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CONSTITUTIVELY PHOTOMORPHOGENIC1 Is Required for the UV-B Response in *Arabidopsis* [W]

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CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) is a negative regulator of photomorphogenesis in *Arabidopsis thaliana*. COP1 functions as an E3 ubiquitin ligase, targeting select proteins for proteasomal degradation in plants as well as in mammals. Among its substrates is the basic domain/leucine zipper (bZIP) transcription factor ELONGATED HYPOCOTYL5 (HY5), one of the key regulators of photomorphogenesis under all light qualities, including UV-B responses required for tolerance to this environmental threat. Here, we report that, in contrast with the situation in visible light, COP1 is a critical positive regulator of responses to low levels of UV-B. We show that in the *cop1-4* mutant, flavonoid accumulation and genome-wide expression changes in response to UV-B are blocked to a large extent. COP1 is required for *HY5* gene activation, and both COP1 and HY5 proteins accumulate in the nucleus under supplementary UV-B. SUPPRESSOR OF PHYTOCHROME A-105 family proteins (SPA1 to SPA4) that are required for COP1 function in dark and visible light are not essential in the response to UV-B. We conclude that COP1 performs a specific and novel role in the plants' photomorphogenic response to UV-B, coordinating HY5-dependent and -independent pathways, which eventually results in UV-B tolerance.

INTRODUCTION

Living organisms have to face exposure to sunlight containing part of the UV-B spectrum (UV-B; 280 to 315 nm), which on the surface of the Earth encompasses the wavelength range above ~290 nm. To adapt optimally to light and to cope with its UV-B component, sessile plants evolved a variety of photoreceptors. The blue and red/far-red range of the visible solar spectrum is monitored by cryptochrome, phototropin, and phytochrome photoreceptors (e.g., Chen et al., 2004), but no photoreceptor(s) specialized to sensing UV-B radiation has yet been identified at the molecular level in plants or any other organism. However, in contrast with animals, for plants, UV-B irradiation is not a mere stress signal but can also serve as an environmental stimulus to direct growth and development (Kim et al., 1998; Boccalandro et al., 2001; Kliebenstein et al., 2002; Brosche and Strid, 2003; Frohnmeyer and Staiger, 2003; Paul and Gwynn-Jones, 2003; Suesslin and Frohnmeyer, 2003; Ulm and Nagy, 2005). This includes hypocotyl growth inhibition, flavonoid accumulation, and specific gene expression changes. A rather well-characterized gene activated by low levels of UV-B is *CHALCONE SYNTHASE* (*CHS*), encoding a key enzyme of phenylpropanoid biosynthesis (Jenkins et al., 2001). On a genome-wide level, recent array analyses for plant responses to UV-B radiation identified a broad range of activated and repressed genes that may now be linked to UV-B responses (e.g., Casati and Walbot, 2004; Ulm et al., 2004; Ulm and Nagy, 2005). On the other hand, genetic insight into players regulating these gene expression changes and physiological responses is rather limited. The *UV-B light insensitive* (*uli*) mutants exhibited reduced hypocotyl growth inhibition and *CHS* gene expression compared with wild-type seedlings after exposure to UV-B (Suesslin and Frohnmeyer, 2003). The *ULI3* gene is predicted to encode a protein with homology to human diacylglycerol kinases but lacking the conserved kinase domain; thus, its exact biochemical function remains to be determined (Suesslin and Frohnmeyer, 2003). Recently, using whole-genome expression profiling, we provided evidence for the existence of a specific pathway mediating transcriptional responses of *Arabidopsis thaliana* to low-level UV-B (Ulm et al., 2004). This pathway does not require known photoreceptors but involves ELONGATED HYPOCOTYL5 (HY5) (Ulm et al., 2004), a basic domain/leucine zipper (bZIP) transcription factor mediating a number of red and blue light photoreceptor-controlled physiological responses (Osterlund et al., 2000; Chen et al., 2004). The *UV resistance locus8* (*uvr8*) mutation blocks gene activation, including *HY5* and *CHS*, and induction of flavonoid biosynthesis by UV-B, resulting in UV-B hypersensitivity (Kliebenstein et al., 2002; Brown et al., 2005). The *UVR8* gene encodes a protein with

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sequence similarity to the human guanine nucleotide exchange factor REGULATOR OF CHROMATIN CONDENSATION1 (Kliebenstein et al., 2002). Recently, it was shown that UVR8 binds histones and is associated with the *HY5* promoter region in vivo (Brown et al., 2005).

The discovery of a HY5 function in UV-B–regulated gene expression indicated that part of the general photomorphogenesis program may contribute to the morphogenic UV-B response. In the dark, the HY5 protein is turned over in the nucleus by the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), a crucial repressor of light signaling (Osterlund et al., 2000; Saijo et al., 2003). In the light, activation of photoreceptors leads to the inactivation and nuclear exclusion of COP1, allowing HY5 stabilization and activation of light-responsive genes (von Arnim and Deng, 1994; Osterlund et al., 2000). Early inactivation of COP1 by visible light occurs most likely through direct interaction with phytochromes and cryptochromes, but in a still unknown molecular fashion (Wang et al., 2001; Yang et al., 2001; Seo et al., 2004). Mutational analysis indicates that nuclear exclusion of COP1 is a rate-limiting step for the establishment of photomorphogenic development (Subramanian et al., 2004). *cop1* mutants display light-grown phenotypes even in complete darkness, including short hypocotyls, open cotyledons, and elevated pigment levels. This constitutive photomorphogenic phenotype exemplifies the COP1 function as a negative regulator of light signaling. In addition to HY5, COP1 was shown to target two other photomorphogenesis-promoting transcription factors, LONG AFTER FAR-RED LIGHT1 and LONG HYPOCOTYL IN FAR-RED1 (HFR1), and the photoreceptor phytochrome A for ubiquitination and proteolysis (Seo et al., 2003, 2004; Duek et al., 2004; Jang et al., 2005; Yang et al., 2005).

COP1 consists of three functional domains: a RING finger required for ligase activity, a coiled-coil for dimerization, and a WD40 repeat domain implicated in the binding of target proteins, such as HY5 (Yi and Deng, 2005). Similarly, in mammals (including humans), the COP1 homolog targets bZIP transcription factors of the Jun family and the transcriptional activator p53 for degradation (Dornan et al., 2004a; Wertz et al., 2004). Moreover, a role of mammalian COP1 in tumorigenesis was recently suggested (Dornan et al., 2004b). However, as yet no genetic data link mammalian COP1 to a specific response in vivo.

In *Arabidopsis*, the E3 ubiquitin ligase activity of the COP1 complex was recently proposed to be supported by direct interaction with the COP9 signalosome and the CDD (for COP10, DDB1, DET1) complexes (Yanagawa et al., 2004). These three complexes are defined by the *COP*/*DET*/*FUS* group of genes that share the *cop* phenotypes when mutated (e.g., Hoecker, 2005; Yi and Deng, 2005). Moreover, COP1-mediated suppression of photomorphogenesis was recently shown to require the plant-specific SUPPRESSOR OF PHYTOCHROME A-105 (SPA) proteins SPA1 to SPA4 (Laubinger et al., 2004). The SPA proteins contain a kinase-like domain, a coiled-coil, and a COP1-like WD40 repeat domain. All SPA proteins were found to interact with COP1 through the coiled-coil domains (Laubinger et al., 2004; Hoecker, 2005), and SPA1 was found to alter the E3 ubiquitin ligase activity of COP1 (Saijo et al., 2003; Seo et al., 2003). In agreement with these results, it was previously reported that COP1 acts as part of a large protein complex and interacts with SPA1 in a light-dependent manner (Saijo et al., 2003). Most strikingly, it was found that the quadruple *spa* mutant exhibits strong *cop* phenotypes almost indistinguishable from *cop1* mutants (Laubinger et al., 2004). Thus, these COP1 regulatory factors seem to have an essential influence on COP1 activity as repressor of light signaling.

Here, we demonstrate a crucial role of COP1 as positive regulator of the photomorphogenic UV-B response in *Arabidopsis*, in contrast with its repressor function in visible light–induced photomorphogenesis. Interestingly, this role of COP1 in the UV-B response is independent of SPA proteins. Moreover, COP1 requirements in UV-B and visible light signaling pathways can be separated in different *cop1* mutant alleles, indicating different structural requirements. We also show that in response to supplementary UV-B, COP1 is required for the activation of *HY5* gene expression, and both proteins accumulate in the nucleus. These two key regulators of photomorphogenesis are thus endowed with a specialized function for UV-B–induced signaling.

RESULTS

HY5 Is Required for UV-B–Mediated Gene Activation and UV-B Tolerance

Our previous work demonstrated the requirement of HY5 in UV-B–responsive gene expression by analyzing a few selected genes in the *hy5-1* null mutant (Ulm et al., 2004). We further assessed this function by gene profiling at the whole-genome level using Affymetrix ATH1 oligonucleotide microarrays. Seven-day-old white light–grown wild-type or *hy5-1 Arabidopsis* seedlings were exposed for 15 min to polychromatic radiation with decreasing short-wave cutoff in the UV range (WG305 = +UV-B; WG327 = −UV-B), and samples were taken 1 h after the onset of irradiation. In total, 68.6% of the early UV-B–activated genes in the Landsberg *erecta* (L*er*) ecotype remain inducible in the *hy5-1* mutant (278 out of 405 genes) (Figure 1A; see Supplemental Table 1 online). From the microarray analysis, it is apparent that HY5 regulates a number of genes that can be connected to UV tolerance, such as *PHR1* and *UVR3* encoding photolyases specific for the repair of CPD- and 6-4-photoproducts, respectively (Britt, 2004), flavonoid biosynthetic genes (including *CHS*, *CHI*, *PAL1*, *PAL2*, and *FLS1*; Winkel-Shirley, 2002), and their transcriptional regulators (e.g., *MYB12*; Mehrtens et al., 2005) (see Supplemental Table 1 online). In agreement with this notion, we found reduced UV-B tolerance in *hy5-1* mutants compared with the wild type (Figure 1B). Thus, we conclude that HY5 regulates the gene expression response required for protection and consequently survival under UV-B.

cop1 Mutants Are Broadly Impaired in UV-B–Mediated Gene Expression, Including *HY5* Activation

The crucial role of HY5 in the UV-B response indicates the use of shared signaling components in response to visible light and UV-B. A prominent regulator of HY5 action in the dark-to-light transition is the E3 ubiquitin ligase COP1; we thus analyzed the UV-B response in *Arabidopsis cop1* mutants. Since null mutants of COP1 are seedling lethal, we resorted to the widely used

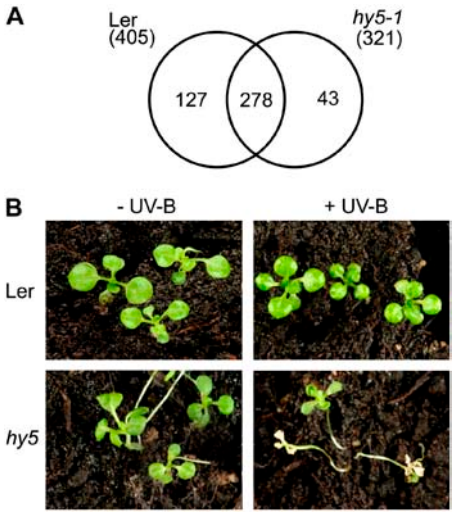

Figure 1. *hy5-1* Is Deficient in UV-B–Activated Gene Expression and UV-B Tolerance.

(A) Numbers of genes defined as responding to UV-B in *hy5-1* and wild-type L*er*. Venn diagram shows the number of genes classified as responding to UV-B in either the wild type only (left; i.e., HY5-dependent genes), the wild type and mutant (center; i.e., HY5-independent genes), or mutant only (right). The corresponding gene list can be found in Supplemental Table 1 online.

(B) HY5 is required for UV-B tolerance. Wild-type and *hy5-1* plants were grown in white light with or without supplementary UV-B. Plants were photographed after 4 weeks. Under supplementary UV-B, 13 plants died and 35 showed necrotic lesions among the *hy5-1* mutants, whereas in the parallel-grown wild-type population, no plant died or was necrotic ($n = 50$). When control plants from both genotypes were grown without supplementary UV-B, all survived without apparent necrosis.

nonlethal *cop1-4* mutant. The *COP1-4* allele has a premature stop codon removing the C-terminal 393 amino acids; the mutant therefore expresses a truncated protein containing only the N-terminal 282 amino acids lacking the WD40 repeats (McNellis et al., 1994b). Surprisingly, we found that the UV-B–mediated activation of a number of selected genes is blocked in the *cop1-4* mutant (Figure 2A), similarly to *hy5* null mutants (Ulm et al., 2004; this work). *HY5* is actually among the UV-B–activated genes that are blocked in *cop1-4* mutants (Figure 2A). In agreement with this, we have also identified COP1 as a UV-B signaling component in an independent genetic approach using a luciferase reporter gene–based screen. Pro_{HY5}:Luc^+ transgenic plants were mutagenized by ethyl methanesulfonate, and seedlings lacking UV-B induction of luciferase activity were identified. Among the isolated recessive mutants, one showed a standard-growth phenotype resembling *cop1* mutants, including dwarf growth, early flowering, and a light-grown habit in dark (McNellis et al., 1994b) (see Figure 2B for the luciferase-reporter phenotype of mutant 0650). In fact, we have fully sequenced the *COP1* gene in that mutant and identified a single C-T transition, changing the Gln-283 codon (CAA) to a Stop codon (TAA), identical to the *COP1-4* mutation described previously (McNellis et al., 1994b). This new *cop1-4* allele, however, was confirmed to be in the Wassilewskija (Ws)/Pro_{HY5}:Luc^+ background by (1) detectable basal luciferase activity in the mutant (at levels comparable to the wild type), (2) UV-B inducibility of the luciferase reporter in the F1 generation after outcrossing to the wild type, and (3) simple sequence length polymorphism analysis differentiating between Columbia (Col) (ecotype of the previously published *cop1-4* allele; McNellis et al., 1994b) and Ws ecotypes (used markers: CIW11, NGA76, and NGA168) (data not shown). The requirement for COP1 in the UV-B response was also confirmed by two other *cop1* mutant alleles: the strong *cop1-1* (McNellis et al., 1994b) (see below) and a new *cop1* allele isolated from a T-DNA collection (SALK_137391; t et al., 2003) (data not shown). Moreover, the *cop1-4* mutant phenotype under UV-B is restored by the expression of a wild-type copy of *COP1* fused with yellow fluorescent protein (YFP) (see below). Thus, our data strongly support the requirement for COP1 in the UV-B response in *Arabidopsis*.

We further assessed the involvement of COP1 in mediating molecular UV-B responses by gene profiling at the whole-genome level using ATH1 oligonucleotide microarrays. Our expression profiling of the *cop1-4* mutant identified a broad impact on UV-B responsiveness. Only 24.7% of the early genes activated in response to low-level UV-B radiation in the Col ecotype are also induced in the *cop1-4* mutant and can therefore be designated as COP1 independent (89 out of 361 genes) (Figure 2C; see Supplemental Table 2 online); in other words, 75.3% of the UV-B–induced genes, including *HY5*, depend on a functional COP1 protein. Thus, both COP1 and HY5 are positive regulators of UV-B–induced gene expression, and COP1 is required for *HY5* gene activation.

Our initial analysis of UV-B–responsive gene expression changes identified a UV-B stress pathway that antagonistically interferes with the postulated UV-B photoreceptor pathway. To test whether or not the UV-B stress pathway (P2 pathway described in Ulm et al., 2004) is affected by COP1 deficiency, we tested selected genes for their response to shorter UV-B wavelength regions. None of the marker genes studied required COP1 under these conditions (see, for example, representative genes *At5g59820* and *At1g19020* in Figure 2A), indicating that COP1 participation is specific for the postulated UV-B photoreceptor pathway and does not involve the more general UV-B stress pathway.

Gene Expression Profiling Identifies the Readout of the UV-B–Activated COP1-HY5 Pathway

The impaired UV-B induction of *HY5* in *cop1-4* prompted us to investigate which part could be tracked to the direct link from COP1 to HY5 activation. We hypothesized that the UV-B–induced genes that require HY5 should be largely included within the group of genes that are dependent on COP1. To be able to directly compare the UV-B response in *hy5-1* and the *cop1-4* mutant, the analysis was focused on genes that are commonly induced in both L*er* and Col ecotypes (total of 320 genes). This restriction allows a conservative estimation of COP1 and HY5 dependence for UV-B–mediated gene induction (see Supplemental Table 3 online). Interestingly, of the COP1-dependent 240 genes shown in Figure 2D, close to half (103 genes; Table 1) also require the HY5 protein, delineating a conservative estimate of the output of the

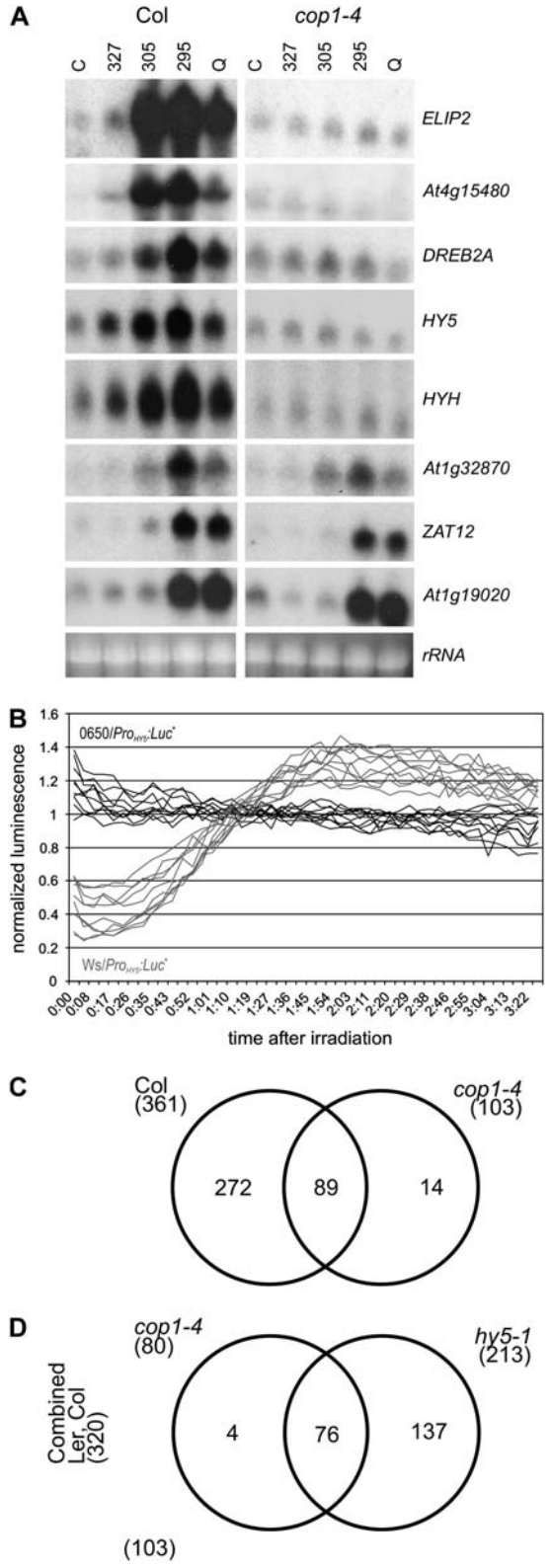

Figure 2. COP1 Is Required for UV-B–Responsive Gene Expression, Including *HY5* Gene Activation.

UV-B–activated COP1-HY5 pathway. As expected, these 103 genes make up a large part of the 107 genes that were found to be blocked in the *hy5-1* mutant, indicating that HY5-dependent genes are also COP1 dependent, in agreement with a sequential action of COP1 and HY5 for this subgroup of genes.

cop1-4 Mutant Plants Have Reduced Tolerance to Supplementary UV-B

As the UV-B hypersensitivity of *hy5-1* indicated the importance of the HY5 regulatory pathway for UV-B tolerance, we tested the *cop1-4* mutant under supplementary UV-B. Similar to *hy5-1*, *cop1-4* mutant plants are impaired in their UV-B tolerance but to a lesser extent, without lethality occurring under our conditions. In contrast with the wild type, the *cop1-4* mutant plants show chlorosis when grown under supplementary UV-B (Figure 3). This might be caused by the block of UV-B–induced genes encoding chloroplast proteins in *cop1-4* (e.g., σ-like factor SIG5, ELIP proteins, and FtsH proteases; see Supplemental Table 2 online). The reduced UV-B tolerance of *cop1-4* doesn't seem to be due to a general weakness linked to dwarf growth, as the phenotypically similar *det1-1* mutant (Pepper et al., 1994; Ma et al., 2003) does not show reduced UV-B tolerance (Figure 3). It is also interesting to note here that supplementary UV-B results in significant shortening of petiole length in the wild type, *cop1-4*/*Pro*$_{35S}$*:YFP-COP1*, and *det1-1*. This phenotype is not apparent in *cop1-4* that already has short petioles under conditions devoid of UV-B (Figure 3).

(A) RNA gel blot analysis of the *cop1-4* mutant compared with its wild type (Col). Total RNA was isolated from 7-d-old white light–grown seedlings at 45 min after 15-min irradiation under a UV-B field with different UV spectra (−UV-B under cutoff WG327; +UV-B with decreasing short-wave cutoff under WG305, WG295, or quartz glass [Q]) or left untreated in the standard growth chamber (control [C]). Blots were sequentially hybridized with specific probes for the indicated genes. Ethidium bromide–stained rRNA is shown as loading control.

(B) New *cop1-4* allele identified in a luciferase-based genetic screen. Normalized data of TopCount luciferase bioluminescence measurements of 10 *Pro*$_{HY5}$*:Luc*$^{+}$ seedlings each after 15 min of UV-B irradiation. Gray: parental *Pro*$_{HY5}$*:Luc*$^{+}$ transgenic line (wild type); black: mutant 0650 (due to *COP1-4* mutation). Note that the data for each seedling were normalized to their mean (absolute values not shown) to facilitate the comparison of induction properties. An absolutely blind mutant is expected to have a normalized luminescence value of 1 over the time course.

(C) Numbers of genes defined as responding to UV-B in the *cop1-4* mutant and wild-type Col. Venn diagram shows the number of genes classified as responding to UV-B in either the wild type only (left; i.e., COP1-dependent genes), the wild type and mutant (center; i.e., COP1-independent genes), or mutant only (right). The corresponding gene list can be found in Supplemental Table 2 online.

(D) Overlap between COP1- and HY5-independent genes. Analysis limited to genes commonly UV-B induced in both wild types Col and L*er*. Venn diagram depicts the relationship of genes induced in *cop1-4* and *hy5-1*. The total number of genes in each category is shown in parentheses. UV-B activation of the 103 genes indicated in the lower left is blocked in both *cop1-4* and *hy5-1* mutants (see Table 1). The corresponding gene list can be found in Supplemental Table 3 online.

COP1 and HY5 Are Required for UV-B–Mediated Hypocotyl Growth Inhibition and Flavonoid Accumulation

Since it is the UV-B pathway in response to low-level UV-B that is blocked in *cop1* and *hy5* mutants, it was of interest to analyze the contributions of COP1 and HY5 to photomorphogenic, non-stress-mediated UV-B responses. A well-established UV-B effect thought to be mediated by the postulated UV-B photoreceptor is the induction of phenylpropanoid biosynthetic pathway components leading to the accumulation of sunscreen flavonoids (e.g., Beggs and Wellmann, 1994; Kucera et al., 2003). To analyze UV-B effects at the organismal level, we use supplemental narrow-band UV-B (311 to 313 nm) irradiation. Continuous irradiation results in hypocotyl growth inhibition and the accumulation of flavonoids in the wild type. Both responses are reduced in the *hy5-1* mutant (Figures 4A and 4B). The absence of a UV-B effect on hypocotyl growth in *cop1-4* is less conclusive under our conditions, as this mutant has already short hypocotyls under white light without UV-B (Figure 4A), but is consistent with a previous report (Kim et al., 1998). More convincingly, we have found that UV-B–induced biosynthesis of flavonoids is strongly reduced in *cop1-4* mutants (Figure 4B). This phenotype is restored to the wild type in three independent *cop1-4*/*Pro*$_{35S}$*:YFP-COP1* lines checked (data not shown).

To further characterize COP1 structural requirement in the physiological UV-B response, we investigated a nonconstitutive photomorphogenic *cop1* allele. *cop1*^{eid6} contains a mutation that changes the conserved His-69 residue of the RING finger motif to a Tyr. *cop1*^{eid6} seedlings display normal etiolated growth in the dark but are strongly hypersensitive to visible light (Dieterle et al., 2003). The latter results in a cop phenotype comparable to *cop1-4* when grown under light, including dwarf growth, early flowering, and elevated pigment levels (Dieterle et al., 2003). In contrast with *cop1-4*, however, light-grown *cop1*^{eid6} mutant seedlings respond similarly to the wild type at the level of flavonoid accumulation under supplementary UV-B (Figure 4B). This difference between the two *cop1* alleles (i.e., similar phenotypes in visible light versus distinct phenotypes under UV-B) indicates different structural requirements and functions of COP1 in the visible and UV-B pathways.

At the molecular level, the *CHS* gene encoding a key enzyme in flavonoid biosynthesis, chalcone synthase, is known to be induced by UV-B (e.g., Jenkins et al., 2001). Moreover, the HY5 protein was previously shown to bind *CHS* promoter sequences in vitro (Ang et al., 1998). Therefore, we analyzed *HY5* and *CHS* gene expression and protein levels in seedlings that were grown for 4 d under continuous light either with or without supplementary UV-B in the same light field (under WG305 and WG327 cutoff, respectively) exactly as used for the flavonoid accumulation experiment in Figure 4B. In addition, seedlings were grown for 4 d without UV-B under a WG327 cutoff filter, which was then exchanged for a WG305 cutoff filter 1 or 6 h before harvesting to analyze the early UV-B response (see also scheme in Figure 4C). Our molecular data show that the flavonoid accumulation phenotypes correlate with the proficiency in activation of the UV-B–responsive *CHS* gene (Figure 4D) and the ability to transcriptionally activate and, as a result, accumulate HY5 protein (Figures 4D and 4E). Thus, in agreement with flavonoid accumulation, the inducibility of *CHS* and *HY5* is maintained in *cop1*^{eid6} mutants, whereas it is severely impaired in *cop1-4* and *hy5-1* mutants (Figure 4D). Residual activation of *CHS* in response to UV-B in *hy5-1* seedlings but not in *cop1-4* seedlings indicates the involvement of a second, HY5-independent pathway downstream of COP1 (Figure 4D).

Our results indicate a sequential action, with COP1 activating *HY5* gene expression and HY5 protein then activating a set of downstream UV-B–responsive genes. To substantiate this notion, we analyzed the temporal relationship of gene activation of *HY5* and HY5-dependent genes, including *CHS. HY5* gene induction is detectable as early as 30 min, with a peak at ~1 h, whereas *CHS* expression is strongly activated after 1 to 2 h and remains on high levels during 12 h of UV-B radiation (Figure 5A). A similar delay is apparent in the case of two other HY5-dependent UV-B–induced genes analyzed, namely, *ELIP2* and *At5g23730* (Figure 5A). On the protein level, HY5 protein elevation is apparent at ~1 to 2 h, from which time on it stays up (Figure 5B), correlating with prolonged *CHS* gene expression (Figure 5A) and CHS protein accumulation (Figure 5B). Thus, UV-B–mediated *HY5* gene activation and HY5 protein accumulation seem to be in temporal correlation with the induction of at least the tested HY5-dependent genes. It is also noteworthy that HY5 protein levels in the wild type are elevated under UV-B at times when *HY5* mRNA levels are back to background levels (see the 24 to 96 h time points; Figures 4D, 4E, 5A, and 5B). This indicates that the HY5 protein is additionally stabilized under these conditions.

COP1 and HY5 Accumulate in the Nucleus in Response to UV-B

In response to visible light, COP1 is excluded from the nucleus where it accumulates in the dark and directs its target proteins, including HY5, to the proteasomal degradation machinery (von Arnim and Deng, 1994; Osterlund et al., 2000; Saijo et al., 2003). This nucleocytoplasmic partitioning of COP1 seems to be required for the dark-to-light growth transition by allowing the HY5 protein to accumulate and light-responsive gene expression to occur (Subramanian et al., 2004; Yi and Deng, 2005). By contrast, we found that both COP1 and HY5 proteins are required for the UV-B response in white light–grown seedlings (Figures 1 to 4). In an attempt to analyze COP1 localization and its possible changes in response to UV-B, we generated stable transgenic lines expressing the YFP-COP1 fusion protein under the control of the constitutive cauliflower mosaic virus 35S promoter in *cop1-4*. It is important to note that expression of the YFP-COP1 protein complements the dark- and light-grown phenotypes of the *cop1-4* mutant to the wild type and, at the molecular level, restores proper HY5 degradation after transfer from light to dark (Figure 6A). This is also true for all the UV-B responses tested, namely, gene expression, hypocotyl growth inhibition, flavonoid induction, tolerance, and HY5 protein accumulation (Figures 3, 6A, and 6C; data not shown). Thus, the YFP-COP1 fusion protein is functional and confers a wild-type photomorphogenic phenotype to *cop1-4*, allowing analysis of subcellular localization in response to UV-B. Interestingly, supplementary UV-B results in nuclear enrichment of the COP1 protein (Figure 6B) in a similar

Table 1. Set of 103 UV-B–Induced Genes That Are Blocked in Both *cop1-4* and *hy5-1* Mutants (Corresponding to Figure 2D)

		Col		*cop1*		L*er*		*hy5*		
Probe Name	Gene Name	Expr.	Fold	Expr.	Fold	Expr.	Fold	Expr.	Fold	Description
245560_at	At4g15480	128	8.6	73	1.0	68	14.9	36	1.1	UDP-glucoronosyl/UDP-glucosyl transferase family (*UGT84A1*)
245126_at	At2g47460	141	4.7	73	−1.1	109	5.2	21	1.2	myb family transcription factor (*MYB12*)
260773_at	At1g78440	26	4.6	21	−1.1	43	4.0	18	1.0	Gibberellin 2-oxidase/GA2-oxidase (*GA2OX1*)
260784_at	At1g06180	134	4.5	71	−1.1	125	4.3	80	2.2	myb family transcription factor (*MYB13*)
254459_at	At4g21200	31	4.4	44	−1.3	31	3.6	17	1.0	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
249063_at	At5g44110	868	4.3	789	−1.1	994	4.7	192	1.3	ABC transporter family protein (*NAP2*/*POP1*)
246966_at	At5g24850	180	4.1	155	−1.1	150	4.1	42	1.6	Cryptochrome 3/dash (*CRY3*/*CRYD*)
248596_at	At5g49330	94	4.1	192	−1.9	70	3.9	74	−1.9	myb family transcription factor (*MYB111*)
251605_at	At3g57830	65	4.1	59	−1.1	70	5.2	78	1.9	Leu-rich repeat transmembrane protein kinase, putative
264752_at	At1g23010	83	4.0	58	1.0	56	3.5	16	−1.1	Multicopper oxidase type I family protein
265634_at	At2g25530	176	4.0	133	1.0	142	4.1	89	1.1	AFG1-like ATPase family protein
257176_s_at	No gene	439	3.8	280	−1.1	414	3.6	292	1.0	Cyclopropane fatty acid synthase, putative (At3g23510/At3g23530)
258167_at	At3g21560	766	3.5	730	−1.2	1114	3.3	358	1.0	UDP-glucosyltransferase, putative (*UGT84A2*)
247463_at	At5g62210	173	3.4	406	1.1	113	3.1	67	−1.3	Embryo-specific protein-related (*ATS3*)
251084_at	At5g01520	137	3.3	73	1.2	129	3.1	75	1.1	Zinc finger (C3HC4-type RING finger) family protein
264906_at	At2g17270	67	3.3	52	1.1	60	5.3	44	1.3	Mitochondrial substrate carrier family protein
249622_at	At5g37550	85	3.1	61	−1.1	82	3.3	22	1.3	Expressed protein
255594_at	At4g01660	317	3.1	369	1.1	276	2.8	164	1.0	ABC1 family (*ATH10*)
260146_at	At1g52770	29	3.1	50	1.1	30	2.3	35	1.2	Phototropic-responsive NPH3 family protein
250794_at	At5g05270	380	3.0	330	−1.1	215	2.8	134	1.1	Chalcone-flavanone isomerase family protein (CHI-like)
258947_at	At3g01830	12	3.0	12	1.1	12	4.4	10	2.7	Calmodulin-related, putative
265665_at	At2g27420	121	3.0	106	−1.1	88	3.1	118	2.3	Cys proteinase, putative
248049_at	At5g56090	162	2.9	170	1.0	166	2.9	91	1.0	Cytochrome oxidase assembly family protein
250882_at	At5g04000	31	2.9	21	1.1	27	2.7	15	1.1	Expressed protein
251984_at	At3g53260	1971	2.9	1379	1.1	923	2.5	927	1.4	Phe ammonia-lyase 2 (*PAL2*)
264637_at	At1g65560	119	2.9	146	−1.1	68	2.7	67	1.1	Allyl alcohol dehydrogenase, putative
266578_at	At2g23910	331	2.9	366	−1.2	143	3.3	25	1.5	Cinnamoyl-CoA reductase-related
259432_at	At1g01520	24	2.8	15	1.1	68	2.6	35	2.2	myb family transcription factor
262853_at	At1g20890	110	2.8	113	−1.1	115	2.1	89	−1.1	Expressed protein
265290_at	At2g22590	94	2.8	41	1.2	41	5.4	22	1.2	Glycosyltransferase family protein (*UGT91A1*)
256296_at	At1g69480	30	2.7	23	1.2	47	2.2	43	1.0	EXS family/ERD1/XPR1/SYG1 family protein
248204_at	At5g54280	146	2.6	85	1.1	170	2.1	148	1.4	Myosin heavy chain, putative
249677_at	At5g35970	2361	2.6	1263	1.0	2472	2.3	1426	1.6	DNA binding protein, putative
261064_at	At1g07510	283	2.6	250	1.0	303	2.7	275	1.1	FtsH protease, putative (*FtsH10*)
265886_at	At2g25620	97	2.6	109	−1.1	127	2.6	87	−1.2	Protein phosphatase 2C, putative
250533_at	At5g08640	1453	2.5	1476	−1.2	868	3.2	139	1.1	Flavonol synthase 1 (*FLS1*)
251827_at	At3g55120	902	2.5	852	1.0	738	2.6	375	1.0	Chalcone-flavanone isomerase/chalcone isomerase (*CHI*/*TT5*)
256186_at	At1g51680	1384	2.5	581	1.0	1205	1.9	1235	−1.1	4-Coumarate–CoA ligase 1/4-coumaroyl-CoA synthase 1 (*4CL1*)
258023_at	At3g19450	809	2.5	593	1.0	723	2.4	657	1.5	Cinnamyl-alcohol dehydrogenase (*CAD*)
245101_at	At2g40890	1084	2.4	698	1.0	989	1.7	1101	1.0	Cytochrome P450 98A3, putative (*CYP98A3*)
246200_at	At4g37240	104	2.4	103	1.2	89	2.2	71	−1.1	Expressed protein
250049_at	At5g17780	204	2.4	164	1.0	148	2.8	75	1.0	Hydrolase, α/β fold family protein
251895_at	At3g54420	240	2.4	160	1.3	168	3.9	205	1.3	Class IV chitinase (*CHIV*)
252383_at	At3g47780	74	2.4	60	1.1	59	2.4	88	1.3	ABC transporter family protein (*ATH6*)
252652_at	At3g44720	313	2.4	192	1.1	152	3.0	146	1.6	Prephenate dehydratase family protein
257081_at	At3g30460	69	2.4	100	1.0	62	3.7	58	1.3	Zinc finger (C3HC4-type RING finger) family protein
257595_at	At3g24750	14	2.4	14	−1.1	15	4.3	15	−1.1	Expressed protein
258227_at	At3g15620	47	2.4	52	1.0	43	2.0	36	−1.2	6-4 Photolyase (*UVR3*)
260336_at	At1g73990	586	2.4	624	−1.1	659	2.6	377	1.1	Peptidase U7 family protein (*SppA*)
260955_at	At1g06000	593	2.4	476	1.0	308	2.8	202	1.0	UDP-glucoronosyl/UDP-glucosyl transferase family (*UGT89C1*)
261607_at	At1g49660	380	2.4	364	1.0	206	1.9	142	1.2	Carbohydrate esterase family 10 protein
261907_at	At1g65060	659	2.4	427	−1.3	308	2.7	125	−1.3	4-Coumarate–CoA ligase 3/4-coumaroyl-CoA synthase 3 (*4CL3*)
263845_at	At2g37040	2074	2.4	1250	1.0	1532	2.5	1240	1.0	Phe ammonia-lyase 1 (*PAL1*)
247751_at	At5g59050	80	2.3	50	1.1	107	1.8	85	1.3	Expressed protein
253219_at	At4g34990	97	2.3	74	−1.4	83	1.5	57	−1.1	myb family transcription factor (*MYB32*)
253879_s_at	No gene	429	2.3	406	1.0	295	2.4	83	1.1	Glycosyltransferase (At4g27560/*UGT79B2*; At4g27570/*UGT79B3*)

(Continued)

Table 1. (continued).

		Col		*cop1*		L*er*		*hy5*		
Probe Name	Gene Name	Expr.	Fold	Expr.	Fold	Expr.	Fold	Expr.	Fold	Description
263796_at	At2g24540	211	2.3	113	−1.1	268	2.1	148	1.9	Kelch repeat-containing F-box family protein
265732_at	At2g01300	47	2.3	41	1.5	43	1.3	61	1.1	Expressed protein
266532_at	At2g16890	69	2.3	69	−1.2	58	1.9	40	1.0	UDP-glucoronosyl/UDP-glucosyl transferase family (*UGT90A1*)
246214_at	At4g36990	188	2.2	147	1.1	312	1.7	200	1.1	Heat shock transcription factor 4 (*HSF4*)
249071_at	At5g44050	150	2.2	93	1.1	117	2.0	114	−1.5	MATE efflux family protein
250207_at	At5g13930	3105	2.2	3772	1.0	2562	2.4	711	1.0	Chalcone synthase/naringenin-chalcone synthase (*CHS*/*TT4*)
252010_at	At3g52740	187	2.2	243	−1.2	244	2.6	113	1.1	Expressed protein
255011_at	At4g10040	408	2.2	391	1.2	513	2.1	509	1.1	Cytochrome c, putative
257147_at	At3g27270	117	2.2	82	1.1	137	1.4	139	1.0	Expressed protein
264148_at	At1g02220	55	2.2	50	1.1	42	2.3	37	1.0	No apical meristem (NAM) family protein
265318_at	At2g22650	185	2.2	123	1.0	122	1.7	100	1.1	FAD-dependent oxidoreductase family protein
266204_at	At2g02410	36	2.2	33	1.1	39	2.3	32	1.2	Expressed protein
266555_at	At2g46270	111	2.2	65	1.0	148	1.9	88	1.1	G-box binding factor 3 (*GBF3*/*bZIP55*)
267070_at	At2g41000	86	2.2	73	1.0	100	2.2	41	1.3	DNAJ heat shock N-terminal domain-containing protein
267470_at	At2g30490	1819	2.2	1385	1.0	1514	2.2	1355	1.2	Cinnamic acid 4-hydroxylase/cytochrome P450 (*C4H*/*CYP73A5*)
245113_at	At2g41660	164	2.1	187	1.0	315	1.7	145	−1.4	Expressed protein
245895_at	At5g09230	323	2.1	293	1.0	350	2.3	225	1.1	Transcriptional regulator Sir2 family protein
248199_at	At5g54170	314	2.1	375	1.5	266	1.8	351	1.6	Expressed protein
253922_at	At4g26850	2140	2.1	1787	−1.2	1775	2.7	2419	1.5	Expressed protein
254210_at	At4g23450	59	2.1	38	−1.1	104	1.6	55	−1.1	Zinc finger (C3HC4-type RING finger) family protein
256451_s_at	No gene	135	2.1	102	1.1	188	1.9	171	1.1	SEC14 cytosolic factor family protein (At1g75170/At5g04780)
256751_at	At3g27170	283	2.1	167	−1.1	298	1.9	239	−1.2	Chloride channel protein (*CLC-b*)
256816_at	At3g21400	136	2.1	135	1.1	126	2.3	112	−1.1	Expressed protein
258315_at	At3g16175	53	2.1	28	1.0	75	2.0	71	1.5	Thioesterase family protein
259076_at	At3g02140	26	2.1	48	1.4	43	3.8	48	1.6	Expressed protein
267624_at	At2g39660	250	2.1	427	1.2	260	1.8	325	1.4	Protein kinase, putative
245090_at	At2g40900	190	2.0	159	1.0	227	1.6	273	−1.3	Nodulin MtN21 family protein
246017_at	At5g10730	209	2.0	199	1.0	195	2.2	169	1.0	Expressed protein
247779_at	At5g58760	134	2.0	73	−1.1	174	2.7	59	1.6	WD40 repeat family protein
248436_at	At5g51220	295	2.0	319	1.0	288	2.0	260	1.1	Ubiquinol-cytochrome c chaperone family protein
250028_at	At5g18130	207	2.0	132	1.4	261	2.0	163	1.1	Expressed protein
250350_at	At5g12010	425	2.0	321	1.0	534	2.0	455	1.0	Expressed protein
255259_at	At4g05020	203	2.0	137	1.2	281	2.2	411	1.2	NADH dehydrogenase-related
257212_at	At3g15090	240	2.0	212	−1.1	272	2.0	123	1.2	Oxidoreductase, zinc binding dehydrogenase family protein
258752_at	At3g09520	54	2.0	67	1.2	72	1.8	79	1.1	Exocyst subunit EXO70 family protein
260153_at	At1g52760	594	2.0	409	−1.1	231	1.4	254	−1.4	Esterase/lipase/thioesterase family protein
247716_at	At5g59350	254	1.9	194	−1.1	348	2.0	299	1.5	Expressed protein
247955_at	At5g56950	321	1.9	327	−1.1	130	2.1	97	1.2	Nucleosome assembly protein (NAP), putative
262945_at	At1g79510	608	1.9	659	1.0	706	2.0	518	1.4	Expressed protein
263787_at	At2g46420	329	1.9	238	−1.1	337	2.1	155	−1.1	Expressed protein
247946_at	At5g57180	351	1.8	283	1.1	306	2.1	335	1.8	Expressed protein
256140_at	At1g48650	322	1.7	304	1.0	261	2.2	248	1.2	Helicase domain-containing protein
259952_at	At1g71400	151	1.5	178	1.3	109	2.3	103	1.5	Disease resistance family protein/LRR family protein
263403_at	At2g04040	113	1.5	160	1.4	47	3.2	94	2.2	MATE efflux family protein
265193_at	At1g05070	362	1.4	345	1.0	398	2.2	350	1.3	Expressed protein
266732_at	At2g03240	192	1.4	162	1.1	250	2.1	199	1.0	EXS family protein/ERD1/XPR1/SYG1 family protein
264261_at	At1g09240	374	1.3	903	1.0	137	2.4	551	−1.1	Nicotianamine synthase, putative

All data shown are from two biological replicates. Control expression level (Expr.) is the average of two array hybridizations under a 327-nm cutoff filter (i.e. −UV-B control). Fold induction (Fold) depicts the ratio of expression levels +UV-B/−UV-B (i.e., WG305/WG327).

time frame as nuclear exclusion happens in the dark-to-light transition (~24 h) (von Arnim and Deng, 1994). We note here that we did not detect any apparent changes in either the number or the size of the inclusion bodies that are formed by cytoplasmic COP1 (see, for example, von Arnim and Deng, 1994), and we confirmed the nuclear accumulation by staining DNA with Hoechst dye (Figure 6B).

In the dark, HY5 is a substrate of COP1 in the nucleus, leading to its ubiquitination and subsequent proteasomal degradation, so we were interested in determining HY5 protein levels under

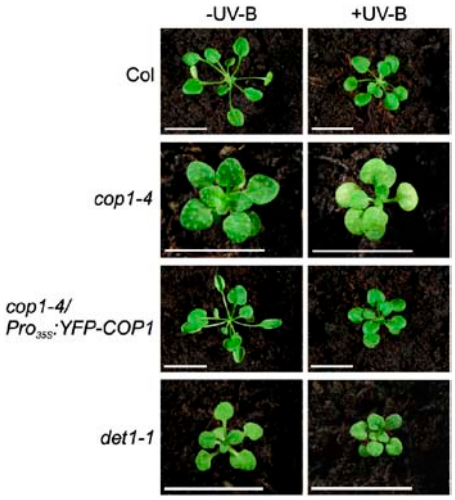

Figure 3. *cop1* Plants Develop Chlorosis under Supplementary UV-B.

Wild-type, *cop1-4*, *cop1-4*/*Pro*$_{35S}$*:YFP-COP1*, and *det1-1* plants were grown in white light with or without supplementary UV-B. Plants were photographed after 5 weeks. Under supplementary UV-B, 37 plants developed chlorosis among the *cop1-4* mutant, whereas among the parallel-grown wild type, *cop1-4*/*Pro*$_{35S}$*:YFP-COP1*, and *det1-1* mutant, no plant was chlorotic ($n = 50$). When control plants from all four genotypes were grown without supplementary UV-B, all survived without apparent chlorosis. Bars = 1 cm.

UV-B conditions when high levels of functional YFP-COP1 are present in the nucleus (Figure 6B). Indeed, we found that endogenous HY5 protein still keeps accumulating in *Pro*$_{35S}$*:YFP-COP1* lines under UV-B to levels similar to the wild type (Figure 6C). As expected from previous work (Oyama et al., 1997) and its function as a transcriptional regulator, the *HY5* promoter-driven HY5-YFP fusion protein accumulates in the nucleus under white light supplemented with UV-B, similar to COP1 (Figure 6D). This observation demonstrates that both of these crucial positive regulators of UV-B responses in *Arabidopsis* act in the nucleus. Thus, our data indicate that the promotion of HY5 degradation by YFP-COP1 is hindered under visible light with supplementary UV-B, even though both proteins reside in the nucleus.

The Function of COP1 in the UV-B Response Is Independent of SPA1 to SPA4 Proteins

The four members of the SPA family of proteins interact with COP1 and are essential for the suppression of photomorphogenesis (Laubinger et al., 2004; Hoecker, 2005). In all responses described so far, SPA proteins seem required for COP1 function (Laubinger et al., 2004). To find out if SPA proteins are also required for COP1 function in the UV-B response, we analyzed UV-B–responsive gene activation in a *spa1 spa2 spa3 spa4* quadruple mutant. In contrast with *cop1-1* and *cop1-4* mutants, UV-B induction of *HY5* and other tested UV-B–responsive genes is not impaired in the *spa* quadruple mutant (Figure 7). This indicates that the SPA proteins are not required; thus, COP1 can function properly without SPA proteins in the UV-B pathway.

DISCUSSION

This study identified COP1 as a crucial promoter of the photomorphogenic UV-B response in *Arabidopsis*. We show that physiological and molecular UV-B responses are impaired in *cop1* mutants and that COP1 accumulates in the nucleus under UV-B. At least part of the UV-B function of COP1 seems to involve HY5, a previously identified positive regulator of UV-B–induced gene expression (Ulm et al., 2004; Brown et al., 2005). Our observations are in sharp contrast with the expectation of a negative regulatory role for COP1 in the control of HY5 protein level as occurs in the dark and in visible light and suggest a different type of relationship between these two proteins in UV-B signaling, with COP1 partly acting through HY5.

COP1 Regulation of UV-B–Dependent Transcription and Photomorphogenesis

Microarray analysis of dark-grown *cop1* mutants revealed an expression profile resembling light-grown wild-type plants, in agreement with their photomorphogenic phenotype in dark (Ma et al., 2002). Moreover, in agreement with genetic and biochemical data, the gene expression regulation attributable to HY5 in the light was included largely within those genes regulated by COP1 in the dark (Ma et al., 2002). Thus, this previous genomic analysis substantiated the genetically defined function of COP1 as a repressor of photomorphogenesis controlling the degradation of transcription factors and thereby the expression of their target genes. It was concluded that the bulk of light-controlled gene expression in wild-type *Arabidopsis* plants could be accounted for by the negative regulation of COP1 activity (Ma et al., 2002).

Using whole-genome profiling, we found that COP1 is responsible for the UV-B–mediated activation of at least three-quarters of the responsive genes in the wild type. Thus, the greater part of UV-B–controlled gene expression in the wild type requires COP1 as a positive regulator. Most interestingly, the broad impact on early gene activation indicates a COP1 function close to the postulated UV-B photoreceptor, similarly to visible light signaling that involves direct interaction of the COP1 protein with cryptochrome and phytochrome photoreceptors (Wang et al., 2001; Yang et al., 2001; Seo et al., 2004).

Target genes of the UV-B pathway through COP1 include *HY5* and *CHS*. As a consequence, the *cop1-4* mutant is impaired in the accumulation of flavonoids and the establishment of tolerance to UV-B. By contrast, under white light, *cop1* mutants display elevated basal levels of *CHS* transcript and flavonoids compared with the wild type (Figures 4B and 4C), which are potential sunscreen metabolites that may interfere with the UV-B response (Li et al., 1993). The phenotype of elevated flavonoid levels in *cop1-4* is shared with *det1-1*, *cop1*^{eid6}, and the *spa* quadruple mutant (Figure 4B; Laubinger et al., 2004). However, whereas *cop1-4* is broadly impaired in UV-B responses, *cop1*^{eid6}, *det1-1*, and the *spa* quadruple mutant behave comparable to the wild type, indicating that the elevated flavonoid levels are not responsible for the observed UV-B phenotype of *cop1-4*. This conclusion is supported by the fact that molecular UV-B responses in double mutants of *cop1-4* and a *chs* knockout mutant

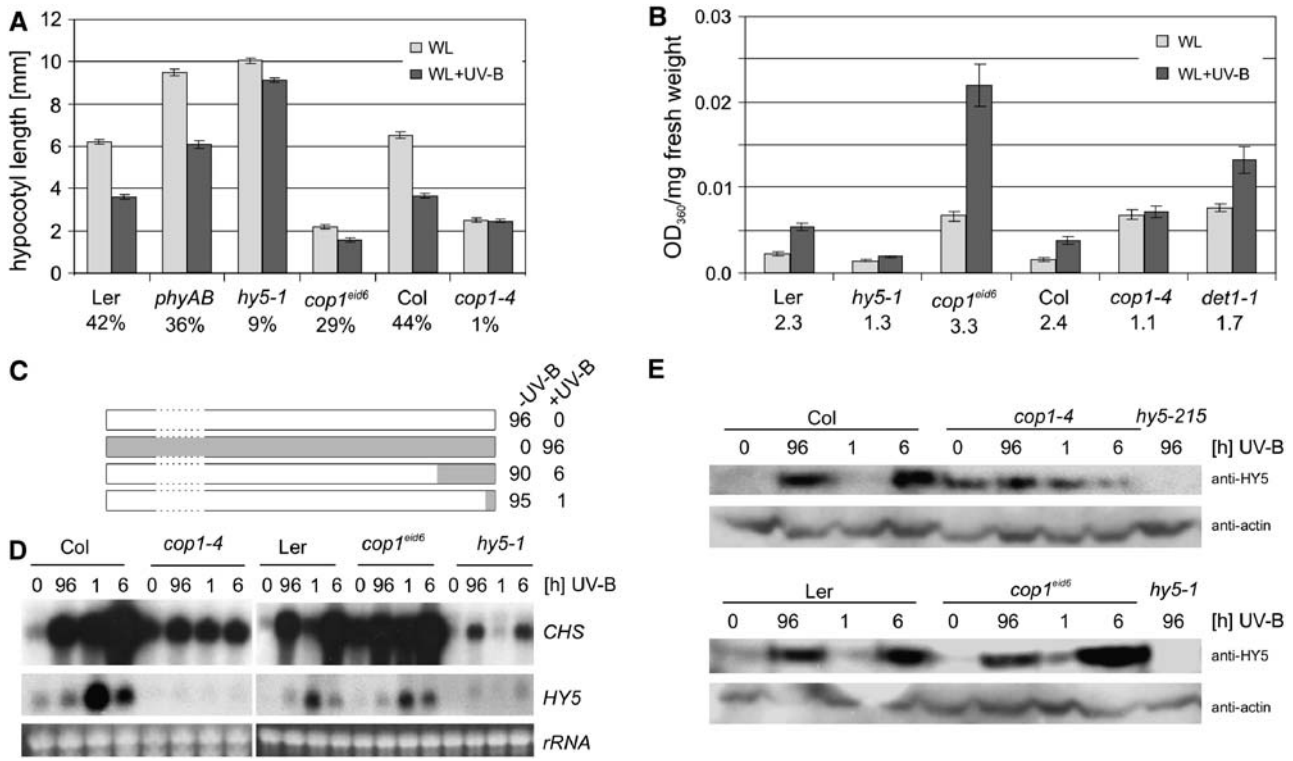

Figure 4. Functional HY5 and COP1 Are Required for UV-B–Responsive Flavonoid Accumulation, Correlating with HY5 Induction Properties.

Seedlings were grown for 4 d under continuous light supplemented with UV-B under a 327-nm cutoff filter (−UV-B) or 305-nm cutoff filter (+UV-B). The 327-nm cutoff filters were exchanged after 4 d for a 305-nm cutoff at 1 h and 6 h before harvesting, where indicated.

(A) Reduced hypocotyl growth inhibition by supplementary UV-B in *hy5-1*. Hypocotyl length was measured from seedlings of *phyA-201 phyB-5*, *hy5-1*, *cop1*^{eid6}, and *cop1-4* mutants, together with their wild types (L*er* and Col), after 4 d of growth under white light (WL) or white light supplemented with UV-B (WL+UV-B). Numbers below bars show the relative hypocotyl growth inhibition by UV-B as a percentage. Bars show SE of the mean ($n = 120$).

(B) UV-B–induced flavonoid accumulation is impaired in *cop1-4* and *hy5-1* mutants but not in *cop1*^{eid6}. Numbers below bars show the fold enrichment values of supplemental +UV-B compared with −UV-B. Bars show SE of the mean ($n = 9$).

(C) Scheme for UV-B treatment. The time under a WG327 cutoff filter (−UV-B) is represented by a white bar and time under a WG305 cutoff filter (+UV-B) by a gray bar. Time is indicated in hours.

(D) Corresponding RNA gel blot showing UV-B–responsive *CHS* and *HY5* gene expression.

(E) UV-B–induced HY5 protein accumulation is impaired in *cop1-4* but not in *cop1*^{eid6}. The protein gel blot was sequentially probed with anti-HY5 and anti-actin antibodies.

(*cop1-4 tt4*; *TT4* = *CHS*) and *cop1-4 TT4* siblings show a similar defect in UV-B signaling (see Supplemental Figure 1 online).

From our results, it is clear that COP1 plays a crucial role in the UV-B response of *Arabidopsis*. Namely, COP1 was found to play a positive regulatory role after UV-B perception. Similarly, a novel function of COP1 as promoter of responses to red light was described recently (Boccalandro et al., 2004), suggesting that COP1 may play opposing roles depending on light conditions. These observations contrast with the function as a repressor of photomorphogenesis (e.g., McNellis et al., 1994a).

COP1 Accumulates in the Nucleus under UV-B Radiation

The subcellular distribution of COP1 is adjusted according to light conditions, with nuclear localization in dark and a drastic reduction of COP1 in the nucleus in light. This light-dependent change in the nuclear abundance of COP1 is a relatively slow process taking ~24 h (von Arnim and Deng, 1994; Yi and Deng, 2005). Nevertheless, recent mutagenesis studies indicated that light-dependent nuclear–cytoplasmic repartitioning of the COP1 protein is a rate-limiting step in light signal transduction (Subramanian et al., 2004).

We show that supplementary UV-B triggers nuclear accumulation of the YFP-COP1 protein, whereas, as expected, filtering out the UV-B radiation results in nuclear exclusion of YFP-COP1. As in white light, the nucleocytoplasmic repartitioning of COP1 under supplementary UV-B is a rather slow process (at least at the detection level), consistently detectable after ~24 h. In both cases, this contrasts with early gene expression changes and the rapid kinetics of target protein stabilization (Osterlund et al., 2000; Duek et al., 2004; Yang et al., 2005). Thus, light seems to impede on COP1-mediated degradation of HFR1, HY5, and other regulatory factors prior to subcellular partitioning. It follows that a mechanism other than nucleocytoplasmic repartitioning

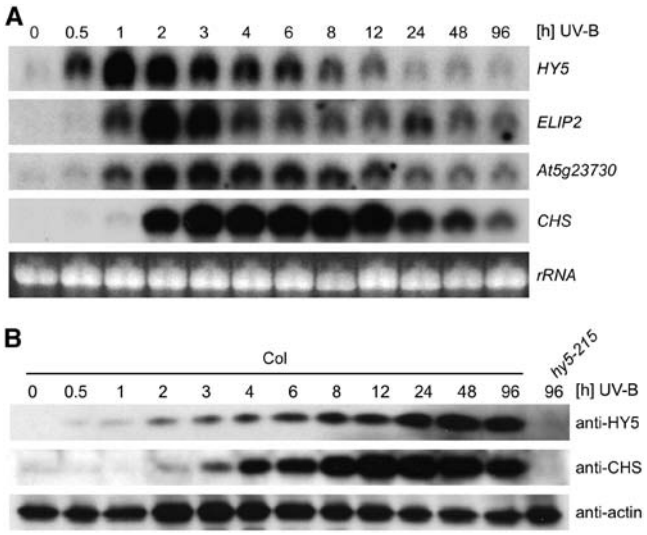

Figure 5. UV-B–Mediated *HY5* Gene Activation and HY5 Protein Accumulation Correlates with Induction of Putative HY5 Target Genes.

(A) RNA gel blot analysis of the wild type (Col). Total RNA was isolated from 4-d-old wild-type seedlings (Col) that were irradiated with UV-B for the indicated times before harvesting. Blots were sequentially hybridized with specific probes for the indicated genes. Ethidium bromide–stained rRNA is shown as loading control.

(B) Corresponding UV-B–responsive accumulation of HY5 and CHS protein in the Col wild type. The protein gel blot was sequentially probed with anti-HY5, anti-CHS, and anti-actin antibodies.

must operate to rapidly regulate COP1 activity. It can, however, be speculated that the change in the localization of COP1 might represent an adaptive long-term response and could also modulate other signaling pathways employing COP1. Notwithstanding this, our data indicate that, similar to white light, the cellular localization of COP1 may play a critical role in mediating UV-B photoreceptor-controlled development in *Arabidopsis*.

It is important to note here that expression of YFP-COP1 in *cop1-4* fully complements the mutant growth phenotype and restores HY5 degradation in dark, indicating that the fusion protein is active as an E3 ubiquitin ligase. Interestingly, we show that UV-B–mediated HY5 accumulation under supplementary UV-B is comparable to the wild type in lines overexpressing YFP-COP1. Because under these conditions both YFP-COP1 and HY5 are localized to the nucleus, the COP1 ubiquitin ligase activity against HY5 must be hindered. This may be due to an alteration of COP1 E3 ligase activity, protein modification influencing target affinity, or association with regulatory proteins.

The UV-B Photomorphogenic Response Is Mechanistically Separate from Light Responses

All *cop1* mutants have an exaggerated photomorphogenic development in the light (Yi and Deng, 2005). Connected to this phenotype are a number of genes that are overexpressed in *cop1* mutants compared with the wild type grown in light, indicating that *cop1* is blocked in the repression of a set of genes (Ma et al., 2003). Thus, it could be hypothesized that supplementary UV-B results in an inactivation of COP1 releasing gene repression. Under the conditions used for our microarray analysis, we identified 575 genes overexpressed in *cop1-4* grown without supplementary UV-B, but only 11 of those overlap with the UV-B–induced genes in the wild type (see Supplemental Table 4 online). Thus, a simple release of repression of light-regulated genes does not seem to be at work in COP1-mediated UV-B signaling.

In contrast with all other *cop1* mutants presently known, the *cop1^{eid6}* allele displays no constitutive photomorphogenic phenotype in darkness. Therefore, it was concluded that the *cop1^{eid6}* allele encodes for a protein whose remaining activity is sufficient for the suppression of photomorphogenesis in dark-grown plants (Dieterle et al., 2003). Importantly, the *cop1^{eid6}* mutant exhibits an extreme hypersensitivity toward all tested light qualities, resulting in an exaggerated photomorphogenic development in the light comparable to other *cop1* mutants, such as *cop1-4* (Dieterle et al., 2003). In contrast with *cop1-4*, however, the *cop1^{eid6}* seedlings seem not affected in the UV-B response leading to *HY5* and *CHS* gene activation and flavonoid accumulation. We conclude that responses to visible light and UV-B have different structural requirements for the COP1 protein.

Next to COP1, the light-regulated HY5 protein degradation via the ubiquitin/26S proteasome system also requires other COP/DET/FUS proteins (Osterlund et al., 2000). One group of proteins that might now be classified as belonging to the COP/DET/FUS class is the SPA proteins. The four SPA proteins interact with COP1 and regulate its activity (Saijo et al., 2003; Seo et al., 2003; Laubinger et al., 2004; Hoecker, 2005). They act partially redundantly, and a quadruple mutant is almost indistinguishable from the strong *cop1-1* allele in the dark and under visible light (Laubinger et al., 2004; our unpublished data). To test whether these proteins also have an overlapping function in UV-B signaling, we investigated selected UV-B phenotypes in a *spa* quadruple mutant (Laubinger et al., 2004). The apparently functional UV-B response in the *spa* quadruple mutant indicates a specific involvement of COP1, independent of its regulatory proteins of the SPA family. This is particularly striking as the *spa* quadruple mutant has a much stronger light phenotype than the weak *cop1-4* mutant that we widely used. It should be noted that *cop1-4* may not be considered a weak allele in UV-B signaling, as the UV-B responsiveness of its target genes seems completely abrogated. Taken together, our data, including our observations on the *cop1^{eid6}* allele, allow clear genetic separation of a COP1 function in UV-B from visible light signaling, in agreement with a specialized and novel function of COP1 in the UV-B response.

Relationship between COP1 and HY5 in the Regulation of UV-B–Dependent Transcription and UV-B Tolerance

The HY5 protein is required for the establishment of UV-B tolerance, as demonstrated by the strongly reduced survival of *hy5* mutants under UV-B radiation, which can be attributed to the lack of the activation of genes encoding proteins required to establish UV-B tolerance (Brown et al., 2005; Figure 1). It is interesting to note that RNA interference–mediated *HY5* knockdown in tomato (*Solanum lycopersicum*) results in cell death phenotypes at various developmental stages of field-grown

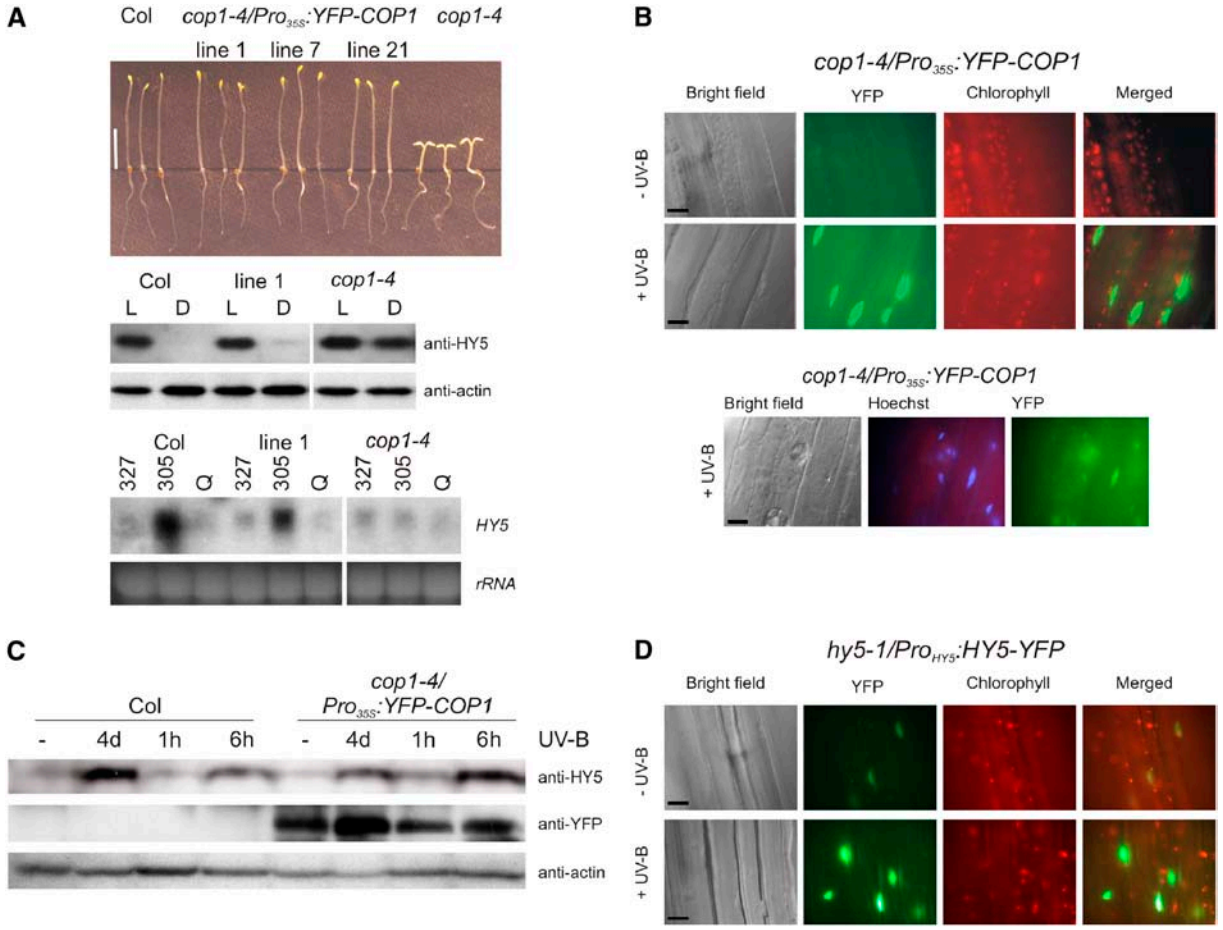

Figure 6. YFP-COP1 Localizes to the Nucleus in Response to UV-B, Not Affecting HY5 Accumulation.

(A) The YFP-COP1 fusion protein is functional and complements the *cop1-4* mutant phenotype. Top: after 6 h of light treatment to synchronize germination, the seedlings were grown for 4 d in the dark before the photograph was taken. Middle: 4-d-old seedlings grown under constant light were either kept in light (L) or transferred to dark (D) for 24 h. The protein gel blot was sequentially analyzed with anti-HY5 and anti-actin antibodies. Bottom: RNA gel blot. Total RNA was isolated from 7-d-old white light–grown seedlings at 45 min after 15-min irradiation under a UV-B field with different UV spectra (−UV-B under cutoff WG327; +UV-B with decreasing short-wave cutoff under WG305 or quartz glass [Q]). Ethidium bromide–stained rRNA is shown as loading control.

(B) to **(D)** Seedlings were grown for 4 d under continuous light supplemented with UV-B under a 327-nm cutoff filter (−UV-B) or 305-nm cutoff (+UV-B). The 327-nm cutoff filters were exchanged after 4 d for a 305-nm cutoff at 1 h and 6 h before harvesting, as indicated **(C)**.

(B) UV-B–responsive accumulation of YFP-COP1 in the nucleus. Nuclear accumulation is confirmed by Hoechst 33342 nuclear stain (bottom panel). Bars = 20 μm.

(C) Accumulation of YFP-COP1 still allows UV-B–induced HY5 protein accumulation. The protein gel blot was sequentially analyzed with anti-HY5, anti-YFP, and anti-actin antibodies.

(D) UV-B–responsive accumulation of HY5-YFP in the nucleus. Bars = 20 μm.

plants (Liu et al., 2004). As speculated before (Liu et al., 2004; Ulm and Nagy, 2005), this phenotype may possibly be due to UV-B hypersensitivity in these plants. Thus, the UV-B signaling pathway culminating in HY5 activation is required for survival under UV-B radiation. Indeed, we found reduced tolerance of *cop1-4* in contrast with the wild type as well, seen as chlorosis under supplementary UV-B. In contrast with *hy5*, however, the UV-B sensitivity of *cop1* mutants was milder and did not result in lethality under the conditions used in our sensitivity assays. This difference may be due to a constitutively elevated level of UV-B tolerance components in the *cop1* mutant due to its stronger light response. Such a higher preformed defense against UV-B radiation is suggested by higher *CHS* gene expression and flavonoid levels that function as sunscreen. As this is also true for *det1-1* mutant plants, the difference in UV-B tolerance between *cop1-4* and *det1-1* suggests that a block in the UV-B–induced pathways contributes to tolerance even under these circumstances. It should also be noted that whereas *hy5-1* is a null mutant, *cop1-4* still expresses the N-terminal part of the protein, even though to a much reduced level (McNellis et al., 1994b). Thus, it cannot be

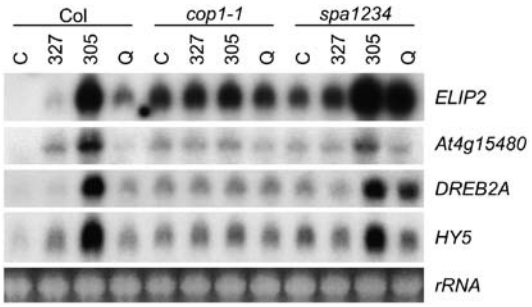

Figure 7. COP1 Function in the UV-B Response Is Independent of SPA Proteins.

RNA gel blot analysis of the *spa1234* quadruple mutant and the *cop1-1* single mutant in comparison with the wild type (Col). Total RNA was isolated from 7-d-old white light–grown seedlings at 45 min after 15-min irradiation under a UV-B field with different UV spectra (−UV-B under cutoff WG327; +UV-B with decreasing short-wave cutoff under WG305 or quartz glass [Q]) or left untreated in the standard growth chamber (control [C]). Blots were sequentially hybridized with specific probes for the indicated genes. Ethidium bromide–stained rRNA is shown as loading control.

formally excluded that the lesser sensitivity of *cop1-4* compared with *hy5-1* is because of a retained function of the COP1-4 truncated protein in UV-B signaling that is independent of early UV-B–induced gene expression. Independent of this, the interpretation of the UV-B tolerance assays is complicated by the mutant phenotypes that might contribute to survival under UV-B, most apparent in the flavonoid content differences. A careful evaluation of the contributions of the preformed and activated UV-B defense mechanisms will be required to dissect the specificities of the different pathways contributing to plant UV-B tolerance.

Our data imply that transcriptional activation and reaccumulation of HY5 following UV-B perception is required for HY5 to fulfil its UV-B signaling function. However, even though 35S promoter–driven overexpression of HY5 complements *hy5* mutant phenotypes under both visible and UV-B light, it does not result in an increased responsiveness to UV-B (data not shown), similar to the case in response to visible light (Ang et al., 1998). Ang et al. (1998) noted that HY5 lacks the Pro- and acid-rich activation domain of several well-characterized bZIP transcription factors and does not activate transcription in yeast by itself. We thus anticipate that HY5 requires interacting partner(s) that are concomitantly induced by UV-B. The identification of these proteins will be aided by the genome-wide UV-B expression profile and will further our understanding of UV-B–responsive gene regulation. Independent of this hypothesis, the temporal relationship with *HY5* activation preceding upregulation of *CHS* mRNA levels is in agreement with a direct relationship supported by HY5 binding to *CHS* promoter elements (Ang et al., 1998).

HY5 was previously described to be required only for the first few days of seedling development in light. This notion was supported by the sharp decrease in HY5 protein levels early after germination in light (Hardtke et al., 2000). Here, we position COP1 into the previously postulated UV-B photoreceptor pathway as an early component upstream of HY5 (Ulm et al., 2004). Thus, in the UV-B response, COP1 is required to relieve HY5 downregulation established during early seedling development, resulting in the reaccumulation of HY5 protein. Therefore, the UV-B function of COP1 might involve the degradation of presently unknown negative regulator(s) of UV-B responses (e.g., a repressor of *HY5* transcription) (Figure 8). Transcriptional regulation through degradation of repressor complexes directly at the promoter was observed for some E3 ligases in mammalian development (Perissi et al., 2004). Alternatively, COP1 may function independently of protein degradation. In agreement with this, human COP1 was reported to repress the transcription activity of c-Jun independently of proteolysis (Bianchi et al., 2003).

Recently, another UV-B pathway component, UVR8, was described to be required for *HY5* gene activation (Brown et al., 2005). In agreement, we have also isolated several new alleles of *uvr8* in our Pro_{HY5}*:Luc*$^{+}$ genetic screen (our unpublished data). UVR8 binds histones in vitro, and green 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). Thus, the functional relationship between COP1 and UVR8 in regulating *HY5* gene expression remains to be determined. Interestingly, however, mutations in both *COP1* and *UVR8* completely block *HY5* activation, indicating that they may function in the same genetic pathway.

In summary, the data presented here describe a crucial role of COP1 as a positive regulator in the UV-B response of *Arabidopsis* (see Figure 8 for a working model). Early UV-B photoreceptor-responsive gene expression changes in *Arabidopsis* are to a large extent COP1 dependent. The COP1 UV-B pathway branches into a HY5-dependent and a HY5-independent part, with HY5 required for UV-B tolerance. Altogether, our data position COP1 as an integration node downstream of the postulated UV-B

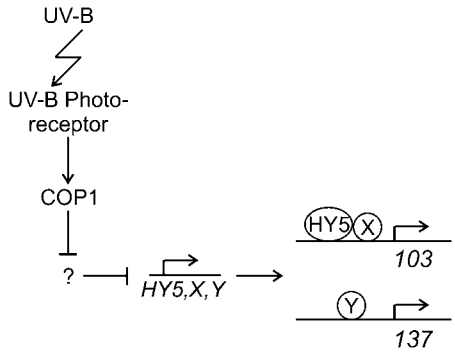

Figure 8. Working Model of an Early Function of COP1 and HY5 in UV-B Signaling.

We propose that UV-B perceived by the postulated UV-B photoreceptor activates COP1 to degrade a repressor of HY5 and other UV-B–responsive genes, including yet unidentified transcription factors X and Y. These transcription factors will act in concert to regulate gene expression, leading to a photomorphogenic UV-B response. As determined by microarray analysis, the 103 COP1- and HY5-dependent and 137 COP1-dependent but HY5-independent genes are indicated (Figure 2D; see Supplemental Table 3 online).

photoreceptor(s), similar to the case in phytochrome and cryptochrome signaling pathways, but with a distinct mode of action.

In view of structural and functional conservation, 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 UV-B–mediated disruption of COP1 interaction with c-Jun and major vault protein in cultured kidney cells (Yi et al., 2005). In contrast with *Saccharomyces*, *Drosophila*, and *Caenorhabditis* that lack obvious COP1 orthologs (Yi and Deng, 2005), *Arabidopsis* allows the genetic dissection of COP1 function in UV-B responses, which eventually might be transferred to other plant and possibly mammalian organisms.

METHODS

Plant Material and Growth Conditions

cop1-1, *cop1-4*, *det1-1*, and *spa1-3 spa2-1 spa3-1 spa4-1* are in the Col ecotype (McNellis et al., 1994b; Pepper et al., 1994; Laubinger et al., 2004), and the Pro_{HY5}:*Luc*$^+$ transgene is in Ws (Ulm et al., 2004), whereas *phyA-201 phyB-5*, *cop1*^{eid6}, and *hy5-1* are in L*er* (Reed et al., 1994; Oyama et al., 1997; Dieterle et al., 2003). Plants were grown exactly as described previously (Ulm et al., 2004).

The *COP1* open reading frame was cloned in frame to the C terminus of the YFP coding sequence under the control of the cauliflower mosaic virus 35S promoter in a pPCV-based binary vector. The genomic clone of *HY5*, including its promoter (759 bp upstream of the translational start ATG), was cloned in frame to the N terminus of YFP. The constructs were verified by sequencing, and *Arabidopsis thaliana* plants were transformed by *Agrobacterium tumefaciens* using the floral dip method (Clough and Bent, 1998). The resulting transgenic lines described in this work were genetically determined to have the transgene integrated at a single locus.

UV-B Irradiation

UV-B irradiation (15 min) for transcriptional responses was conducted with broadband UV-B lamps (Philips TL40W/12RS) exactly as described previously (Ulm et al., 2004).

For flavonoid induction, hypocotyl growth inhibition, and corresponding RNA gel blot analysis, plants were grown under continuous irradiation in a white-light field with Osram L18W/30 tubes (3.6 μmol m^{-2} s^{-1}; measured with a LI-250 Light Meter; LI-COR Biosciences) supplemented with Philips TL20W/01RS narrowband UV-B tubes (1.5 μmol m^{-2} s^{-1}; measured with a VLX-3W UV Light Meter equipped with a CX-312 sensor; Vilber Lourmat). The UV-B range was modulated by the use of 3-mm transmission cutoff filters of the WG series with half-maximal transmission at the indicated wavelength (WG295, WG305, WG327, and WG345; Schott Glaswerke) or unfiltered through a 3-mm quartz plate, as described previously (Ulm et al., 2004).

For UV-B sensitivity assays, 50 seeds were sown onto soil with equal spacing in each pot, stratified at 4°C for 4 d, and then transferred to a standard growth chamber (Controlled Environments) with 9-h-dark/15-h-light cycle, 24°C, and 80% relative humidity. After 4 d, the seedlings were grown further under a UV-B field in the same chamber consisting of four warm white light tubes (Osram L18W/30) and two narrow-band UV-B tubes (Philips TL20W/01RS). The fluence rate of the white light was 60 μmol m^{-2} s^{-1}, and UV-B irradiance was 2.6 μmol m^{-2} s^{-1}. The incident light was filtered through a layer of 3-mm 305-nm cutoff filters (half-maximal transmission at 305 nm) for UV-B–treated pots, giving 1.6 μmol m^{-2} s^{-1} irradiance at 312 nm, whereas additional 327-nm cutoff filters (half-maximal transmission at 327 nm) were used for the control pots.

Gene Expression Analysis

Arabidopsis RNA was isolated with the Plant RNeasy kit (Qiagen) according to the manufacturer's instructions. Gene-specific probes were amplified by PCR from *Arabidopsis* cDNA using the following primers: At1g19020 (5′-ATACAATAATGAACGGAACA-3′ and 5′-AACTCGAACAATTTACCACA-3′), At1g32870 (5′-GCTCAAGAGGTGTGGTGG-3′ and 5′-GAAGGAACAGGGTTTAGG-3′), *HYH* (5′-ATGTCTCTCCAACGACCC-3′ and 5′-TTAGTGATTGTCATCAGT-3′), *ELIP2* (5′-CAACTCCATCTCACTTCTC-3′ and 5′-TCACACTGTTTAAAACTC-3′), At4g15480 (5′-CTCTGTTTCATTCCCTAC-3′ and 5′-CACAATCATCCCTTTACC-3′), *DREB2A* (5′-AGAGGAGTTAGGCAAAGG-3′ and 5′-GTTGAGGCTTTGTAGCGG-3′), *HY5* (5′-ATGCAGGAACAAGCGACT-3′ and 5′-TCAAAGGCTTGCATCAGC-3′), *CHS* (5′-CCTCAAGGAAAACCCACAC-3′ and 5′-CACTGAAAAGAGCCTGACC-3′), At5g23730 (5′-CCGGCGAAACTTAGTAGTC-3′ and 5′-CTTGAAGAAAGTCATTCCCA-3′), and *ZAT12* (5′-ATCATCACAACTACTATC-3′ and 5′-ACAAATCTCCAATGCTAC-3′). The PCR fragments were cloned into the pCR2.1-TOPO vector (Invitrogen) and verified by sequencing. RNA gel blot analysis was performed as described previously (Ulm et al., 2004).

For the microarray analysis, a 10-μg aliquot of total RNA was reverse transcribed using the Affymetrix cDNA synthesis kit according to the manufacturer's instructions (Affymetrix). The oligonucleotide used for priming was 5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(T)24-3′ (Genset Oligo) as recommended by Affymetrix. Double-stranded cDNA was purified by phenol:chloroform extraction, and the aqueous phase was removed by centrifugation through Phase-lock Gel (Eppendorf). The cDNA was then in vitro transcribed in the presence of biotinylated UTP to give labeled cRNA using the Affymetrix IVT kit. The cRNA was purified using RNAeasy cleanup columns (Qiagen). To improve the recovery from the columns, the elution water was spun into the matrix at 27*g* and then left to stand for 1 min prior to the standard 8000*g* centrifugation recommended by Qiagen. The cRNA was fragmented by heating in 1× fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM KOAc, and 30 mM MgOAc) as recommended by Affymetrix. Ten micrograms of the fragmented cRNA was hybridized to ATH1-120501 GeneChips (Affymetrix) using the standard procedure specified by the manufacturer (45°C; 16 h). Hybridization was controlled using the GeneChip eukaryotic hybridization control kit (Affymetrix). Washing and staining were performed in an Affymetrix Fluidics Station 450, and the samples were scanned in an Affymetrix GeneChip 3000 scanner according to the manufacturer's instructions. Expression values were estimated using the Robust Multichip Average algorithm (Irizarry et al., 2003) as implemented in the RMAExpress software package (http://stat-www.berkeley.edu/~bolstad/RMAExpress/RMAExpress.html). The expression analysis was performed using GeneSpring 6.2 (Silicon Genetics). Changes in gene expression were identified by requiring that a gene reaches a minimum expression level of 50 in at least one condition, change in expression level by at least twofold relative to the UV-B control in one or more UV-B–treated samples, and pass a one-way analysis of variance ($P < 0.05$) with a Benjamini and Hochberg false discovery rate multiple-testing correction. The origin of the significant changes was estimated by performing a Tukey post hoc analysis, and the resulting gene lists were saved for further analysis.

Luciferase Activity Measurements

Pro_{HY5}:*Luc*$^+$ transgenic seedlings were grown for 6 d under axenic standard conditions and transferred to 96-well plates containing Murashige and Skoog medium (Sigma-Aldrich) with 1% sucrose and 0.8% agar. Twenty microliters of 2.5 mM luciferin (Duchefa Biochemie) was added to each well and then the plants were put back into the standard growth chamber overnight. The next day, the plants were irradiated with UV-B for 15 min as described above, before luminescence was measured in a

TopCount Microplate Scintillation and Luminescence Counter (Packard BioScience).

Flavonoid and Hypocotyl Measurements

For each flavonoid measurement, triplicates of 50 4-d-old seedlings were counted and snap frozen in liquid nitrogen. Flavonoids were extracted according to Kucera et al. (2003). For hypocotyl growth inhibition experiments, hypocotyl lengths of at least 30 seedlings were measured. Experiments were performed in at least three independent biological repetitions.

Antiserum Production and Immunoblot Analysis

Polyclonal HY5 antibodies (Eurogentec) were raised against recombinant His_6-tagged HY5 protein that was expressed in *Escherichia coli* from the pQE30 expression vector and purified by nickel-nitrilotriacetic acid metal-affinity chromatography according to the manufacturer's instructions (Qiagen).

For protein gel blot analysis, total cellular proteins (30 μg) were separated by electrophoresis in 12% SDS-polyacrylamide gel and electrophoretically transferred to a polyvinylidene difluoride membrane according to the manufacturer's instructions (Bio-Rad). We used polyclonal anti-HY5, polyclonal anti-CHS (Santa Cruz Biotechnology), and monoclonal anti-GFP (BAbCO) as primary antibodies, with horseradish peroxidase–conjugated anti-rabbit, anti-goat, and anti-mouse immunoglobulins (DAKO), respectively, as secondary antibodies. Signal detection was performed as described in the ECL Western detection kit (Amersham Biosciences). Equal loading was confirmed for each membrane by immunoblot analysis using an anti-actin (I-19) primary antibody generated against the C terminus of human actin that can be used to detect a broad range of actin isoforms in different organisms (Santa Cruz Biotechnology).

Microscopical Analysis

For epifluorescence and light microscopy, the seedlings were transferred to glass slides and analyzed with an Axioskop II microscope (Zeiss). Nuclear DNA staining was performed using Hoechst 33342 stain according to the manufacturer's instructions (Invitrogen). Excitation and detection of YFP were performed with a standard YFP filter set (AHF Analysentechnik). Documentation of representative cells was performed by photography during the first 2 min of microscopic analysis using a digital Axiocam camera system (Zeiss). Cellular localizations were confirmed in at least three independent experiments and three transgenic lines.

Accession Numbers

Affymetrix microarray expression data reported in this article have been deposited in the EMBL-EBI ArrayExpress database (accession no. E-MEXP-557). Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g19020, At1g32870, At2g32950 (*COP1*), At3g17609 (*HYH*), At4g14690 (*ELIP2*), At4g15480, At5g05410 (*DREB2A*), At5g11260 (*HY5*), At5g13930 (*CHS*), At5g23730, and At5g59820 (*ZAT12*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. UV-B–Mediated Gene Induction of the Marker Genes Tested Is Not Affected in *tt4* Mutants.

Supplemental Table 1. Comparison of Early, Low-Level UV-B–Induced Genes in the L*er* Wild Type and the *hy5-1* Mutant (Corresponding to Figure 1A).

Supplemental Table 2. Comparison of Early, Low-Level UV-B–Induced Genes in the Col Wild Type and the *cop1-4* Mutant (Corresponding to Figure 2C).

Supplemental Table 3. Comparison of Early, Low-Level UV-B–Induced Genes in *hy5-1*, *cop1-4*, and the Wild Type (Corresponding to Figure 2D).

Supplemental Table 4. Genes That Are Over- or Underexpressed in *cop1-4* Compared with the Col Wild Type under a 327-nm Cutoff Filter (−UV-B).

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