seedlings that shows the induction of 100 genes in response to low-level UV-B after 1 hour (Figure 5.1.A and Ulm et

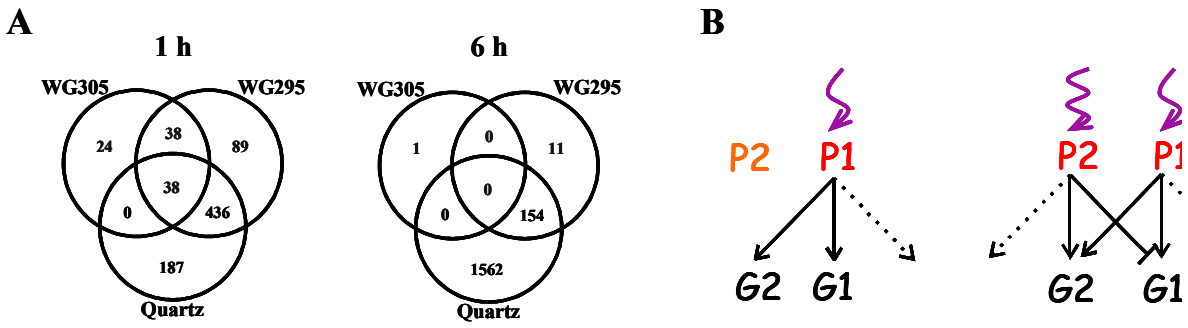

Figure 5.1. UV-B activated early transcriptional responses
(A) Venn diagrams based on microarray results showing the distribution of genes that are at least 2-fold induced under different UV-B spectra in the broadband UV-field. (after Ulm et al., 2004)
(B) Simplified model of antagonistic regulation by shorter wavelength UV-B. Left: events triggered by longer wavelength UV-B (i.e. under 305 nm cut-off). Right: events triggered by shorter wavelength UV-B (i.e. unfiltered UV-B under quartz glass). (after Ulm et al., 2004)

al., 2004). These up-regulated genes include transcriptional regulators involved in light signalling (*HY5, HYH*), abiotic stress responses (*DREB2A, ZAT12*) or of unknown functions (*NAM-like*), and also genes functioning in metabolism (*At4g15480*) and stress responses (*ELIP2*) as well as an unclassified gene (*At5g52250*) (Figure 4.1.1.). These genes were used as pathway marker genes in this work. It is noteworthy that a number of UV-B-responsive promoter and luciferase-reporter-gene fusion constructs revealed that the UV-B-dependent regulation of these transcripts in fact occurs at the level of transcription rather than at mRNA stability (Ulm et al., 2004). It is apparent that a considerable portion of the UV-B induced genes are known transcription factors. On the global transcriptome level, more than 30% of the early UV-B induced genes were shown to be annotated as transcription regulators (Ulm et al., 2004). This overrepresentation of transcription factors among the transiently activated

early UV-B induced genes may be indicative of the early activation of a transcriptional network to regulate UV-B responses, similarly to the case of light induced transcriptional regulatory pathways (Tepperman et al., 2001; Jiao et al., 2007).

The broadband UV-B field provides the opportunity to analyse the transcriptional effects of diverse UV-B ranges. We used a series of decreasing short-wave transmission cut-off filters to exclude shorter UV-B ranges thus generating four different UV-B spectra (Figure 3.1.). Light transmitted by the WG327 cut-off filter served as minus UV-B control; the low-level UV-B treatment widely used in this study was generated with WG305 cut-offs; WG295 was used for the shorter wavelength (stronger) UV-B treatments and finally, a quartz glass to apply unfiltered UV-B irradiation provided by the broadband lamps (strongest UV-B). In this way all regions of the spectra other than the UV-B part were held constant, thus the application of shorter wavelength cut-off filters eventuate the extension of the irradiation to the shorter wavelength ranges. It should be noted that under the WG327 cut-offs UV-B treatment still results in a slight induction of most of the UV-B induced genes compared to non-irradiated controls. WG327 cut-offs still traject a small amount of UV-B light between 310-320 nm which could be responsible for this small activation (Figure 3.1. left). In addition, the absorption spectra of a putative UV-B photoreceptor is unknown, therefore it can not be excluded that it may perceive photons of longer wavelength ranges outside of the conventionally defined UV-B range (i.e. 280-320nm).

The UV-B-activated genes fall into two groups according to their transcriptional activation profile under longer UV-B wavelength ranges (WG305) versus shorter wavelength ranges (WG295 and quartz). As expected, transcription of a subset of genes is further induced and kept on high level under shorter wavelength UV-B (e.g. *At1g19020* and *At5g59820* on Figure 4.1.1.A and C). However surprisingly, the other subset of genes display reduced transcript accumulation in shorter wavelength UV-B (quartz) treated samples compared to the low-level UV-B-treated ones (WG305) (e.g. *HY5* and *ELIP2*; Figure 4.1.1.A and C). Since the longer wavelength parts of the spectrum are identical in the WG305, WG295 and quartz treatments (see Figure 3.1.) it seems that the low-level UV-B triggered gene induction is further induced in one subset, whereas it is antagonised in another subset of genes by to shorter wavelength UV-B. These findings are also reflected by microarray analysis where 38% of the UV-B induced genes belong to the former “quartz induced”, and 62% to the latter, “quartz antagonised” class of genes (Figure 5.1.A and Ulm et al., 2004). Based on these findings, we postulate the existence of at least two, interacting UV-B perception and signalling pathways (Figure 5.1.B). One is activated by longer wavelength (lower level) UV-B (P1) and a second pathway is triggered by the shorter wavelength (stronger) part of the UV-

B spectrum (P2). When both pathways are activated, the latter negatively interferes with the former, restraining the transcriptional induction of a subset of genes up-regulated by the former pathway (G1). The P1 perception system may illustrate a specific UV-B photoreceptor, whereas P2 system may indirectly respond to UV-B stimulus through more general cellular stress pathways, or alternatively P2 may also represent a distinct UV-B photoreceptor.

5.1.1. Early UV-B responses are independent of known photoreceptors

RNA gel blot analysis of photoreceptor mutants demonstrated that the UV-B triggered gene activation is independent of phytochromes, cryptochromes and phototropins, therefore of the known red/far-red, blue and UV-A light sensing receptors (Figure 4.1.1.C, and D). The bZIP transcription factor *HY5* is known to be transcriptionally induced by light in a phytochrome-dependent manner (Tepperman et al., 2001; Quail, 2002a). Interestingly, however, the UV-B induction of *HY5* is clearly independent of the phytochromes. Additionally, the closest HY5-homologue, *HYH* is also UV-B inducible independently of the known photoreceptors. These finding strongly indicates that UV-B induced transcription is activated through a specific UV-B photoreceptor or photoreceptors. Interestingly, our results also anticipate a possible crosstalk between the UV-B and the cryptochrome pathway. The hyper-responsiveness of UV-B induced genes in longer wavelength ranges (i.e.WG327 and WG305) in cryptochrome mutants indicates a negative regulatory role for this photoreceptor pathway on UV-B responses (Figure 4.1.1.C). Furthermore, loss of cryptochromes results in enhancement of the antagonistic effect of shorter wavelength ranges (i.e. quartz treatment, Figure 4.1.1.C), suggesting that the cryptochrome pathway negatively regulates the antagonistic part of the P2 UV-B pathway as well, possibly by affecting the component that integrates this antagonistic P2 into the P1-G1 pathway. As the “quartz induced” genes do not show hyper-responsiveness in shorter wavelength ranges (i.e. WG295 and quartz), the cryptochrome pathway does not seem to effect the G2 regulating part of the P2 pathway.

5.1.2. Low-level UV-B responses are not related to general DNA damage responses

The two major UV-B induced DNA damage products are the cyclobutane pyrimidine dimers (CPDs) and the pyrimidine (6-4) pyrimidinon dimers (6-4 products), with the former being the most prominent DNA lesion (Landry et al., 1997). The *Arabidopsis* type II photolyase mutant *uvr2* that is deficient in the photoreactivation of CPDs has been reported to be hypersensitive to UV-B irradiation (Landry et al., 1997). Also double mutants of *uvr1uvr2* and *uvr1uvr3* were shown to exhibit increased UV-B sensitivity (Jiang et al., 1997). These latter mutants are deficient in the dark repair of 6-4 products due to the *uvr1* mutation, and

additionally the former is unable for the photorepair CPDs and the latter for the 6-4 products. Therefore if the observed UV-B responses described herein are due to general DNA damage, then these mutants are expected to show hypersensitivity under our conditions as well.

On the contrary, both *uvr1uvr2* and *uvr1uvr3* double mutants exhibit wild type-level gene-induction in response to UV-B, which argues against that particular DNA damage would be the cause for the activation of these genes in wild types (Figure 4.1.1.E). Furthermore the antagonistic effect of the shorter wavelength ranges as well as the shorter wavelength UV-B triggered gene induction were also shown to be unaltered in *uvr2* single mutants (Ulm et al., 2004). This further suggests that also for the P2 pathway DNA damage is unconnected to gene induction and "quartz antagonism".

Gene expression profiling demonstrated that 6 h after the 15 min irradiation under weak UV-B (i.e. WG305 cut-off) only 1 gene is induced in contrast to the 165 up-regulated transcripts after WG295 and particularly the 1716 activated genes after quartz treatment (Figure 5.1.). This clearly indicates the lack of sustained cellular effects after the low-level irradiation (WG305), therefore this treatment is considered to be marginal that triggers transient transcriptional changes with negligible damage. In support of this it is interesting to note that such short and low-level UV-B irradiation does not result in any visible phenotype under our conditions. Furthermore, no significant enhancement of the hypocotyl growth inhibition phenotype was observed in *uvr1uvr2* and *uvr1uvr3* double mutants compared to wild type under the narrowband UV-field (data not shown).

It is also of note that the UV-B irradiation used for these treatment are regarded as very low (WG305) to low (quartz glass) UV-B levels according to recently proposed categories (Brosche and Strid, 2003). In line with this, even in the case of the quartz treatment the "intermediate and high-level UV-B marker genes" according to the model of Broche and Strid (2003) remains un-induced (Ulm et al., 2004). Taken these findings together we conclude that under our conditions the UV-B responses are not connected to general DNA damage responses.

5.2. HY5 and COP1 are major positive regulators of UV-B signalling

5.2.1. HY5 is an essential activator of UV-B responses

A key regulator of photomorphogenic development is the bZIP transcription factor HY5 that is required for the proper development of plants during dark-to-light transition. HY5 is only required for the first few days of seedling development in light, which is indicated by the sharp reduction of HY5 protein levels short after germination (Hardtke et al., 2000). Furthermore, except for the phenotypes of young seedlings, *hy5* mutants develop normally

without showing any significant developmental defect compared to wild-types in adult stage, further suggesting the lack of a predominant HY5-role in later stages of plant development.

Our results on four- and seven-day-old *Arabidopsis* seedlings of Col, Ler and Ws accessions demonstrate that different UV-B treatments result in the fast and transient induction of *HY5* gene expression (Figures 4.1.1.A and C, 4.2.4.B, 4.2.5.A, 4.2.6.C and D, 4.3.2.B and C). Furthermore, protein gel blot analysis of endogenous HY5 protein and cell biological assay of a HY5-YFP fusion demonstrate the UV-B triggered nuclear enrichment of HY5 protein (Figures 4.2.1., 4.2.5.B and 4.3.2.C and Appendices 1 and 2B). These results suggest a specific role for HY5 in UV-B. In fact, investigation of different *hy5* mutant alleles confirmed the substantial role of HY5 in UV-B responses. We show that HY5 is essential for the activation of a set of UV-B-responsive genes which on the whole-transcriptome-level accounts for nearly 33% of the UV-B-induced transcripts (Figures 4.1.1.C, 4.2.4.A and B, 4.2.6.C and D and Oravecz et al., 2006). These include other transcriptional regulators, the induction of which is in temporal correlation with HY5 protein accumulation (Figures 4.1.1.C and 4.2.5.A). These results may be indicative of a UV-B induced transcriptional regulatory cascade.

The HY5-dependent transcriptional activation of UV-B-induced genes involves *CHS* gene induction and protein accumulation. Timewise, HY5 protein accumulation precedes *CHS* mRNA and subsequent protein accumulation. Additionally, other biosynthetic enzymes of the flavonoid pathways are also induced by UV-B in a HY5-dependent manner (Table 4.2.2.). In agreement with this we found HY5 to be required for the UV-B induced flavonoid production and for the establishment of UV-B tolerance (Figure 5.4.1.). Moreover, another physiological response to UV-B, the hypocotyl growth inhibition was also shown to require functional HY5.

Taken these together our data position HY5 into the UV-B pathway as a crucial positive regulator. Therefore we propose that the transcriptional induction and reaccumulation of HY5 protein upon UV-B stimulus is required to fulfil its regulatory function in UV-B. This includes the up-regulation of downstream genes in the UV-B pathway ultimately leading to the activation of UV-protective mechanisms, such as the induction of CSH that leads to elevated flavonoid production and UV-B tolerance.

5.2.2. Additional regulators of UV-B induced HY5 dependent transcription.

The predominant role of HY5 in regulating UV-B induced transcription is apparent. However, a subset of the HY5 dependent genes do not seem to be regulated exclusively by HY5 with regard to their UV-B induced transcription, as demonstrated by the residual

transcript activation of these genes in different *hy5* mutants (Figures 4.1.1.C, 4.2.4.A and B). This indicates that other components of the UV-B pathway may also participate in the regulation of this set of genes. One candidate as such a component is the closest HY5 homologue, HYH that has been shown to form heterodimers with HY5 (Holm et al., 2002). HYH has also been demonstrated to predominantly regulate blue-light responses by controlling partially overlapping target genes with HY5 (Holm et al., 2002). Therefore we investigated the role of HYH in UV-B signalling as well. We show that *HYH* is UV-B inducible independently of HY5, and that the remaining responsiveness of some HY5-dependent genes in *hy5* mutants appears to be mediated in part by HYH (Figures 4.1.1.C, 4.2.4.A and B). On the other hand, although the additional loss of HYH in a *hy5* mutant results in the reduction of the residual responsiveness of for example *At4g14690*, the *hyh hy5* double mutant still retains a marginal UV-B inducibility of this gene under certain conditions (Figure 4.2.4.B). In addition, no considerable alteration in the flavonoid induction phenotype of the *hyh-1* mutant is detectable and also *hyh hy5* double mutant fails to exhibit an enhancement of the *hy5* mutant phenotype (Figure 4.2.4.C).

Our results therefore suggest that albeit the regulatory role of HYH is apparent, it seem to contribute to the UV-B responses to a lesser degree. It is also reasonable to speculate therefore that beside HYH other factors may also contribute to the regulatory function of HY5. These factors could then be largely included among the HY5 independent components of the UV-B pathway which then envisions a possible crosstalk between the HY5-dependent and -independent branches of the UV-B pathway.

5.2.3. The E3 Ubiquitin Ligase COP1 is a Positive Regulator of UV-B signalling partly acting through the activation of *HY5*

Regulation of HY5 protein stability is a key mechanism for the suppression of photomorphogenic development under dark conditions. The crucial regulator responsible for the ubiquitylation and subsequent 26S proteasomal degradation of HY5 protein in darkness is the E3 ubiquitin ligase COP1 (Osterlund et al., 2000). Beside HY5, COP1 has been shown to target other positive regulators of light signalling for proteasomal degradation including HYH, and also other transcription factors (Holm et al., 2002; Seo et al., 2003; Duek et al., 2004; Jang et al., 2005; Yang et al., 2005). COP1 acts as a master switch that negatively regulates photomorphogenesis by eliminating positive regulators of light responses and that is inactivated upon light perception (Yi and Deng, 2005).

This tight connection between HY5 and COP1 in light signalling and the established function of HY5 in UV-B queries a potential role of COP1 in UV-B responses as well. In fact, the epifluorescence microscopic analysis of YFP-COP1 fusion protein revealed the nuclear

enrichment of COP1 in response to UV-B irradiation in a similar timeframe that has been reported for the light triggered nuclear exclusion of COP1 (Appendices 2A and 3) (von Arnim and Deng, 1994). Furthermore, the removal of the UV-B stimulus leads to the depletion of the nuclear YFP-COP1 that can be reverted with a repeated UV-B treatment resulting in the reaccumulation of the fusion protein in the nucleus. These dynamic changes of COP1 levels following the presence or absence of UV-B anticipates a specific role of COP1 in UV-B responses.

Interestingly, the nucleocytoplasmic repartitioning of COP1 in response to the presence or absence of supplementary UV-B is a relatively slow process, similarly to the situation in light-signalling (von Arnim and Deng, 1994; Yi and Deng, 2005). In both cases however, it contrasts the rapid gene expression changes and fast kinetics of target protein stabilisation (Osterlund et al., 2000; Duek et al., 2004; Yang et al., 2005). Therefore, light seem to inactivate the photomorphogenesis-suppressing function of COP1 by a yet unknown, but presumably fast acting mechanism prior to subcellular partitioning. A potential mechanism of such sort could involve the well established direct interaction of COP1 and the cryptochromes (Yi and Deng, 2005). To clarify whether similar photoreceptor-COP1 interaction has a role in UV-B signalling has to wait the identification of the UV-B photoreceptor. Notwithstanding, our data indicates that similar to white light, the subcellular localization of COP1 may play critical role in the regulation of UV-B mediated responses, potentially representing a long-term adaptation mechanism.

Surprisingly we found that both in the weak *cop1-4* and the strong *cop1-1* allele the UV-B triggered gene activation is broadly impaired (Figures 4.3.1. and 4.3.8.), suggesting that COP1 is required for UV-B induced transcription to occur. This inference is strongly supported by microarray data demonstrating that the presence of functional COP1 is in fact obligatory for the 75% of the UV-B induced genes to be able to respond to UV-B (Oravecz et al., 2006). Albeit this finding is in good agreement with enhanced nuclear abundance of COP1 in UV-B, it contrasts its established repressor function in light. In addition, *HY5* was found to be one of the COP1 dependent genes (Figure 4.3.1.), and consistent with this, COP1 was also shown to be required for the UV-B induced HY5 protein accumulation (Figure 4.3.2.C). These findings are astonishing, considering that HY5 is one of the targets destined for degradation by COP1 function in light signalling. Further support of these results come from an independent genetic screening approach, performed by Agnieszka Brezinska in our group, that aimed at the discovery of mutants deficient in the UV-B induced activation of *HY5* promoter. Through this screen, a mutant *cop* allele was found in Ws that is identical to the *cop1-4* in Col (Oravecz et al., 2006).

In agreement with the positive regulatory role of COP1 on *HY5* transcription, the comparative analysis of COP1 and HY5 dependent UV-B induced genes revealed that almost all of the HY5 dependent genes are COP1 dependent as well (Figure 4.1.1.C and 4.3.1.) (Oravecz et al., 2006), including those that can be linked to the establishment of UV-B tolerance (Table 4.2.2.). This suggests a sequential regulatory role of COP1 and HY5 in UV-B, and the commonly regulated set of COP1- and HY5-dependent genes may represent the readout of the UV-B activated COP1-HY5 pathway culminating in for example flavonoid biosynthesis.

The apparent lack of residual activation of COP1 dependent genes in *cop1* mutants contrasts the remaining inducibility of HY5 dependent genes in *hy5* mutants (compare Figures 4.3.1. and 4.3.8. to 4.1.1.C and 4.2.4.A and B). This difference further supports the broader impact of COP1 on UV-B induced genes that is clearly demonstrated by microarray data (i.e. 75% of the genes are COP1, whereas 33% are HY5 dependent) (Oravecz et al., 2006). Moreover this may also argue that the postulated components modulating HY5 dependent gene activation, such as HYH (see section 5.2.2.), are positioned in the COP1 dependent HY5 independent rather than in the COP1 independent branch of the pathway, at least for the genes analysed.

Our RNA gel blot analysis of genes that are not antagonized by the shorter wavelength quartz treatment shows that these genes are UV-B induced independently of both COP1 and HY5 (Figures 4.1.1.C and 4.3.1.). This suggests that these genes may act in the more general UV-B stress pathway (P2-G2 on Figure 5.1.). Taken these together, we propose that both COP1 and HY5 are positive regulators of UV-B signalling with a distinct mechanistic relationship between them compared to the light responses.

5.3. Mechanistic comparison of photomorphogenic UV-B and white light responses

The essential role of COP1 and HY5 in both UV-B and light signalling exemplifies the involvement of shared components in the two biological processes. However, the independence of UV-B induced gene activation on the visible light photoreceptors, as well as the positive regulatory role of COP1 in UV-B indicates that the two pathways are operating in mechanistically different ways.

In light signalling, the loss of the repressor function of COP1 results in the overexpression of a large number of genes in *cop1* mutants compared to wild types grown in white light (Ma et al., 2003; Yi and Deng, 2005). This raises the possibility that COP1 inactivation by UV-B stimulus could be the reason for the transcriptional up-regulation of the COP1 dependent UV-

B-induced genes. Thus UV-B would act to inactivate COP1 and therefore would generally induce COP1 repressed genes. However, the UV-B induced nuclear enrichment of COP1 renders its inactivation unlikely. More importantly, in both *cop1-4* and *cop1-1* mutants the analysed UV-B induced genes are not overexpressed (except *ELIP2* in *cop1-1*) (Figure 4.3.8.), suggesting that their transcription is not repressed through the functional COP1 protein. Moreover, according to a comparative microarray analysis out of the 575 overexpressed genes in white light-grown *cop1-4* mutant only 11 belongs to the UV-B induced genes in wild type (Oravecz et al., 2006). Therefore simple release of COP1 repression does not seem to be the regulatory mechanism for UV-B controlled gene activation.

It is interesting to note that under our conditions UV-B treatments do not seem to result in significant changes in *COP1* mRNA levels according to both microarray and RNA gel blot analysis (Ulm et al., 2004; Oravecz et al., 2006 and data not shown). The YFP-COP1 protein level on the contrary seems to be slightly increased in response to UV-B stimulus, raising the possibility of COP1 protein-stabilization in UV-B. The significance of this finding, however is not yet clear.

5.3.1. Different structural requirements of COP1 in UV-B and light signalling: two *cop1* alleles with opposing functionality in UV-B

Contrary to other *cop1* alleles, $cop1^{eid6}$ mutants exhibit wild-type growth phenotypes in darkness, which led to the conclusion that the residual activity of the mutant protein is sufficient in the suppression of photomorphogenesis in darkness (Dieterle et al., 2003). In contrast to the case in darkness, $cop1^{eid6}$ mutants are strongly hypersensitive to light displaying exaggerated photomorphogenic growth similar to the other *cop1* mutants, like *cop1-4* (Dieterle et al., 2003). In agreement with this, we found $cop1^{eid6}$ proficiency in HY5 degradation upon light-to-dark transfer (data not shown), whereas light grown $cop1^{eid6}$ mutants accumulates elevated HY5 protein levels compared to the wild type similarly to *cop1-4* (Figure 4.3.2.C). In sharp contrast to the similar deficiency of *cop1-4* and $cop1^{eid6}$ in light responses, the UV-B responses in $cop1^{eid6}$ seem to operate normally (contrary to *cop1-4*) as demonstrated by its proficiency in *HY5* and *CHS* gene activation and protein accumulation ultimately leading to elevated flavonoid production (Figure 4.3.2.A to C). This striking difference between the two *cop1* alleles may indicate different structural requirements of COP1 for UV-B and white light signalling. The highly modular functionality of COP1 in light signalling has been reported (Stacey et al., 2000). According to our data on $cop1^{eid6}$, the WD40 repeat domain seems to be essential for proper function in UV-B, whereas in the same responses, mutations in the RING domain may be tolerated. On the contrary, intact RING

domain may be required for COP1 function in white light. It is interesting to note that all of the lethal *cop1* alleles that still producing detectable amount of mutant protein contain mutation in the WD40 domain. Loss of the entire WD40 domain in *cop1-4* however results in a weak, viable allele (McNellis et al., 1994b). Thus it seems that it is better for COP1 not to have the entire WD40 domain at all than to have a defective one. A possible explanation for this is that the loss of the WD40 domain may be compensated in the cell by a COP1 interacting protein that provides functional WD40 repeats through coiled-coil domain mediated heterodimerization (McNellis et al., 1994b). It follows, that the severe UV-B phenotypes of *cop1-4* would be indicative of the lack of such compensatory protein for COP1 in UV-B. It should be noted that *cop1-4* may not be considered as a weak allele in UV-B signalling as the UV responses in this mutant are completely abrogated.

5.3.2. Known regulators of COP1 function do not modulate UV-B response.

The SPA proteins form a four member family of COP1-like WD40 repeat containing proteins. They have been shown to interact with COP1 through their coiled-coil domain, and regulate its activity. SPA1 through SPA4 proteins have partially redundant role in light signalling and the *spa1234* quadruple mutant has constitutive photomorphogenic phenotype as severe as has the strong *cop1-1* allele, indicating that they are required for COP1 to function properly in light (Saijo et al., 2003; Seo et al., 2003; Laubinger et al., 2004; Hoecker, 2005). Our RNA gel blot analysis of UV-B induced genes in *spa1234* demonstrates however the apparently functional UV-B response in the quadruple mutant (Figure 4.3.8.). This is particularly striking as the *spa* quadruple mutant has a much stronger light phenotype than the weak *cop1-4* allele (Laubinger et al., 2004). Thus these results indicate that COP1 functions independently of the SPA proteins in UV-B, and also that the SPA proteins do not have a role in UV-B signalling despite of the specific involvement of COP1 in it. It can therefore also be envisioned that the SPA proteins are not sufficient to provide alternative WD40 domain for the truncated *cop1-4* under UV-B conditions.

COP1 belongs to the COP/DET/FUS loci, other members of which also share the constitutive photomorphogenic phenotype. These are genes encoding components of two large protein complexes: the evolutionary-conserved eight-subunit COP9 signalosome (CSN), and the CUL4-RBX1-CDD complex, both of which have predominant role in light signalling (Schwechheimer, 2004; Yi and Deng, 2005; Chen et al., 2006).

The CSN has been implicated in the regulation of ubiquitin-proteasome-mediated protein degradation (e.g., Wei and Deng, 2003). Its deneddylating activity conferred by the fifth subunit (CSN5) towards Cullin-containing E3 ubiquitin ligases is required for the suppression

of photomorphogenesis in dark-grown plants (Dohmann et al., 2005). However, the activation of a set of UV-B-induced genes in a *csn5a* mutant indicates that UV-B mediated signalling does not involve the COP9 signalosome (Figure 4.3.8.). This finding further demonstrates the mechanistic discreteness of UV-B and light signalling. It should be noted, that the CSN5 protein is encoded by two genes (*CSN5A* and *CSN5B*) that act redundantly (Dohmann et al., 2005). Notwithstanding, it is the loss of *CSN5A* that leads to severe cop phenotype, whereas *csn5b* mutants has only comparably subtle defects (Dohmann et al., 2005). This suggests that mainly CSN5A contributes to the CSN5 function in light. Still, it formally can not be excluded, that *CSN5B* is responsible for the UV-B responses in the *csn5a* mutant. This would then indicate the selective utilisation of the two homologues in light versus UV-B responses. Furthermore, it has been shown that in contrast to the loss of other subunits of the CSN, the elimination of CSN5 does not lead to the disruption of the remaining CSN complex, and the *csn5* knockouts still retain a CSN5-free CSN complex (Kwok et al., 1998; Dohmann et al., 2005). Therefore it remains possible, that this CSN5-free complex is able to mediate UV-B responses, which would indicate that the deneddylating activity of CSN would not be required for such activity. That in turn would be again indicative of a different mode of regulation in light and in UV-B, this time with regard to the structure and function of the CSN. Further analysis of *csn5a csn5b* double mutants and other CSN subunit mutants should help to clarify these questions.

The CUL4-RBX1-CDD complex has been proposed to act as an E3 ubiquitin ligase to support COP1 to ubiquitylate photomorphogenesis promoting factors (Yanagawa et al., 2004; Chen et al., 2006). Loss of the DET1 component of this complex has been reported to destabilize it, as in *det1-1* mutant the CUL4-RBX1-CDD complex is no longer detectable (Yanagawa et al., 2004; Chen et al., 2006). In UV-B however, the transcripts of the UV-B induced genes accumulate to similar levels in the *det1-1* mutant as in wild-type (Figure 4.3.8.). Furthermore *det1-1* is proficient in activating flavonoid production, and consistently exhibits UV-B tolerance in the presence of UV-B irradiation (Figures 4.3.2.A and 5.4.1.A). These findings indicate that the tested UV-B responses are operable in the absence of functional DET1, and thus might not require the complex. Some of the genes albeit exhibit reduced inducibility in the *det1-1* mutant compared to the wild-type, indicating that DET1 may have minor role in regulating UV-B responses independent of CUL4-RBX1-CDD.

Taken together our results on *spa1234, csn5a, det1-1* and $cop1^{eid6}$ let us to conclude that besides the employment of shared components like COP1 and HY5, UV-B and visible light signalling operates in mechanistically distinct manner that is consistent with a specialised and novel role of COP1 in UV-B responses.

5.4. COP1-HY5: regulators of UV-B induced transcription and UV-B tolerance

5.4.1. COP1 and HY5 mediated regulation of UV-tolerance components

The positive regulatory role of COP1 and HY5 on UV-B induced gene expression includes the transcriptional activation of genes that may be linked to defence mechanisms against the destructive effects of UV irradiation. These genes include DNA-damage repair enzymes, components of the phenylpropanoid-biosynthesis-pathway and their regulators (Table 4.2.2.). In support of this we demonstrate that both COP1 and HY5 are required for the UV-B induced transcriptional activation and protein accumulation of the key enzyme of flavonoid biosynthesis CHS that in turn results in the elevated production of flavonoid sunscreen metabolites (Figure 4.3.2.A to C). Therefore we conclude that the sequential regulatory action of COP1 and HY5 controls the activation of UV-protective components in response to UV-B stimulus.

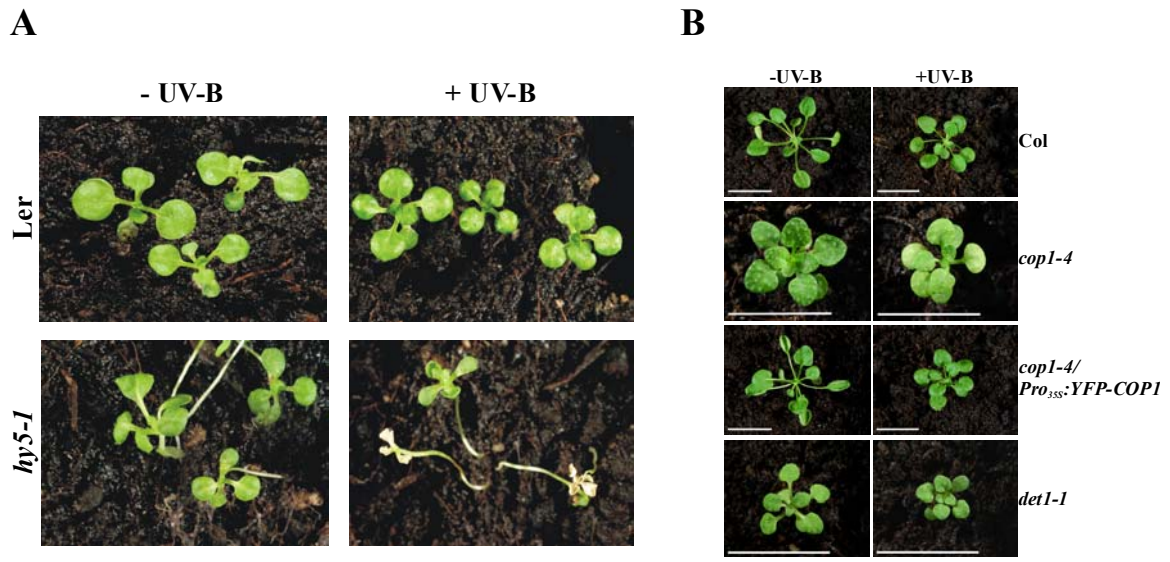

Figure 5.4.1. Reduced UV-B tolerance of *hy5-1* and *cop1-4* mutants
(A) Ler and *hy5-1* plants were grown for 28 days in 9h light/15h dark cycles under the narrowband UV field in UV-B supplemented white light (WG305 cutoff) or in white light only (WG327 cutoff). 26% of the mutants died and 70% showed necrotic lesions whereas none of the wild-types died or was necrotic under supplementary UV-B. In the absence of supplementary UV-B all plants of both genotypes survived without apparent necrosis. (after Oravecz et al., 2006, data kindly contributed by Zoltán Máté [ABC, Gödöllõ])
(B) Col, *cop1-4*, *cop1-4/Pro$_{35S}$:YFP-COP1* and *det1-1* plants were grown for 35 days in 9h light/15h dark cycles under the narrowband UV field in UV-B supplemented white light (WG305 cutoff) or in white light only (WG327 cutoff). 74% of the *cop1-4* mutants developed chlorosis whereas none of the plants from the other 3 genotypes were chlorotic under supplementary UV-B. In the absence of supplementary UV-B all plants of all 4 genotypes survived without apparent necrosis. Bars = 1 cm. . (after Oravecz et al., 2006, data kindly contributed by Zoltán Máté [ABC, Gödöllõ])

Further evidences in this regard were provided by UV-B tolerance assays carried out in collaboration with Dr. Zoltán Máté at ABC Gödöllő. These experiments revealed that *hy5-1* mutants develop necrotic lesions and show increased mortality in supplementary UV-B. An

independent study published by Brown et al (2005) also presented similarly severe defects for *hy5-1* in UV-B. Furthermore, in field-grown tomato (*Solanum lycopersicum*), RNA interference-mediated *HY5* knockdown leads to cell-death phenotypes at different developmental stages (Liu et al., 2004). Similar to *hy5-1*, *cop1-4* mutants also showed decreased UV-B tolerance, though to a lesser extent, which is illustrated by the increased development of chlorosis under supplementary UV-B conditions (Oravecz et al., 2006 and Figure 5.4.1.). It should be noted that the reduced tolerance of *cop1-4* does not seem to be the consequence of general weakness due to its dwarf growth phenotype, as the phenotypically similar *det1-1* does not exhibit reduced UV-B tolerance, as discussed before (section 5.3.2.). Taken together, we conclude that the UV-B pathway regulating COP1 and culminating in HY5 activation is required for the survival of plants under UV-B irradiation.

Our data demonstrate that the enhanced CHS accumulation as well as the flavonoid induction in response to UV-B is abolished or strongly reduced in both *cop1* and *hy5* mutants (Figure 4.3.2.A to C). It is interesting to note however, that a weak but still significant activation of *CHS* gene transcription and flavonoid production is detectable in *hy5-1*, whereas no such residual induction is apparent in *cop1-4* (Figure 4.3.2.A and B). This may be indicative of a separate branch of the UV-B pathway under COP1 that is independent of HY5. In fact, flavanone 3-hydroxylase (F3H, *At3g51240*), an enzyme of the flavonoid biosynthetic pathway was found to be induced independently of HY5 but still under the regulation of COP1 (Table 4.2.2.).

It should also be noted that a slight increase in the HY5 and CHS protein level is observable in the *cop1-4* mutant after four days of UV-B irradiation (Figure 4.3.2.C). Importantly, this increase is absent after 6 hours of UV-B treatment, however this 6 hours UV-B irradiation is clearly sufficient to trigger HY5 and CHS production in wild-types and *cop1*^{eid6} (Figure 4.3.2.C). Several explanations may be possible for this observation. McNellis and co-workers (1994b) demonstrated that *cop1-4* mutants still express the truncated COP1-4 protein, though to a much reduced level compared to the wild-type. Therefore it is possible, that the residual HY5 and CHS induction is the result of some retained function of the truncated COP1-4 protein which function affects late UV-B processes and which is independent of the early transcriptional responses. Alternatively, the COP1 independent part of the UV-B pathway made up by the 1/4 of the UV-B induced genes may contribute to the regulation of HY5 and CHS protein levels in these late responses. The postulate of such an alternative branch of the UV-B pathway, which acts independently of the early COP1-HY5 response and which affects rather late processes would explain why the residual *CHS* induction in *hy5-1* mutants is not detectable after 15 min UV-B treatment whereas it is clearly

observable after 6 hours of continuous irradiation (see Section 4.3.3.. and compare Table 4.2.2. and Figure 4.3.2.B).

In *cop1-4*, the exaggerated light responses include constitutively increased *CHS* gene expression and protein accumulation that in turn lead to constantly enhanced production of UV-protective flavonoids (Figure 4.3.2.A to C). Being potential sunscreen metabolites, flavonoids may interfere with UV-B responses (Li et al., 1993) The elevated protective-pigment production-phenotype of *cop1-4* is shared with *det1-1, cop1*^{eid6} and *spa1234* (Figure 4.3.2.A, Laubinger et al., 2004), whereas in contrast to *cop1-4* the latter three mutants exhibit wild-type UV-B responses on both molecular and physiological levels (Figures 4.3.2.A to C, 4.3.8. and 5.2). This indicates that the elevated flavonoid content is not the reason for the UV-B phenotypes observed in *cop1-4*. Analysis of *tt4 cop1-4* double mutant seedlings that lacks functional CHS (CHS=TT4) further substantiate this statement, as the transcriptional UV-B response of the double mutant is comparable to that of the *TT4 cop1-4* single mutant (Figure 4.3.2.D). Similar results were obtained with *tt5 cop1-4* double mutants (data not shown) (TT5 = CHI [CHALCONE ISOMERASE]). In line with these results, besides the common phenotype of elevated flavonoid content of *cop1-4* and *det1-1*, *cop1-4* mutants still exhibit reduced UV-B tolerance compared to *det1-1* (Figure 5.2B). It is clear that the flavonoid content in *cop1-4* is enhanced independently of UV-B. It is also apparent that these seedlings fail to respond to UV-B and thus to activate other components of the defence mechanisms, such as the DNA damage repair enzymes (Table 4.2.2.). This therefore may render *cop1-4* seedlings more sensitive to UV-B irradiation compared to *det1-1*, which is proficient in activating its UV-B pathway. However the already elevated level of flavonoids in *cop1-4* may absorb a considerable portion of the UV-B photons, providing a certain level of protection that may grant the milder sensitivity compared to the high rate of mortality of *hy5-1* (Figure 5.4.1.). Alternatively it is also possible that the already hypothesized retained function of the truncated COP1-4 (see above) may contribute to activate defence in late UV-B responses.

5.4.2. HY5 may cooperate with other UV-B activated factors to directly regulate transcription in UV-B

HY5 has been previously shown to bind to *CHS* promoter and regulate its activity in a light dependent manner (Ang et al., 1998; Lee et al., 2007). In agreement with this, we demonstrate that also in UV-B, HY5 mediates *CHS* activation (Figures 4.2.4.B, 4.2.6.D, 4.3.2.B). This, together with the temporal correlation of *CHS* gene induction following the elevated HY5 protein production upon UV-B stimulus (Figure 4.2.5.), is in agreement with a direct regulatory relationship between HY5 and *CHS* expression in UV-B.

HY5 overexpression complements both the light and the UV-B phenotypes, including *CHS* activation, of a *hy5* mutant (Figure 4.2.6.). However, overexpression of HY5 alone is not sufficient to induce its UV-B-regulated targets in white light, indicating that the UV-B stimulus is necessary for HY5 to specifically regulate these genes (Figure 4.2.6.A to D). Furthermore we show that even in the presence of UV-B stimulus the elevated HY5 protein amount confers only wild type-level response in the overexpressor (Figure 4.2.6.C and D). These findings are consistent with a previous report demonstrating that HY5 overexpression alone does not result in visible light-hypersensitivity (Ang et al., 1998). Interestingly, Ang et al (1998) pointed out that HY5 lacks the proline-rich and acid-rich activation domain characteristic of several bZIP transcription factors, and that HY5 cannot activate transcription by itself in yeast. These findings indicate that additional, concomitantly UV-B activated factors that could provide functional activation domain may be required to cooperate with HY5 to regulate UV-B induced transcription. Further genetic and molecular analysis of UV-B induced known or putative transcription factors may lead to the identification of such regulators.

5.4.3. UV-B regulated alteration of COP1 function to transcriptionally induce and additionally stabilize HY5 protein

In support of their specific positive regulatory role in UV-B both COP1 and HY5 were found to be enriched in the nuclei of UV-B treated seedlings (Appendix 2.2.). Additionally, under the same conditions plants expressing high level of functional YFP-COP1 keep accumulating wild-type level of endogenous HY5 protein, although both components reside in the nucleus (Figure 4.3.4.E and Appendix 2.2.). This strongly suggests that the COP1 ubiquitin ligase activity that targets HY5 for degradation is hindered in UV-B, providing an additional example for the distinct mechanistic regulation of UV-B and light responses (see also Section 5.3.).

Following its fast transcriptional induction HY5 protein accumulates to high level in the presence of continuous UV-B. However, when the transient mRNA accumulation has already decreased, HY5 protein amount is still retained at high levels (Figure 4.2.5.). Furthermore, in HY5 overexpressing seedlings, where the transcription rate remains unaltered, HY5 protein accumulates to slightly elevated levels in supplementary UV-B compared to white light (data not shown). These findings strongly suggest that HY5 protein is stabilized under these conditions, which correlates well with the assumption of COP1 dependent HY5 degradation being hindered under UV-B.

Interestingly, the subsequent cessation of UV-B treatment results in the fast degradation of HY5 protein in wild type, a HY5 overexpressor and a YFP-COP1 overexpressor line

(Figure 4.3.7.A top, B). From our previous results it is clear that under these conditions (i.e. 4 days of UV-B treatment) the *HY5* transcript level is already returned to close to the background level of white-light grown seedlings (Figures 4.2.5.A and 4.3.2.B). Furthermore, in the HY5 overexpressor, the *HY5* transcript accumulation is held at constant level (Figure 4.2.6.C). Therefore, changes in the HY5 protein levels after the removal of UV-B are most probably not due to reduced *HY5* transcription. However, precise determination of *HY5* transcript levels by real-time RT-PCR could further substantiate this notion. It also should be noted that in the YFP-COP1 overexpressor no enhanced and/or faster degradation of HY5 could be observed after the removal of UV-B (Figure 4.3.7.B bottom). However these findings are less conclusive due to the partial complementation conferred by the YFP-COP1 construct. Analysis of an untagged COP1 overexpressor will clarify this issue. Notwithstanding this, our data indicate that the removal of UV-B stimulus results in the rapid and proteasome dependent degradation of HY5 protein (Figure 4.3.7.A top, B and C), which process seem to require functional COP1 (Figure 4.3.7.A bottom).

Therefore it is reasonable to assume that UV-B acts to inactivate COP1 degradatory function on HY5, whereas the removal of UV-B stimulus restores this function. How this regulatory switch is achieved remains to be answered. The direct interaction of COP1 and HY5 under UV-B conditions is still an open question. A tempting possibility for the modification of COP1 function is the specific alteration of the COP1 E3 ubiquitin ligase activity, or perhaps the association of COP1 with other regulatory protein(s). Alternatively, UV-B specific modification of HY5 protein influencing target affinity may also be the reason for increased HY5 stability under UV-B. Interestingly, phosphorylation of HY5 in its COP1 interacting domain has been demonstrated to regulate both HY5 stability and activity in light signalling. The phosphorylated HY5 protein appears to be more resistant to COP1 mediated destabilization, though at the same time has weaker biological activity (Hardtke et al., 2000).

Our data indicates that COP1 is also required for the UV-B mediated transcriptional activation of *HY5* and other UV-B-responsive genes. It remains however unclear, how exactly COP1 can regulate these genes in response to UV-B. Given the known function of COP1 as an E3 ligase promoting the degradation of transcriptional regulators, a feasible mode of action might be the UV-B triggered destabilisation of unknown repressor(s) of UV-B-responsive genes. Regulation of transcription by E3 ligases through degradation of repressors directly at the promoter has been reported in mammalian development (Perissi et al., 2004). However, COP1 has also been shown to regulate the activity of the c-Jun transcription factor independently of proteolysis in mammalian cells (Bianchi et al., 2003). Furthermore, E3 ligase mediated ubiquitylation of transactivator domains has been reported to be required for

transcriptional activation (Conaway et al., 2002). Thus, COP1 mediated activation of transcription regulators by ubiquitylation is also an alternative. This is especially intriguing as COP1 mediated monoubiquitylation of HY5 has previously been demonstrated *in vitro* (Saijo et al., 2003).

It should be noted that COP1 has been reported to act as part of a large protein complex of about 700 kDa in light signalling (Saijo et al., 2003). It is therefore reasonable to envision that UV-B may trigger the recruitment of UV-B specific components to COP1, resulting in the assembly of an altered COP1 complex that acts specifically in UV-B signalling. This complex may then posses the altered potential functionalities discussed above that are proficient in mediating the UV-B induced signalling pathways.

Recently, another pathway component, UVR8, was described to be required for *HY5* gene activation (Brown et al., 2005). UVR8 binds histones *in vitro*, and fluorescent protein-UVR8 was found to associate with the *HY5* promoter *in vivo* using chromatin immunoprecipitation assays, indicating a direct involvement of UVR8 in *HY5* gene activation (Brown et al., 2005). Interestingly, mutations in both *COP1* and *UVR8* completely block *HY5* activation, indicating that they may function in the same genetic pathway. The functional relationship between COP1 and UVR8 still remains to be determined. It would be, therefore, interesting to analyse, whether the two proteins interact directly and/or incorporate into the same complexes, and regulate the transcription of their potentially common target genes directly at the promoters.

Several studies demonstrated the structural and functional conservation of COP1 among plants and mammals (see Section 2.1.8. and Yi and Deng, 2005). It is tempting to speculate that human COP1 might also have a function in UV-B responses in human skin cells. A first hint comes from the recent demonstration of a UV-B mediated disruption of COP1 interaction with c-Jun and major vault protein (MVP) in cultured kidney cells (Yi et al., 2005). Further studies in mammalian cells demonstrated that the ATM protein kinase phosphorilates COP1 and stimulates its rapid autodegradation in response to DNA damage (Dornan et al., 2006). This phosphorylation event on COP1 is also necessary and sufficient to disrupt the COP1-p53 interaction and thus to stabilise p53 and allow it to execute its tumor suppressor function. Furthermore, ionizing radiation was shown to trigger ATM-dependent nuclear export of COP1 (Dornan et al., 2006). These findings raise the intriguing possibility that phosphorylation of the *Arabidopsis* COP1 may also serve as a mechanism to regulate its function in UV-B signalling.

The findings that COP1 seems to be involved in different UV-B responses in both mammalian cells and *Arabidopsis* might have special importance with regard to the elucidation of COP1 UV-B function in mammals. *Arabidopsis* allows the genetic dissection

of COP1 function in UV-B responses, which eventually might be transferred to other plant and possibly mammalian organisms. This is a specially tempting possibility, as the best studied animal model organisms, *Saccharomyces, Drosophila* and *Caenorhabditis*, lack obvious COP1 orthologs (Yi and Deng, 2005).

5.4.4. A working model for COP1 and HY5 function in UV-B signalling

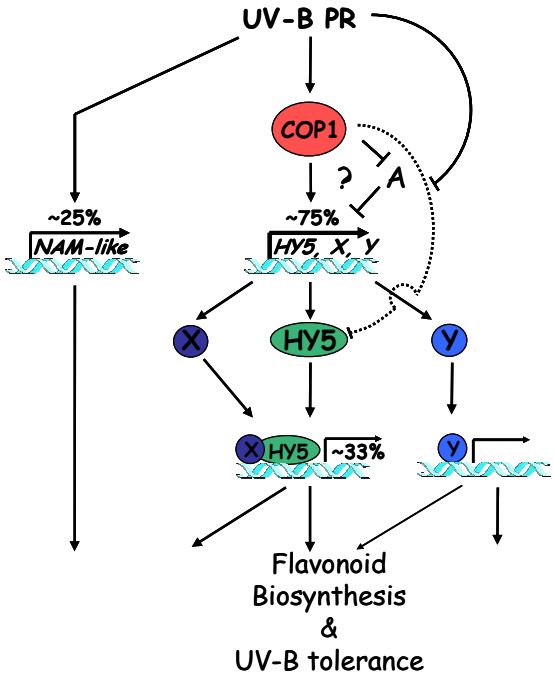

Figure 5.4.4. A simplified model of the function of COP1 and HY5 in UV-B signalling
We propose that UV-B perceived by the postulated UV-B photoreceptor (PR) activates COP1 that is required for the activation of the majority (75%) of the UV-B-induced genes. The COP1-independent branch of the UV-B pathway, with a proposed example gene acting in it (*NAM-like*), is also indicated. COP1 may act either through the degradation of a yet unidentified repressor (A) or through a direct activation mechanism to up-regulate the UV-B induced genes, such as *HY5*, *X* and *Y*. These transcription factors will act in concert to regulate gene expression leading to photomorphogenic UV-B responses, such as flavonoid biosynthesis. The HY5-dependent branch of the pathway regulating 1/3 (33%) of the UV-B induced genes, that largely involve the flavonoid biosynthetic pathway, is also indicated. The model also indicates the UV-B-regulated inhibition of the degradatory function of COP1 against HY5. Arrows indicate positive regulatory steps, whereas blocked arrows suggest inhibitory steps.

In summary, the data presented here describe the crucial regulatory roles of COP1 and HY5 as positive regulators in the UV-B responses of *Arabidopsis* (Figure 5.4.4.). The early UV-B photoreceptor-responsive gene expression changes are to a large extent COP1, and to a smaller extent HY5 dependent. The COP1 UV-B pathway branches into a HY5-dependent and a HY5-independent part, with the former regulating the establishment of UV-B tolerance. Altogether, our data position COP1 as an integration node downstream of the postulated UV-B photoreceptor(s), similar to the case of phytochrome and cryptochrome signalling, but with a distinct mode of action.

Though our data introduce a novel and important part of the UV-B perception and signalling pathway in *Arabidopsis*, a lot more remains to be done to further understand the importance of this pathway as well as the different molecular mechanisms involved in it. The microarray analyses performed in our laboratory provide firm bases to investigate further potential components of UV-B signalling by the application of several reverse genetic, molecular genetic and biochemical approaches. The establishment of forward genetic screens based on these results may lead to the identification of the enigmatic UV-B photoreceptor. Furthermore, the exact relationship of COP1 and HY5, including the question

whether they directly interact in UV-B or not, also remains to be elucidated. Biochemical and cell biological approaches may help to clarify this point. Likewise, biochemical and cell biological analysis of potential alternative COP1 complexes in UV-B, that may possibly include for example UVR8, is also an important aspect of further UV-B research. The investigation of these and also further questions should greatly broaden our knowledge of UV-B perception and signal transduction.

6. SUMMARY

UV-B radiation is an intrinsic part of the light that reaches Earth's surface. Plants, as photoautotroph and sessile organisms are inevitably exposed to this part of the light spectrum. The detrimental effects of UV-B are well known. However, low levels of such irradiation can also serve as an environmental cue, and thus can evoke specific regulatory responses similarly to the visible part of the electromagnetic spectrum that is perceived by specific photoreceptors. The analysis of plants' morphogenic responses to UV-B irradiation is a relatively new field of plant molecular biology research. The components involved in the perception and signal transduction of this environmental stimulus are largely unknown, in sharp contrast to the signalling mechanisms in visible light.

The main goal of our work was to gain further understanding of the molecular mechanisms underlying the photomorphogenic UV-B responses in the model plant *Arabidopsis thaliana*. We sought to describe the transcriptional changes in response to low levels of UV-B irradiation and to identify and characterise new components of the signal transduction pathways regulating these responses.

We demonstrate by a series of RNA gel blot analyses of young seedlings that low levels of UV-B irradiation results in the early and transient induction of a set of genes, several of which encodes transcription factors. These findings are consistent with a complex regulatory network similarly to the case of visible/UV-A-light-responsive signal transduction pathways. A subset of these UV-B-responsive genes exhibit weaker induction in stronger (i.e. shorter wavelength) UV-B, whereas the other subset remains unchanged or is further induced under such conditions. This differential regulation of the two subsets of UV-B-induced genes is indicative of the presence and interaction of at least two UV-B perception and signalling pathways.

Further RNA gel blot analyses of compound photoreceptor mutants *phyAphyB*, *cry1cry2* and *phot1phot2* revealed that the molecular UV-B responses are independent of all the known photoreceptors of *Arabidopsis*. This strongly supports the notion that these responses are mediated through a specific UV-B photoreceptor. However, changes in the expression pattern of a set of UV-B-induced genes in the *cty1cty2* double mutant indicate a possible interaction between the cryptochrome and the UV-B pathways. The comparable transcriptional activation of the low-level UV-B induced genes in DNA damage repair mutants *uvr1uvr2* and *uvr1uvr3* supports that the UV-B responses under investigation are independent of general DNA damage pathways.

We found through the expression analyses that a major positive regulator of photomorphogenic development, the bZIP transcription factor HY5 is induced by low level UV-B irradiation. We additionally show by Western blot analysis that abundant amount of HY5 protein is produced upon UV-B stimulus. Epifluorescent microscopic examination of *HY5* promoter driven HY5-YFP fusion protein further confirmed the accumulation of HY5 protein to high levels in the nuclei of UV-B treated seedlings. Moreover, analysis of *hy5* null mutants clearly showed that HY5 is required for the UV-B mediated activation of a set of UV-B induced genes, which largely involves components and regulators of the phenylpropanoid biosynthetic pathways that produces UV-protective "sunscreen" pigments. Results of RNA gel blot analyses also showed that components other than HY5 may also contribute to the regulation of UV-B responses, yet to a miner extent. One of such a component is the closest homolog of HY5, HYH.

We further assessed HY5 function in UV-B through stable transgenic lines ectopically overexpressing HY5 protein from the CaMV 35S strong constitutive promoter. We confirmed the functionality of the construct by hypocotyl length comparison and RNA gel blot analysis, which showed the complementation of both the light and UV-B phenotypes of *hy5* mutant. Overexpression of HY5 however did not cause hyper-responsiveness to UV-B, suggesting that HY5 alone is not sufficient to trigger the UV-B response. Therefore we suggest that additional regulators are required to cooperate with HY5 to activate other UV-B responsive genes.

Based on the tight regulatory relationship of HY5 and the E3 ubiquitin ligase COP1 in light signalling, that involves the COP1 mediated promotion of proteasomal degradation of HY5 by mediating its specific ubiquitylation in the absence of light stimulus, prompted us to investigate the potential role of COP1 in UV-B responses. In fact, epifluorescent microscopy revealed that the functional YFP-COP1 protein accumulates to high levels in the nuclei of UV-B treated seedlings, and this nuclear abundance of COP1 follows the presence or absence of UV-B stimulus. RNA gel blot analysis of *cop1* mutants revealed the impairment of the transcriptional activation of a broad range of UV-B induced genes, including HY5-dependent genes and HY5 itself. This finding suggests a sequential regulatory action of COP1 and HY5 in the control of a subset of UV-B induced genes. In fact, RNA gel blot and Western blot analysis together with flavonoid-content measurement of *hy5-1* and *cop1-4* mutants revealed that UV-B induced induction of *CHS* gene activation and protein accumulation, and the resulting elevated flavonoid production requires the transcriptional induction and protein production of HY5, which in turn requires functional COP1. This pathway is thus required for the establishment of plant defence against the damaging effects of UV-B, assuring survival

under these conditions. Therefore, in sharp contrast to its negative regulatory function in light signalling, COP1 act as a crucial positive component in UV-B.

Our RNA gel blot, Western blot and flavonoid-production analyses of the non-constitutive photomorphogenic *cop1* allele, *cop1*^{eid6} revealed that though this mutant has a hypersensitive mutant phenotype in light, it is not impaired in its UV-B responses. This finding is indicative of different structural requirements of COP1 in UV-B and light signalling.

We also showed by Western blot analysis that in addition to its transcriptional activation, HY5 protein is further stabilized under sustained UV-B treatments. Epifluorescent microscopic analysis demonstrated that under the same conditions both COP1 and HY5 accumulates to high levels to the nucleus. Additionally, Western bolt results show that in the abundant presence of functional YFP-COP1 protein, HY5 accumulates to wild-type levels in response to UV-B stimulus. These results strongly suggest that the COP1 mediated HY5 degradation is hindered in UV-B. Therefore we propose that UV-B modulates COP1 function to: a) induce *HY5* transcription by a yet unknown mechanism that may involve the UV-B induced degradation of a transcriptional repressor of HY5 or the direct activation of positively acting transcription factors possibly by monoubiquitylation; b) to ensure HY5 protein accumulation through hindering the degradatory function of COP1 on HY5.

We also analysed *spa1234* quadruple mutant and *csn5a* and *det1-1* single mutants by RNA gel blots. Non of these were significantly impaired in their transcriptional UV-B responses, suggesting that the main regulators of COP1 function in light signalling (the SPA proteins, the CSN and the CUL4-RBX1-CDD E3 ubiquitin ligase) do not have a role in UV-B signalling. Thus UV-B and light signalling pathways use shared components but with distinct mechanisms.

Taken all together, in this study we describe the transcriptional responses to UV-B irradiation in the model plant *Arabidopsis thaliana*. Our results place the bZIP transcription factor HY5 into the UV-B signalling pathways as an essential positive regulatory component. Furthermore, we demonstrate that the E3 ubiquitin ligase COP1, a major negative regulator of HY5 function in visible light signalling pathways, acts as an essential positive component of UV-B signalling, partly through the regulation of HY5. Therefore we anticipate a novel function of COP1 that is exploited in the control of UV-B responses. Our data further suggest that the UV-B induced regulatory pathway operating through the sequential regulatory action of COP1 and HY5 is essential for the establishment of plant defence mechanisms against the detrimental effects of UV-B.

ÖSSZEFOGLALÁS

Az ultraviola-B (UV-B) sugárzás a földfelszínre jutó napfény rendkívül lényeges részét képezi. A növények, mint fotoautotróf és helyhez kötött szervezetek szükségszerűen kitettek a fényspektrum ezen tartományának is. Az UV sugárzásnak az élő szervezetekre gyakorolt káros hatásai jól ismertek. Az alacsony szintű (hosszabb hullámhosszúságú, kis dózisú) UV-B sugárzás azonban nemcsak környezeti stressz tényező, hanem a fejlődést specifikusan szabályozó információs szignál is. Így hatás a növényekre specifikus szabályozási mechanizmusokat is indukál, hasonlóan a látható és UV-A fényhez. A növények UV-B sugárzásra adott morfogenikus válaszreakcióinak vizsgálata a növényi molekuláris biológiai kutatásoknak egy viszonylag új területe. Ezen környezeti tényező érzékelésében és jelátvitelében szerepet játszó komponensek ezidáig alig ismertek, ellentétben a látható fény érzékelésének és jelátvitelének szereplőivel.

Munkánk fő célja volt, hogy jobban megismerjük az *Arabidopsis thaliana* modell növény UV-B sugárzásra adott fotomorfogenikus válaszreakcióinak molekuláris hátterét. Megkíséreltük az alacsony szintű UV-B sugárzás által kiváltott transzkripciós változások karakterizálását, és az ezen változásokat szabályozó, új jelátviteli komponensek azonosítását és jellemzését. Eredményeink és következtetéseink az alábbiakban kerülnek rövid felsorolásra.

Csíranövények RNS gél blot vizsgálataival igazoltuk, hogy az alacsony szintű UV-B sugárzás az *Arabidopsis* gének egy csoportjának gyors és átmeneti indukcióját eredményezi. Bemutattuk, hogy ezen gének jelentős része transzkripciós faktorokat kódol. Eredmények így alátámasztják egy UV-B indukálta, összetett szabályozási hálózat működését, hasonlóan a látható fény/UV-A jelátviteli rendszerhez. Az UV-B indukálta gének egy alcsoportja gyengébb indukcióval válaszol az erősebb (rovidebb hullámhosszú) UV-B sugárzásra, míg ugyanerre a stimulusra egy másik alcsoport indukciója változatlan marad. A két géncsoport eltérő szabályozása két különböző, egymással kölcsönható UV-B érzékelő és jelatviteli rendszer jelenlétére utal.

Különböző fotoreceptor null mutánasok (*phyAphyB*, *cry1cry2*, *phot1phot2*) RNS gél blot analízisével igazoltuk, hogy a vizsgált molekuláris UV-B válaszok függetlenek az ismert fotoreceptoroktól, alátámasztva, hogy ezen válaszreakciókat egy specifikus UV-B fotoreceptor(ok) szabályozhatja. Mindamellett az UV-B indukálta gének egy csoportjának expressziós mintázat változása a *cry1cry2* kettős mutánsban a kriptokróm és az UV-B jelátviteli kaszkádok lehetséges kölcsönhtására utal. A DNS javító mechanizmusok

mutánasainak a vad típúshoz hasonló transzkripciós válaszreakciója azt mutatja, hogy az általunk vizsgált folyamatok függetlenek az általános DNS károsodást közvetítő jelátviteltől.

A génexpressziós vizsgálataink kimutatták, hogy a fotomorfogenezis kulcsfontosságú szabályozójának, a *HY5* transzkripciós faktornak az expressziója alacsony szintű UV-B sugárzás hatására aktiválódik. Western blot és epifluoreszcens mikroszkópiás vizsgálatokkal kimutattuk továbbá, hogy az endogén HY5 fehérje és a rekombináns HY5-YFP fúziós fehérje is nagy mennyiségben termelődik az UV-B kezelt növények sejtmagjaiban. *hy5* null mutánsok vizsgálatával igazoltuk, hogy a HY5 szükséges az UV-B indukálta gének egy csoportjának transzkripciós aktivációjához. Ezek közé tartoznak különböző szabályozói és komponensei (pl. *CHS*) a fenilpropanoid bioszintézisnek, mely az UV-B-t abszorbeáló, ún. fényvédő pigmnetek (pl. flavonoidok) termelését, és így az UV-B sugárzás káros hatásainak kivédését biztosítja. További RNS gél blot vizsgálatokkal kimutattuk, hogy bár kisebb mértékben, de a HY5 mellett más fehérjék is (pl. HYH) részt vesznek a HY5-függő gének szabályozásában.

A HY5-nak az UV-B jelátvitelben betöltött szerepét olyan stabil transzgénikus vonalak segítségével vizsgáltuk tovább, melyek túltermelik a HY5 fehérjét. Hipokotil növekedés gátlás vizsgálatával és RNS gél blot analízissel igazoltuk a transzgéneknek köszönhető komplementációt. Eredményeink alapján a HY5 fehérje túltermelése önmagában nem elégséges megnövekedett UV-B válasz kiváltásához, vagyis más szabályozó komponensek HY5-val való együttműködése szükséges a HY5 által szabályozott gének UV-B általi aktiválásához.

A látható fény által közvetített jelátvitelben a HY5 fehérje a COP1 E3 típusú ubiquitin ligáz poszttranszlációs szabályozása alatt áll. Fény hiányában a COP1 biztosítja a HY5 poli-ubiquitilálását, és proteaszómális lebontását, blokkolva így a fotomorfogenikus program kifejeződését. Ezekre az ismeretekre alapozva megvizsgáltuk a COP1 lehetséges szerepét az UV-B jelátvitelben. Epifluoreszcens mikroszkópiás vizsgálatokkal kimutattuk, hogy a funkcionális YFP-COP1 fehérje feldúsul az UV-B kezelt csíranövények sejtmagjaiban, és a fehérjének ezen megemelkedett vagy csökkent szintje követi az UV-B stimulus jelenlétét vagy hiányát. Különböző *cop1* mutánsok RNS gél blot vizsgálatával kimutattuk továbbá, hogy a COP1 nélkülözhetetlen az UV-B indukálta gének jelentős részének aktivációjához, beleértve a HY5-függő géneket és magát a HY5-ot is. Ezen eredmények a COP1 és a HY5 UV-B jelátvitelben betöltött egymást követő szabályozó szerepére utalnak. *cop1-4* és *hy5-1* mutánsokon végzett RNS gél blot és Western blot vizsgálatok valamit flavonoid termelés mérések kimutatták, hogy a *CHS* gén aktivációjához és megemelkedett fehérje termeléséhez, valamint az ebből adódó megnövekedett flavonoid akkumulációhoz a *HY5* gén indukciója és fehérje termelése szükséges, ami viszont funkcionális COP1 fehérjét igényel. A COP1-HY5

útvonal tehát szükséges az UV-B sugárzás károsító hatásaival szembeni védelem kialakításához és így a növények túléléséhez. Mindezek alapján a COP1 egy létfontosságú pozitív szabályozó az UV-B jelátvitelben, ellentétben az eddig ismert, látható fény által közvetített folyamatokban betöltött negatív szabályozó szerepével.

A *cop1*^{eid6} allélről RNS gél blot, Western blot és flavonoid termelés vizsgélatokkal kimutattuk, hogy bár a *cop1*^{eid6} mutánsnak látható fényben hiperszenzitív cop fenotípusa van, az UV-B válaszok tekintetében vad fenotípust mutat. Mindez arra utal, hogy a COP1 különböző szerkezeti elemeinek eltérő szerepe van az UV-B és a látható fény közvetítette jelátvitelben.

Western blot analízisekkel igazoltuk, hogy a transzkripciós aktiváción túl a HY5 fehérjeszinten is stabilizálódik a folyamatos UV-B kezelés során. Ennek megfelelően az UV-B stimulus megszűnése a HY5 fehérje gyors degradációját eredményezi. Epifluoreszcens mikroszkópiás vizsgálatokkal kimutattuk, hogy azonos körülmények között mind a HY5, mind a COP1 fehérje feldúsul az UV-B kezelt növények sejtmagjaiban. Western blot vizsgálataink igazolták továbbá, hogy a funkcionális YFP-COP1 fehérje túltermelése ellenére a HY5 fehérje UV-B hatására a vad típussal megegyező mértékben termelődik. Ezen eredmények a COP1-nek a HY5 ellen irányuló degradatív funkciójának az UV-B által történő gátlására utalnak. Mindezek alapján azt feltételezzük, hogy az UV-B módosítja a COP1 funkcióját, ezáltal: a) indukálja a *HY5* (és más UV-B indukálta gének) expresszióját egy eddig ismeretlen mechanizmuson keresztül, b) a COP1-nek a HY5 ellen irányuló degradatív funkciójának gátlásán keresztül biztosítja a HY5 fehérje stabilitását.

A *spa1234*, *csn5a* és *det1-1* mutánsok RNS gél blot vizsgálatával igazoltuk, hogy az ismert, a látható fény jelátvitelében is szerepet játszó, a COP1 működéséhez szükséges fehérje komplexek a COP1 UV-B-ben betöltött szerepének szabályozásában nem vesznek részt. Mindez megerősíti, hogy az UV-B és a látható fény jelátviteli folyamatai megosztott komponenseket használnak de eltérő mechanizmusokon keresztül.

Összefoglalva, ezen dolgozatban jellemzzük az UV-B sugárzás által kiváltott transzkripciós változásokat *Arabidopsis thaliana*ban. Eredményeink a HY5 bZIP transzkripciós faktort, mint nélkülözhetetlen pozitív szabályozó komponenst, az UV-B jelátviteli kaszkád elemi közé helyezik. Bemutatjuk, hogy a COP1 E3 ubiquitin ligáz, ellentétben az eddig ismert funkciójával, mint szintén nélkülözhetetlen pozitív szabályozó vesz részt az UV-B jelátvitelben. Ez egyben előre vetíti a COP1-nek egy új, UV-B által szabályozott funkcióját is. Eredményeink alátámasztják továbbá, hogy az UV-B által indukált COP1-HY5 szabályozó útvonal elengedhetetlen a károsító UV-B sugárzás elleni védelem kialakításához, az UV tolerancia kifejlődéséhez.

APPENDIX

A1. Literature cited

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List of scientific publications by the author in the topic of this dissertation

Oravecz, A., Baumann, A., Mate, Z., Brzezinska, A., Molinier, J., Oakeley, E.J., Adam, E., Schafer, E., Nagy, F., and Ulm, R. (2006). CONSTITUTIVELY PHOTOMORPHOGENIC1 is required for the UV-B response in Arabidopsis. Plant Cell **18,** 1975-1990.

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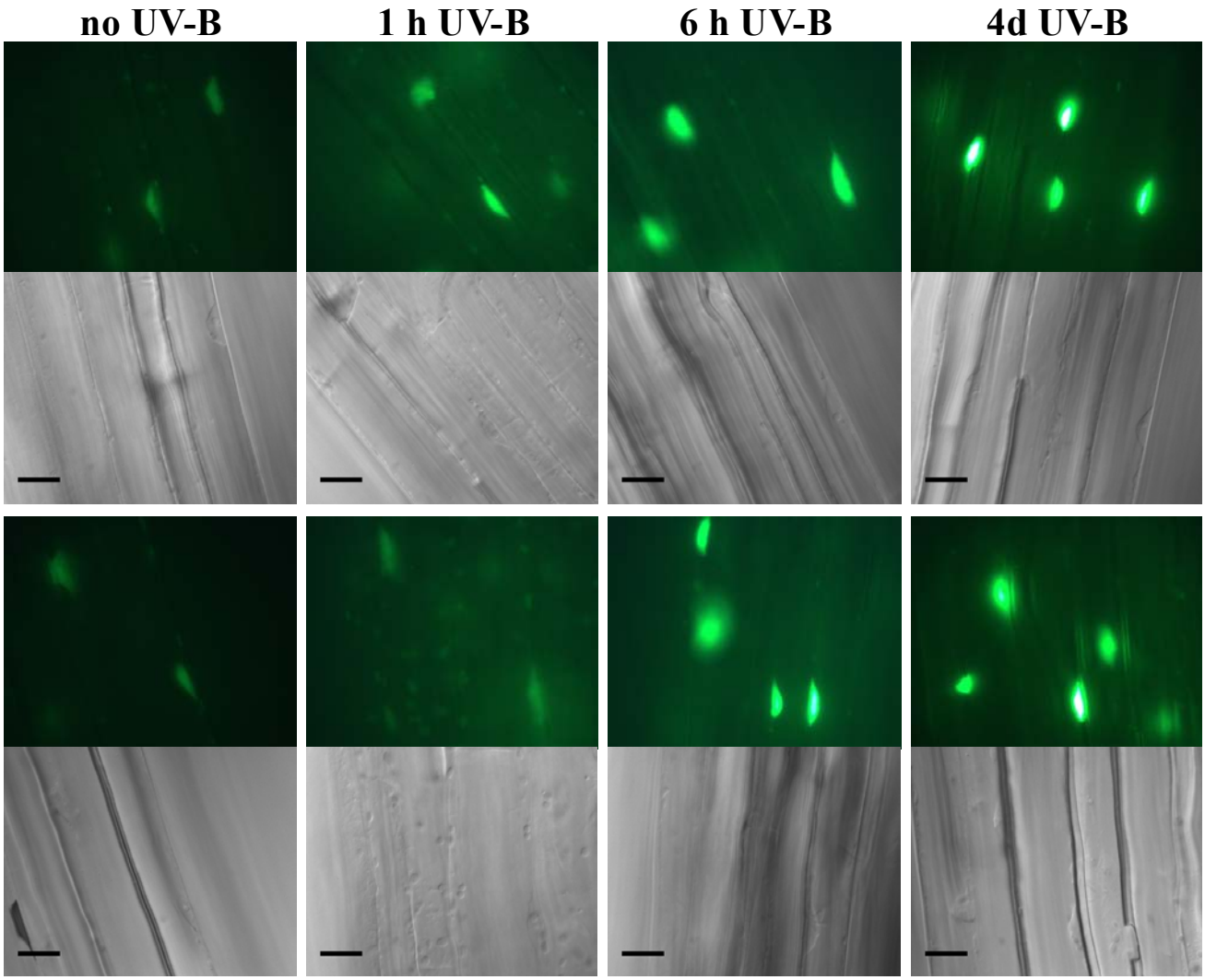
A2. Photographs of microscopy

Appendix 2.1. HY5-YFP exhibits enhanced nuclear accumulation in response to supplementary UV-B.
Seedlings of the *hy5-1/Pro$_{HY5}$:HY5-YFP* line 10 were grown for four days in the narrowband UV-field either in UV-B supplemented white light under WG305 cut-off (4d UV-B) or in white light only under WG327 cut-off (no UV-B). 1 and 6 h supplementary UV-B treated samples (1 h and 6 h UV-B) were generated by exchanging the WG327 cut-off to WG305 1 and 6 h before microscopic analysis. Two representative digital photographs taken under the epifluorescent microscope are shown for each condition. Bright field DIC images are greyscale, coloured images were taken with the YFP filter-set. For the acquisition of YFP fluorescent images 5548 ms exposure time was used for all images. Bars = 20 μm.

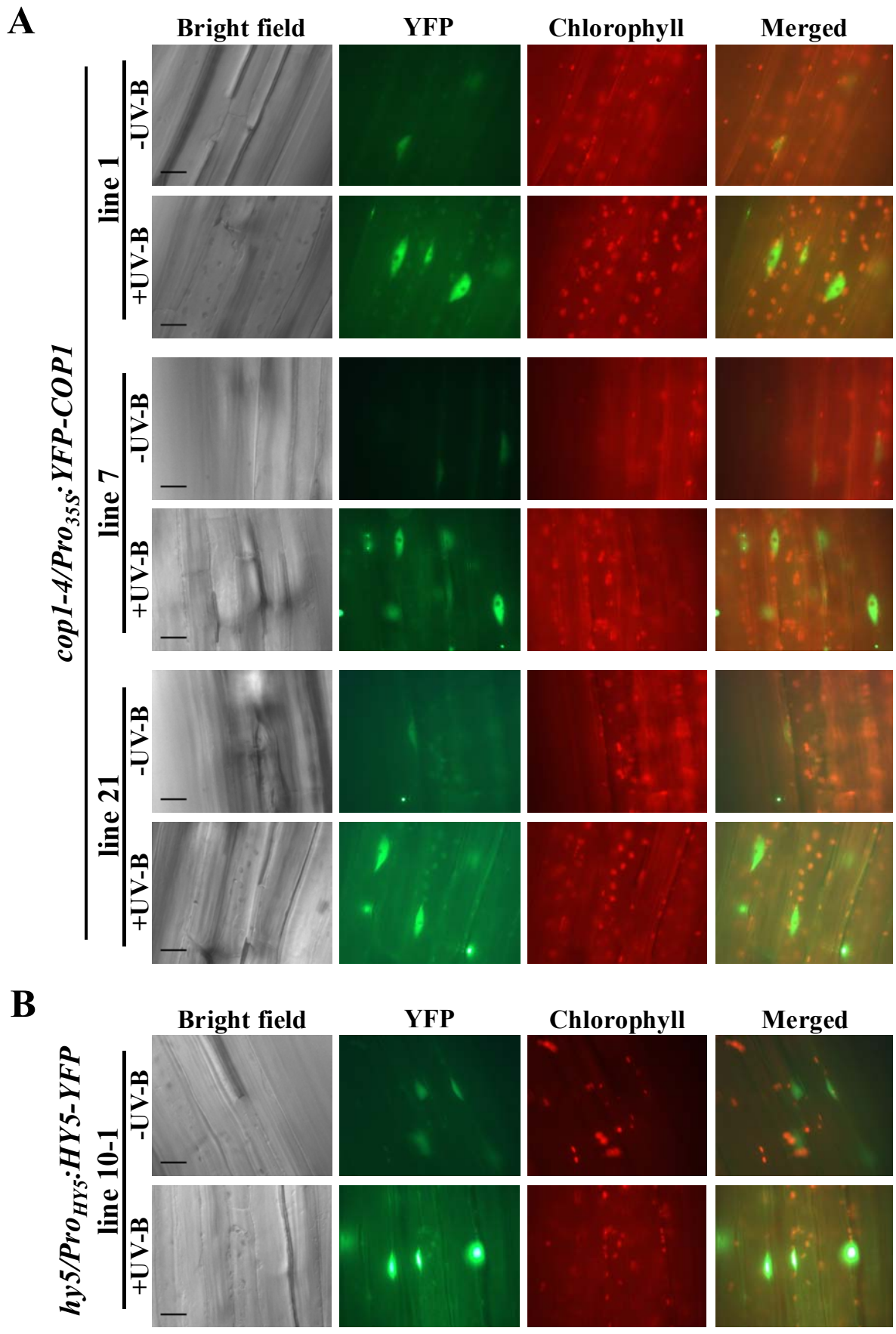
Appendix 2.2. UV-B induces nuclear enrichment of both YFP-COP1 and HY5-YFP
Seedlings were grown for four days in the narrowband UV-field in UV-B supplemented white light under WG305 cut-offs (+UV-B) or in white light only under WG327 cut-offs (-UV-B). Seedlings were analysed by epifluorescent microscopy. From left to right: bright field DIC images; images taken with the YFP (green) and the Rhodamine (Chlorophyll; red) filter set and a superimposed image of the later two (merged). Bars = 20 μm
(A) Images of three independent *cop1-4/Pro$_{35S}$:YFP-COP1* lines (line 1, 7 and 21). YFP images were taken with the following exposure times: 5548 ms for line 1, 7475 ms for line 7 and 6760 ms for line 21.
(B) Images of *hy5-1/Pro$_{HY5}$:HY5-YFP* line 10. Exposure time: 5548 ms.

Appendix 2.3. Nuclear abundance of YFP-COP1 is dynamically changed according to the presence or absence of UV-B stimulus
cop1-4/Pro$_{35S}$:YFP-COP1 line 21 seedlings were grown for four days in the narrowband UV-field either in UV-B supplemented white light under WG305 cut-offs or in white light only under WG327 cut-offs. Half of both sets of samples were left under the original conditions (continuous UV-B and continuous WL, respectively). On the other half of both sets of samples the cut-off filters were exchanged to the opposite (i.e. WG327 to WG305 [plus UV-B], and WG305 to WG327 [UV-B removal]). Seedlings from all four sets of samples were analysed by epifluorescent microscopy at the indicated time-points counted from the filter exchange. Bright field DIC images are greyscale, coloured images were taken with the YFP filter-set. Exposure time for all YFP images = 5027 ms. Bars = 20 μm.

Appendix 2.1.



Appendix 2.2.


A
Bright field
YFP
Chlorophyll
Merged
cop1-4/Pro35S:YFP-COP1
line 1
line 7
line 21
-UV-B
+UV-B
B
hy5/ProHY5:HY5-YFP
line 10-1

Appendix 2.3.

4 d supplementary UV-B
(WG305)
4d WL
(WG327)
continuous UV-B
(WG305)
remove UV-B
(WG327)
plus UV-B
(WG305)
continuous WL
(WG327)
0.5 h
1 h
3 h
6 h
12 h
18 h
1 d
2 d
3 d
1
2
3
4
5
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