Plant photoreceptors and their signaling components compete for COP1 binding via VP peptide motifs

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Abstract

Plants sense different parts of the sun’s light spectrum using distinct photoreceptors, which signal through the E3 ubiquitin ligase COP1. Here, we analyze why many COP1-interacting transcription factors and photoreceptors harbor sequence-divergent Val-Pro (VP) motifs that bind COP1 with different binding affinities. Crystal structures of the VP motifs of the UV-B photoreceptor UVR8 and the transcription factor HY5 in complex with COP1, quantitative binding assays, and reverse genetic experiments together suggest that UVR8 and HY5 compete for COP1. Photoactivation of UVR8 leads to high-affinity cooperative binding of its VP motif and its photosensing core to COP1, preventing COP1 binding to its substrate HY5. UVR8–VP motif chimeras suggest that UV-B signaling specificity resides in the UVR8 photosensory core. Different COP1–VP peptide motif complexes highlight sequence fingerprints required for COP1 targeting. The blue-light photoreceptorsCRY1 and CRY2 also compete with transcription factors for COP1 binding using similar VP motifs. Thus, our work reveals that different photoreceptors and their signaling components compete for COP1 via a conserved mechanism to control different light signaling cascades.

Keywords COP1; E3 ubiquitin ligase; light signaling; UV-B receptor; UVR8

Subject Categories Plant Biology

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Introduction

Flowering plants etiolate in darkness, manifested by the rapid elongation of the embryonic stem, the hypocotyl, and closed and underdeveloped embryonic leaves, the cotyledons. Under light and upon photoreceptor activation, seedlings de-etiolate and display a photomorphogenic phenotype, characterized by a short hypocotyl and open green cotyledons, enabling a photosynthetic lifestyle (Gommers & Monte, 2018). The constitutively photomorphogenic 1 (cop1) mutant displays a light-grown phenotype in the dark, including a short hypocotyl, and open and expanded cotyledons. COP1 is thus a crucial repressor of photomorphogenesis (Deng et al., 1991). COP1 contains an N-terminal zinc finger, a central coiled-coil, and a C-terminal WD40 domain, which is essential for proper COP1 function (Deng et al., 1992; McNellis et al., 1994). Light-activated phytochrome, cryptochrome, and UVR8 photoreceptors inhibit COP1’s activity (von Arnim & Deng, 1994; Hoecker, 2017; Podolec & Ulm, 2018). Although COP1 can act as a stand-alone E3 ubiquitin ligase in vitro (Saijo et al., 2003; See et al., 2003), it forms higher-order complexes in vivo, for example, with SUPPRESSOR OF PHYA-105 (SPA) proteins (Hoecker & Quail, 2001; Zhu et al., 2008; Ordoñez-Herrera et al., 2015). COP1 can also act as a substrate adaptor in CULLIN4–DAMAGED DNA BINDING PROTEIN 1 (CUL4–DDB1)-based heteromeric E3 ubiquitin ligase complexes (Chen et al., 2010). These different complexes may finetune COP1’s activity toward different substrates (Ren et al., 2019). COP1 regulates gene expression and plays a central role as a repressor of photomorphogenesis by directly modulating the stability of transcription factors that control the expression of light-regulated genes (Lau & Deng, 2012; Podolec & Ulm, 2018). For example, the bZIP transcription factor ELONGATED HYPOCOTYL 5 (HY5) acts antagonistically with COP1 (Ang et al., 1998). COP1 binding to HY5 leads to its subsequent degradation via the 26S proteasome in darkness, a process that is inhibited by light (Osterlund et al., 2000).

In addition to HY5, other COP1 targets have been identified including transcriptional regulators, such as the HY5 homolog HYH (Holm et al., 2002), CONSTANS (CO) and other members of the BBX protein family (Jang et al., 2008; Liu et al., 2008; Khanna et al., 2009; Xu et al., 2016; Lin et al., 2018; Ordoñez-Herrera et al., 2018), and others such as LONG HYPOCOTYL IN FAR-RED 1 (HFR1) (Jang et al., 2005; Yang et al., 2005) and SHI-RELATED SEQUENCE 5 (SRSS) (Yuan et al., 2018). It has been suggested that specific Val-Pro (VP)-peptide motifs with a core sequence V-P-E/D-Φ-G, where Φ designated a hydrophobic residue, are able to bind the COP1...
...to interact with downstream transcription factors and upstream components suggests that COP1 may use a common targeting mechanism of COP1 substrates (Holm et al., 2003; Müller & Bouly, 2015). The WD40 domains of human and Arabidopsis COP1 directly interact with VP-containing peptides (Uljon et al., 2016). Such a VP peptide motif can be found in the UVr8 C-terminus that is not part of the UV-B-sensing β-propeller domain (Fig 1A; Kliebenstein et al., 2002; Rizzini et al., 2011; Christie et al., 2012; Wu et al., 2012), but is essential for UV-B signaling (Cloix et al., 2012; Yin et al., 2001). Indeed, CR1 and CR2 also contain potential VP peptide motifs within their CCT domains, but their function in blue-light signaling has not been established (Lin & Shalitin, 2003; Müller & Bouly, 2015). The presence of VP peptide motifs in different light signaling components suggests that COP1 may use a common targeting mechanism to interact with downstream transcription factors and upstream photoreceptors. Here, we present structural, quantitative biochemical, and genetic evidence for a VP peptide based competition mechanism, enabling COP1 to play a crucial role in different photoreceptor pathways in plants.

Results

The COP1 WD40 domain binds VP motifs from UVr8 and HY5

The WD40 domains of human and Arabidopsis COP1 directly interact with VP-containing peptides (Uljon et al., 2016). Such a VP peptide motif can be found in the UVr8 C-terminus that is not part of the UV-B-sensing β-propeller domain (Fig 1A; Kliebenstein et al., 2002; Rizzini et al., 2011; Christie et al., 2012; Wu et al., 2012), but is essential for UV-B signaling (Cloix et al., 2012; Yin et al., 2001). HY5 (Oyama et al., 1997), which is a COP1 target acting downstream of UVr8 in the UV-B signaling pathway (Ulm et al., 2004; Brown et al., 2005; Oravec et al., 2006; Binkert et al., 2014), also contains a VP peptide motif (Fig 1A; Holm et al., 2001). UV-B absorption leads to UVr8 monomerization, COP1 binding, and subsequent stabilization of HY5 (Favory et al., 2009; Rizzini et al., 2011; Huang et al., 2013). Mutation of the HY5 VP pair to alanine (AA) stabilizes the HY5 protein (Holm et al., 2001).

In order to compare how the VP peptide motifs from different plant light signaling components bind COP1, we quantified the interaction of the UVr8 and HY5 VP peptides with the recombinant Arabidopsis COP1 WD40 domain (termed COP1 thereafter) using isothermal titration calorimetry (ITC). We find that both peptides bind COP1 with micromolar affinity and with HY5~48 binding ~8 times stronger than UVr8~413 (Fig 1B). Next, we solved crystal structures of the COP1 WD40 domain–VP peptide complexes representing UVr8~413–COP1 and HY5~48–COP1 interactions, to 1.3 Å resolution (Fig 1C). Structural superposition of the two complexes (r.m.s.d. is ~0.2 Å comparing 149 corresponding Cα atoms) reveals an overall conserved mode of VP peptide binding (r.m.s.d is ~1.2 Å comparing six corresponding Cα atoms), with the central VP residues making hydrophobic interactions with COP1 Tyr467 and COP1 Pro597 (buried surface area is ~500 Å² in COP1; Fig 1D and Appendix Fig S1). COP1~422 and COP1~441 form hydrogen bonds and salt bridges with either UVr8 Tyr407 or HY5 Tyr41, both being anchored to the COP1 WD40 core (Fig 1C and D, and Appendix Fig S1), as previously seen for the corresponding TRIB1 Gin356 residue in the COP1–TRIB1 peptide complex (Uljon et al., 2016). In our HY5~48–COP1 structure, an additional salt bridge is formed between HY5 Glu45 and COP1 His528 (Fig 1D). In the peptides, the residues surrounding the VP core adopt different conformations in UVr8 and HY5, which may explain their different binding affinities (Fig 1B and C). We tested this by mutating residues Lys422, Tyr441, and Thr467 in the VP peptide binding pocket of COP1. Mutation of COP1 Tyr441 to alanine abolishes binding of COP1 to the UVr8 core and greatly reduces binding to the HY5 peptide (Fig 1B and E), in good agreement with our structures (Fig 1D). The COP1~422Ala mutant binds HY5~48 as wild type, but increases the binding affinity of UVr8~413 ~10-fold (Fig 1B and E). Interestingly, COP1~422Ala interacts with full-length UVr8 also in the absence of UV-B in yeast two-hybrid assays, which is not detectable for wild type COP1 (Appendix Fig S2A; Rizzini et al., 2011). Moreover, COP1~422Ala also interacts more strongly with the constitutively interacting UVr8~441 fragment (corresponding to the C-terminal UVr8 tail containing the VP motif) when compared to wild type COP1 in yeast two-hybrid assays (Appendix Fig S2B). In contrast, COP1~441Ala and COP1~467Ala show reduced interaction to both UVr8 and HY5 (Appendix Fig S2). A UVr8~413–COP1~441Ala complex structure reveals the UVr8 VP peptide in a different conformation, with UVr8~406–413 bending at the surface of the VP-binding pocket (Appendix Fig S3A–E). In contrast, a structure of HY5~48–COP1~422Ala closely resembles the wild type complex (Appendix Fig S3F).

We next assessed the impact of COP1 VP peptide binding pocket mutants in UV-B signaling assays in planta. The seedling-lethal cop1-5 null mutant can be complemented by expression of YFP–COP1 driven by the CaMV 3555 promoter. We introduced COP1 mutations into this construct and isolated transgenic lines in the cop1-5 background. All lines expressed the YFP-fusion protein and complemented the seedling lethality of cop1-5 (Figs 1F and EV1A). We found that cop1-5/Pro3555–YFP–COP1~467Ala and cop1-5/Pro3555–YFP–COP1~422Ala transgenic lines have constitutively shorter hypocotyls when compared to wild type or cop1-5/Pro3555–YFP–COP1 plants (Fig 1F and G), in agreement with previous work (Holm et al., 2001), suggesting partially impaired COP1 activity. This is similar to the phenotype of cop1-4 (Figs 1F and G, and EV1A–E), a weak cop1 allele that is viable but fully impaired in UVr8-mediated UV-B signaling (McNellis et al., 1994; Oravec et al., 2006; Favory et al., 2009). In contrast, cop1-5/Pro3555–YFP–COP1~441Ala showed an elongated hypocotyl phenotype when compared to wild type (Fig 1G and F), suggesting enhanced COP1 activity.
Figure 1.
Importantly, in contrast to YFP-COP1, transcriptional responses for UV-B-induced marker genes like HY5, RUP2, ELIP2, and CHS are clearly abolished in YFP-COP1<sup>Y422A</sup>, YFP-COP1<sup>T441A</sup>, or YFP-COP1<sup>T467A</sup> after 2 h of UV-B treatment (Figs 1H and I, and EV1B and C). These responses represent an early read-out of UV-B signaling, which has been previously linked to UVR8-COP1-HY5 (Brown et al., 2009; Oravecz et al., 2006; Favory et al., 2009; Binkert et al., 2014). Surprisingly, however, the YFP-COP1<sup>Y422A</sup> line showed strongly reduced UV-B levels (Fig EV1A), despite showing normal UVR8 transcript levels (Fig EV1F and G), precluding any conclusion of the mutation’s effect on UV-B signaling per se. In contrast, YFP-COP1<sup>T441A</sup> and YFP-COP1<sup>T467A</sup> were impaired in UV-B signaling, despite showing wild type UVR8 protein levels (Fig EV1A). This indicates strongly reduced UV-B signaling, in agreement with the reduced affinity of the COP1 mutant proteins versus UVR8<sup>406-413</sup> in vitro (Fig 1E). Together, our crystallographic, quantitative biochemical, and functional assays suggest that UVR8 and HY5 can specifically interact with the COP1 WD40 domain using sequence-divergent VP motifs and that mutations in the COP1 VP-binding site can modulate these interactions and impair UV-B signaling.

**High-affinity, cooperative binding of photoactivated UVR8**

HY5 levels are stabilized in a UVR8-dependent manner under UV-B light (Favory et al., 2009; Huang et al., 2013). We hypothesized that COP1 is inactivated under UV-B light, by activated UVR8 preventing HY5 from interacting with COP1. Our analysis of the isolated VP peptide motifs of UVR8 and HY5 suggests that UVR8 cannot efficiently compete with HY5 for COP1 binding. However, it has been previously found that the UVR8 β-propeller core can interact independently of its VP motif with the COP1 WD40 domain (Yin et al., 2015). We thus quantified the interaction of UV-B-activated full-length UVR8 with the COP1 WD40 domain. Recombinant UVR8 expressed in insect cells was purified to homogeneity, monomerized under UV-B, and analyzed in ITC and grating-coupled interferometry (GCI) binding assays. We found that UV-B-activated full-length UVR8 binds COP1 with a dissociation constant (K<sub>d</sub>) of ~150 nM in both quantitative assays (Fig 2A and B) and ~10 times stronger than non-photoactivated UVR8 (Appendix Fig S4A). This ~1,000-fold increase in binding affinity compared to the UVR8<sup>406-413</sup> peptide indicates cooperative binding of the UVR8 β-propeller core and the VP peptide motif. In line with this, UV-B-activated UVR8 monomers interact with the COP1 WD40 domain in analytical size-exclusion chromatography experiments, while the non-activated UVR8 dimer shows no interaction in this assay (Fig EV2A).

As the interaction of full-length UVR8 is markedly stronger than the isolated UVR8 VP peptide, we next dissected the contributions of the individual UVR8 domains to COP1 binding (Fig 2C). We find that the UV-B-activated UVR8 β-propeller core (UVR8<sup>12-381</sup>) binds COP1 with a K<sub>d</sub> of ~0.5 µM and interacts with the COP1 WD40 domain in size-exclusion chromatography experiments (Fig EV2B and Appendix Fig S4B). The interaction is strengthened when the C-terminus is extended to include the VP peptide motif (UVR8<sup>12-415</sup>; Appendix Fig S4B and C). Mutation of the UVR8 VP peptide motif to alanines results in ~20-fold reduced binding affinity when compared to the wild type protein (Fig 2D). However, the mutant photoreceptor is still able to form complexes with the COP1 WD40 domain in size-exclusion chromatography assays (Fig EV2C). We could not detect sufficient binding enthalpies to monitor the binding of UVR8<sup>12-381</sup> to COP1 in ITC assays nor detectable signals in GCI experiments in the absence of UV-B (Appendix Fig SS). The COP1<sup>Y422A</sup> mutant binds UV-B-activated full-length UVR8 with wild type affinity, while COP1<sup>T467A</sup> binds ~5 times more weakly (Appendix Fig S6A and B). Mutations targeting both COP1 and the UVR8 C-terminal VP peptide motif decrease their binding affinity even further (Appendix Fig S6C). Thus, full-length UVR8 uses both its β-propeller photoreceptor core and its C-terminal VP peptide to cooperatively bind the COP1 WD40 domain when activated by UV-B light.
We next asked whether UV-B-activated full-length UVR8 could compete with HY5 for binding to COP1. We produced the full-length HY5 protein in insect cells and found that it binds the COP1 WD40 domain with a $K_d$ of $\sim 1\text{mM}$ in GCI assays (Fig 2E). For comparison, the isolated HY5 VP peptide binds COP1 with a $K_d$ of $\sim 20\text{mM}$ (Fig 1B). This would indicate that only the UV-B-activated UVR8 and not ground-state UVR8 ($K_d \sim 150\text{nM}$ versus $\sim 1\text{mM}$, see above) can efficiently compete with HY5 for COP1 binding. We tested this hypothesis in yeast 3-hybrid experiments. We confirmed that HY5 interacts with COP1 in the absence of UVR8 and that this interaction is specifically abolished in the presence of UVR8 and UV-B light (Fig 2F). We conclude that UV-B-activated UVR8...
efficiently competes with HY5 for COP1 binding in yeast cells, thereby impairing the COP1–HY5 interaction under UV-B. The UVR8ValPro/AlaAla and UVR8129–476 mutants cannot interfere with the COP1–HY5 interaction in yeast cells (Fig 2F), suggesting that a functional UVR8 VP peptide motif is required to compete off HY5 from COP1, in agreement with our biochemical assays.

**UVR8–VP peptide chimeras trigger UV-B signaling in planta**

Our findings suggest that UVR8 requires both its UV-B-sensing core and its VP peptide motif for high-affinity COP1 binding and that the UVR8 VP peptide can inhibit the interaction of HY5 with COP1 (Figs 1 and 2; Yin et al., 2015). This led us to speculate that any VP peptide with sufficient binding affinity for COP1 could functionally replace the endogenous VP motif in the UVR8 C-terminus in vivo. We generated chimeric proteins in which the UVR8 core domain is fused to VP-containing sequences from plant and human COP1 substrates, namely HY5 and TRB1 (Fig 3A). Arabidopsis uvr8-7 null mutants expressing these chimeric proteins show complementation of the hypocotyl and anthocyanin phenotypes under UV-B, suggesting that all tested UVR8 chimeras are functional (Figs 3B–D, and EV3). Early UV-B marker genes are also up-regulated in the lines after UV-B exposure, demonstrating that these UVR8 chimeras are functional photocaptors, although to different levels (Fig 3E). In line with this, the UVR8ValPro/AlaAla chimera can displace HY5 from COP1 in yeast 3-hybrid assays (Fig 3F). All the UVR8 chimeras can bind COP1 with affinities comparable to wild type (Figs 3G and EV3) and are dimers in vitro that monomerize under UV-B light (Fig 3H and Appendix Fig S7D). Together, these experiments reinforce the notion that divergent VP peptide motifs compete with each other for binding to the COP1 WD40 domain.

**Sequence-divergent VP peptide motifs are recognized by the COP1 WD40 domain**

Our protein engineering experiments prompted us to map core VP peptide motifs in other plant light signaling components, including the COP1-interacting blue-light photoreceptors CRY1 and CRY2 (Yang et al., 2000, 2018a; Wang et al., 2001; Yu et al., 2007) and the transcription factors HYH, CO/BBX1, COL3/BBX4, SALT TOLERANCE (STO/BBX24) (Holm et al., 2002; Datta et al., 2006; Jang et al., 2008; Liu et al., 2008; Yan et al., 2011), and HFR1 (Duke et al., 2004; Jang et al., 2005; Yang et al., 2005). We mapped putative VP motifs in all these proteins and assessed their binding affinities to the COP1 WD40 domain (Fig 4A and B). We could detect binding for most of the peptide motifs in ITC assays, with dissociation constants in the mid-micromolar range (Figs 4A and EV4). Next, we obtained crystal structures for the different peptide bound to COP1 (1.3–2.0 Å resolution, see Tables EV1 and EV2, Figs 4E and F, and EV4) to compare their peptide binding modes (Fig 4C). We found that all peptides bind in a similar configuration with the VP forming the center of the binding site (r.m.s.d. between the different peptides range from ~0.3 to 1.5 Å, comparing 5 or 6 corresponding Cα atoms). Chemically diverse amino acids (Tyr/Arg/ Gin) map to the ~3 and ~2 position and often deeply insert into the COP1 binding cleft, acting as anchor residues (Fig 4C). This suggests that the COP1 WD40 domain has high structural plasticity, being able to accommodate sequence-divergent VP-containing peptides.

To investigate this property of COP1, we quantified the interaction of different VP peptides with our COP1ValPro/AlaAla mutant protein. As for UVR8 (Fig 1B and E), COP1ValPro/AlaAla showed increased binding affinity for some peptides such as those representing the COL3287–294 and CO366–373 VP motifs, while it reduced binding to others, such as to CRY1544–552 and CRY2527–535 (Fig 4A and D). These observations may be rationalized by an enlarged VP-binding pocket in the COP1ValPro/AlaAla mutant, increasing accessibility for the COL3287–294 anchor residue, and potentially abolishing interactions with CRY1Asp545 (Fig 4E and F). In yeast 3-hybrid assays, we find that, similar to HY5 (Fig 2F), UV-B-activated UVR8 can efficiently compete with HYH and an N-terminal fragment of HFR1 for binding to COP1 (Fig EV5). Taken together, VP peptide motifs of cryptochrome photoreceptors and diverse COP1 transcription factor targets all bind to the COP1 WD40 domain and UVR8 is able to compete with COP1 partners for binding.
Figure 3.
The plasticity of the COP1 WD40 domain is illustrated by the variable modes of binding for sequence-divergent VP motifs found in different plant light signaling components. The COP1<sup>1,442Ala</sup> mutation can modulate the interaction with different VP peptide (Fig 4A). We noted that the <i>cpl-5/Pro<sub>353</sub>:YFP-COP1<sup>1,442Ala</sup></i> but not other COP1 mutants show delayed flowering when grown in long days (Fig 5A–D). This phenotype has been previously associated with mutant plants that lack the COP1 substrate CO (Fig 5B; Putterill et al., 1995; Jang et al., 2008; Liu et al., 2008).

We thus hypothesized that in COP1<sup>1,442Ala</sup> plants, binding and subsequent degradation of CO may be altered under long-day conditions. In vitro, we found that the CO VP peptide binds COP1<sup>1,442Ala</sup> ~ 4 times stronger than wild type COP1 (Fig 5F). The same mutation in COP1 strongly reduces (~ 30 times) binding of the CRV2 VP peptide in vitro (Fig 5F). It is of note that, in contrast to UVR8 (Fig 1F), CRV2 levels are not altered in the COP1<sup>1,442Ala</sup> background (Fig 5E). Thus, the late- flowering phenotype of the COP1<sup>1,442Ala</sup> mutant background: reduced affinity to CRV2, enhanced binding to CO—both consistent with the late- flowering phenotype. In line with this, we find that only recombina nt light-activated full-length CRV2 binds wild type COP1 with nanomolar affinity in quantitative GCl experiments (Fig 5G and Appendix Fig S8). This ~ 200-fold increase in binding affinity over the isolated CRV2 VP peptide strongly suggests that UVR8 and CRV2 both use a cooperative binding mechanism to target COP1. As a control, we tested a fragment of the CRV2 C-terminus containing the VP motif, the NC80 domain (CRV2<sup>486-565</sup>; Yu et al., 2007). We found that NC80 binds COP1 with an affinity comparable to the isolated CRV2<sup>527-535</sup> VP peptide assayed by ITC (Fig 5F and H). Together, the COP1<sup>1,442Ala</sup> phenotypes and our biochemical assays suggest that different plant photoreceptors may use a light-induced cooperative binding mechanism, preventing COP1 from targeting downstream light signaling partners for degradation.

Discussion

The COP1 E3 ubiquitin ligase is a central signaling hub that integrates inputs from plant light- sensing photoreceptors. There is strong evidence that the UV-B-sensing photoreceptor UVR8, the blue-light receptors CRY1 and CRY2, and the red/far-red discriminating photoreceptors all regulate COP1 activity (Hoecker, 2017; Podolec & Ulm, 2018). The regulation of COP1 by photoreceptors enables a broad range of photomorphogenic responses, including de- etiolation, cotyledon expansion, and transition to flowering, as well as UV-B light acclimation (Lau & Deng, 2012; Jenkins, 2017; Yin & Ulm, 2017; Gommers & Monte, 2018). Here, we have dissected at the structural, biochemical, and genetic levels how the activated UVR8 and cryptochrome photoreceptors impinge on COP1 activity, by interacting with its central WD40 domain, resulting in the stabilization of COP1 substrate transcription factors. For both types of photoreceptors, interaction through a linear VP motif and a folded, light-regulated interaction domain leads to cooperative, high-affinity binding of the activated photoreceptor to COP1. We propose that in response to UV-B light, UVR8 dimers monomerize, exposing a new interaction surface that binds to the COP1 WD40 domain and releases the UVR8 C-terminal VP motif from structural restraints that prevent its interaction with COP1 in the absence of UV-B (Yin et al., 2015; Heilmann et al., 2016; Camacho et al., 2019; Wu et al., 2019). Similarly, the VP motif in the CCT domain of cryptochromes may become exposed and available for interaction upon blue-light activation of the photoreceptor ( Müller & Bouly, 2015; Wang et al., 2018). Because UVR8 and CRV2 are very
A

| Peptide | vs. | WT | Lys422Ala |
|---------|-----|----|-----------|
| UVR8    | 190 ± 40 | 16 ± 1 |
| HY5     | 23 ± 7 | 23 ± 10 |
| COL3    | 39 ± 0.4 | 7.8 ± 0.6 |
| CO      | 74 ± 20 | 21 ± 10 |
| CRY1    | 3.0 ± 0.3 | 76 ± 20 |
| CRY2    | 21 ± 2 | 560 ± 100 |

STO: no detectable binding
HYH: no detectable binding
HFR1: no detectable binding

B

| Peptide | WT | Lys422Ala |
|---------|----|-----------|
| UVR8    | 190 ± 40 | 16 ± 1 |
| HY5     | 23 ± 7 | 23 ± 10 |
| COL3    | 39 ± 0.4 | 7.8 ± 0.6 |
| CO      | 74 ± 20 | 21 ± 10 |
| CRY1    | 3.0 ± 0.3 | 76 ± 20 |
| CRY2    | 21 ± 2 | 560 ± 100 |

STO: no detectable binding
HYH: no detectable binding
HFR1: no detectable binding

Figure 4.
different in structure and domain composition, they likely use distinct interaction surfaces to target the COP1 WD40 domain, in addition to the VP peptide motifs. The cooperative, high-affinity mode of binding enables UVR8 and cryptochromes to efficiently displace downstream signaling components such as HY5, HYH, HFR1, and CO in a light-dependent manner. Structure-guided mutations in the COP1 WD40 binding cleft resulted in the identification of the COP1^Lys422Ala mutant, which displays flowering phenotypes, and COP1^Tyr441Ala and COP1^Trp467Ala, which shows UV-B signaling phenotypes, that are all consistent with our competition model. Similar mutations have previously been shown to affect hypocotyl elongation in white light (Holm et al., 2001). It is interesting to note that the COP1^Tyr441Ala mutant still shows UV-B-dependent hypocotyl growth inhibition even though the COP1^Tyr441Ala protein is impaired in its interaction with UVR8. This UV-B response is possibly due to a recently described COP1-independent UVR8 activity involving direct interaction and inhibition of transcription factors that promote hypocotyl elongation (Liang et al., 2018; Yang et al., 2018b). Moreover, we cannot exclude that the UVR8 interaction with COP1 that is independent of the UVR8 C-terminal WD40 domain (Fig 2D, Appendix Fig S4B and Fig EV2B; see also Yin et al., 2015) can affect COP1^Tyr441Ala activity sufficiently to see a long-term phenotype such as hypocotyl growth inhibition. Likewise, partial complementation of COP1^Trp467Ala reveals that it may not be impaired in interacting with all COP1 substrates and that it may still target other photomorphogenesis-promoting factors for degradation or retains an activity that is independent of the VP peptide binding pocket. Unexpectedly, COP1^Lys422Ala rendered the UVR8 protein unstable, preventing conclusive analysis of the effect of this COP1 mutant on UV-B signaling in vivo. Moreover, the mechanism behind UVR8 protein instability remains to be determined. Independent of this, it is interesting to note that the hy4-9 mutant, which replaces the proline in the CRY1 VP peptide motif with leucine, does not show inhibition of hypocotyl elongation under blue light (Ahmad et al., 1995). Similarly, mutations of the UVR8 VP peptide motif or C-terminal truncations (including the uvr8-2 allele, which has a premature stop codon at Trp400) all strongly impair UV-B signaling (Brown et al., 2005; Cloix et al., 2012; Yin et al., 2015). We now report quantitative biochemical and crystallographic analyses that reveal that UVR8 and cryptochrome photoreceptors and their downstream transcription factors all make use of VP-containing peptide motifs to target a central binding cleft in the COP1 WD40 domain. VP-containing peptides were previously identified based upon a core signature motif E-S-D-E-x-x-x-V-P-[E/D]-Φ-G, where Φ designated a hydrophobic residue (Holm et al., 2001; Uljon et al., 2016). Our structural analyses of a diverse set of VP-containing peptides now reveal that COP1 has evolved a highly plastic VP-binding pocket, which enables sequence-divergent VP motifs from different plant light signaling components to compete with each other for COP1 binding. It is reasonable to assume that many more bona fide VP

Figure 4. Many COP1 substrates and interacting photoreceptors contain VP peptides that can bind the COP1 WD40 domain.

A Table summarizing affinities (K_d, dissociation constant) of VP-containing peptides versus the COP1 WD40 domain (WT) and COP1^Lys422Ala as determined by ITC. All values are μM (± standard deviation).
B A sequence comparison of VP peptide motifs that interact with the COP1 WD40 domain. The VP core is colored in red and the anchor residues in orange. The peptide sequences are numbered on a register where the V in the VP is green.
C (Top panel) Superposition of the VP peptide binding modes of the HYS (green), COL3 (orange), and STO (gray) peptides depicted in ball-and-stick representation when bound to the COP1 WD40 domain as observed in their respective X-ray crystal structures. (Bottom panel) A comparison of the HYS, COL3, STO, CRY1, and UVR8 peptides highlights the chemically diverse anchor residues (orange) and their variant positions.
D ITC assays between the COL3 VP peptide (left) and CRF1 VP peptide (right) versus the COP1 WD40 and COP1^Lys422Ala, with a table summarizing their corresponding affinities. The following concentrations were typically used (titrant into cell): COL3-COP1 (2.40 μM in 195 μM); COL3-COP1^Lys422Ala (1.50 μM in 175 μM); CRF1-COP1 (750 μM in 75 μM); and CRF1-COP1^Lys422Ala (1,500 μM in 175 μM). The inset shows the dissociation constant (K_d). The stoichiometries of binding (N) for COL3-COP1 = 0.75 ± 0.2; COL3-COP1^Lys422Ala = 0.90 ± 0.2; CRF1-COP1 = 0.99 ± 0.3; CRF1-COP1^Lys422Ala = 0.98 ± 0.1 (all measurements ± standard deviation).
E, F The crystal structure of the (E) COL3 peptide and (F) CRF1 peptide bound to the COP1 WD40 domain. The peptides are depicted in ball-and-stick representation. Selected residues from COP1 are depicted in gray in stick representation.
motifs may exist and our structures now provide sequence fingerprints to enable their bioinformatic discovery.

Interestingly, although we predict that at least some of our COP1 mutant variants (e.g., Trp467Ala) completely disrupt the interaction with VP motif harboring COP1 targets, all COP1 variants can complement the cop1-5 seedling-lethal phenotype and largely the cop phenotype in darkness (Holm et al, 2001; and this work, Fig EV1D and E). This could imply that a significant part of COP1

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**Figure 5.**

A. 39 days in long days

B. 39 days in long days

C. Days to Flower in long days

D. Number of Leaves at Flowering

E. cop1-5/Pro35S::YFP-COP1

F. COP1 (ligand) vs. CRY2 peptide (syringe) vs. COP1

G. COP1 (ligand) vs. CRY2 peptide (syringe) vs. COP1

H. COP1 (ligand) vs. CRY2 peptide (syringe) vs. COP1
activity is independent from the VP-mediated destabilization of photomorphogenesis-promoting transcription factors. It has been recently suggested that part of the cop1 phenotype could be explained by COP1-mediated stabilization of PIFs (Pham et al., 2018). Our COP1 lines could be used to gain further insight into this aspect of COP1 activity.

Human COP1 prefers to bind phosphorylated substrates, and their post-translational regulation may also be relevant in plants (Hardtke et al., 2000; Uljon et al., 2016). In this respect, it is noteworthy that the full-length COP1 protein may exist as an oligomer as well as in complex with other light-signaling proteins, such as SPA proteins (Seo et al., 2003; Huang et al., 2013; Sheerin et al., 2015; Holtkotte et al., 2017). The four SPA protein family members share a similar domain architecture with COP1, consisting of an N-terminal kinase-like domain, a central coiled-coil domain, and a C-terminal WD40 domain (~45% amino acid identity with the COP1 WD40 domain), and are partially redundant in their activities (Yang & Wang, 2006; Ordoñez-Herrera et al., 2015). Mutations in the SPA1 WD40 domain residues Lys767 and Trp812, which correspond to COP1 residues Lys422 and Trp467, cannot complement the spa1-3 mutant (Yang & Wang, 2006). These higher-order complexes are known to be part of some but not all light-signaling pathways and could thus encode additional determinants for signaling specificity (Hoekker, 2017; Podolec & Ulm, 2018). In addition to the competition mechanism presented here, it has been observed that active cryptochrome and phytochrome receptors directly interact with SPA proteins and thereby separate COP1 from SPA proteins, which results in COP1 inactivation (Lian et al., 2011; Liu et al., 2011; Zuo et al., 2011; Lu et al., 2015; Sheerin et al., 2015). However, early UV85 signaling is independent of SPA proteins (Oravecz et al., 2006) and may thus rely exclusively on the competition mechanism described here. For cryptochrome signaling, the VP-mediated competition and COP1-SPA disruption mechanisms are obviously not mutually exclusive but likely function in parallel in vivo to reinforce COP1-SPA E3 ligase inactivation in blue-light signaling. Reconstitution of a photoreceptor–COP1/SPA signaling complex may offer new insights into these different targeting mechanisms in the future.

Materials and Methods

Cell culture, strains, plasmids, and antibodies

Sf9 cell culture

*Spodoptera frugiperda* Sf9 cells (Thermo Fisher) were cultured in SF-4 Baculo Express insect cell medium (Bioconcept).

Yeast strains

The following *Saccharomyces cerevisiae* reporter strains were used: L40 (MATα trp1 leu2 his3 ade2 lys2::lexA-HIS3 URA3::lexA-lacZ GAL4) (Vojtek & Hollenberg, 1995), Y190 (MATα ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3 112 gal4A gal80A cyh2/2 LYS2::GAL1UAS-HIS3TATA-HIS3 MEL1 URA3::GAL1UAS-GAL1TATA-lacZ), and Y187 (MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3 112 gal4A met+ gal80A URA3::GAL1UAS-GAL1TATA-MEL1) (Yeast Protocols Handbook, Clontech).

Plants

**cop1-4** (Oravec et al., 2006) **cop1-5** (McNellis et al., 1994), **cop1-5/Pro35S:YFP-COP1, cop1-5/Pro35S:YFP-COP1Lys422Ala**, **cop1-5/Pro35S:YFP-COP1Tyr441Ala**, **cop1-5/Pro35S:YFP-COP1Trp467Ala** (this work), **uvr8-7** (Favory et al., 2009), **uvr8-7/Pro35S:UVR8HY5**, and **uvr8-7/Pro35S:UVR8HY5S** (this work) are in the Arabidopsis thaliana Wassilewskija (Ws) accession. The cry2-1 (Guo et al., 1998) mutant is in the Columbia accession. The co-11 allele was generated in the Ws accession (this work) using CRISPR/Cas9 technology (Wang et al., 2015).

Plasmids

All constructs and plasmids used are listed as described below and in the Appendix Table S1.

Antibodies

All antibodies used are listed as described below and in the Appendix Table S1.

Protein expression and purification

All COP1, UVR8, HY5, and CRY2 proteins were produced as follows: The desired coding sequence was PCR amplified (see Appendix Table S1 for primers) or Ncol/NotI digested from codon-optimized genes (GeneArt or Twist Biosciences) for expression in Sf9 cells. Chimeric UVR88 constructs were PCR amplified directly from vectors used for yeast 3-hybrid assays (see below). All constructs except CRY2NC80 were cloned into a modified pFastBac (Geneva Biotech) insect cell expression vector, via Ncol/NotI restriction enzyme sites or by Gibson assembly (Gibson et al., 2009). The modified pFastBac vector contains a tandem N-terminal His10-Twin-Strep-tags followed by a TEV (tobacco etch virus protease) cleavage site. CRY2NC80 was cloned into a modified pET-28 a (+) vector (Novagen) containing a tandem N-terminal His10-Twin-Strep-tags followed by a TEV (tobacco etch virus protease) cleavage site by Gibson assembly. Mutagenesis was performed using an enhanced plasmid mutagenesis protocol (Liu & Naismith, 2008).

pFastBac constructs were transformed into DH10MultiBac cells (Geneva Biotech), white colonies indicating successful recombination were selected, and bacmids were purified by the alkaline lysis method. Sf9 cells were transfected with the desired bacmid with Profectin (AB Vector). eYFP-positive cells were observed after induction with IPTG at a final concentration of 0.2 mM, and then amplified for 20 min and stored at 20°C. CRY2NC80 was produced in transformed Rosetta (DE3) pLysS (Novagen) cells. Escherichia coli were grown in 2xYT broth with kanamycin. One liter of broth was inoculated with 20 ml of a saturated overnight pre-culture, grown at 37°C until OD600 ~ 0.5, induced with IPTG at a final concentration of 0.2 mM, and then shaken for another 16 h at 18°C. The cell pellet was harvested by centrifugation at 2,000 × g for 20 min and stored at -20°C.

CRY2NC80 was produced in transformed Rosetta (DE3) pLysS (Novagen) cells. Escherichia coli were grown in 2xYT broth with kanamycin. One liter of broth was inoculated with 20 ml of a saturated overnight pre-culture, grown at 37°C until OD600 ~ 0.5, induced with IPTG at a final concentration of 0.2 mM, and then shaken for another 16 h at 18°C. The cell pellet was harvested by centrifugation at 2,000 × g for 20 min and stored at -20°C.

Every 1 l of Sf9 or bacterial cell culture was dissolved in 25 ml of Buffer A (300 mM NaCl, 20 mM HEPES 7.4, 2 mM BME), supplemented with glycerol (10% v/v), 5 μl turbonuclease, and 1
Roche cComplete Protease Inhibitor tablets. Dissolved pellets were lysed by sonication, and insoluble materials were separated by centrifugation at 60,000 \( \times g \) for 1 h at 4°C. The supernatant was filtered through tandem 1- and 0.45-\( \mu \)m filters before Ni\(^{2+}\)-affinity purification (HisTrap excel, GE Healthcare). Ni\(^{2+}\)-bound proteins were washed with Buffer A and eluted directly into a coupled Strep-Tactin Superflow XT column (IBA) by Buffer B (500 mM NaCl, 500 mM imidazole pH 7.4, 20 mM HEPES pH 7.4). Twin-Strep-tagged-bound proteins on the Strep-Tactin column were washed with Buffer A and eluted with 1\(^{\text{st}}\) Buffer BXT (IBA). Proteins were cleaved overnight at 4°C with TEV protease. Cleaved proteins were subsequently purified from the protease and affinity tag by a second Ni\(^{2+}\)-affinity column or by gel filtration on a Superdex 200 Increase 10/300 GL column (GE Healthcare). Proteins were concentrated to 3–10 mg/ml and either used immediately or aliquoted and frozen directly at –80°C. Typical purifications were from 2 to 5 l of cell pellet.

All protein concentrations were measured by absorption at 280 nm and calculated from their molar extinction coefficients. Molecular weights of all proteins were confirmed by MALDI-TOF mass spectrometry. SDS–PAGE gels to assess protein purity are shown in Appendix Fig S7.

For UVR8 monomerization and activation by UV-B, proteins were diluted to their final assay concentrations (as indicated in the figure legends) in Eppendorf tubes and exposed to 60 min at max intensity (69 mA) under UV-B LEDs (Roithner Lasertechnik GmbH) on ice.

For CRY2 activation, proteins were diluted to their final assay concentrations (as indicated in the figure legends) in Eppendorf tubes and exposed to 10 min of fluorescent light on ice.

**Analytical size-exclusion chromatography**

Gel filtration experiments were performed using a Superdex 200 Increase 10/300 GL column (GE Healthcare) pre-equilibrated in 150 mM NaCl, 20 mM HEPES 7.4, and 2 mM BME. Peptides were synthesized and delivered as lyophilized powder (Peptide Specialty Labs GmbH) and dissolved directly in buffer. The peptides were centrifuged at 14,000 \( \times g \) for 10 min, and only the supernatant was used. The dissolved peptide concentrations were calculated based upon their absorbance at 280 nm and their corresponding molar extinction coefficient. Typical experiments consisted of titrations of 20 injections of 2 \( \mu l \) of each peptide into the cell containing COP1 at a 10-fold lower concentration. Typical concentrations for the titrant were between 500 and 3,000 \( \mu M \) for experiments depending on the affinity. Experiments were performed at 25°C and a stirring speed of 1,000 rpm on an ITC200 instrument (GE Healthcare). All data were processed using Origin 7.0 and fit to a one-site binding model after background buffer subtraction.

**Grating-coupled interferometry (GCI)**

The Creoptix WAVE system (Creoptix AG), a label-free surface biosensor, was used to perform GCI experiments. All experiments were performed on 2PCH or 4PCH WAVEchips (quasi-planar polycarbonate surface; Creoptix AG). After a borate buffer conditioning (100 mM sodium borate pH 9.0, 1 M NaCl; Xantec), COP1 (ligand) was immobilized on the chip surface using standard amine-coupling: 7 min activation [1:1 mix of 400 mM N-(3-dimethylaminopropyl)-N’-ethylenedicarbodiimide hydrochloride and 100 mM N-hydroxysuccinimide (both Xantec)], injection of COP1 (10 \( \mu g/ml \)) in 10 mM sodium acetate pH 5.0 (Sigma) until the desired density was reached, and final quenching with 1 M ethanolamine pH 8.0 for 7 min (Xantec). Since the analyte CRY2 showed non-specific binding on the surface, BSA (0.5% in 10 mM sodium acetate, pH 5.0; BSA from Roche) was used to passivate the surface between the injection of COP1 and ethanolamine quenching. For a typical experiment, the analyte (UVR8/CRY2) was injected in a 1:3 dilution series (highest concentrations as indicated in the figure legends) in 150 mM NaCl, 20 mM HEPES 7.4, and 2 mM BME at 25°C. Blank injections were used for double referencing and a dimethyl sulfoxide (DMSO) calibration curve for bulk correction. Analysis and correction of the obtained data were performed using the Creoptix WAVEcontrol software (applied corrections: X and Y offset; DMSO calibration; double referencing), and a one-to-one binding model or a heterogenous ligand model with bulk correction was used to fit all experiments.

**Protein crystallization and data collection**

Crystals of co-complexes of HYS\(^{29–48}\)–COP1\(^{349–675}\), UVR8\(^{406–413}\)–COP1\(^{349–675}\), HYS\(^{29–48}\)–COP1\(^{349–675}\), Lys422Ala, UVR8\(^{406–413}\)–COP1\(^{349–675}\), Lys422Ala, and COL3\(^{287–294}\)–COP1\(^{349–675}\) were grown in sitting drops and appeared after several days at 20°C when 5 mg/ml of COP1 supplemented with three- to 10-fold molar excess in peptide was mixed with twofold (v/v) more mother liquor (1:2 ratio; protein:buffer) containing 2 M (NH\(_4\))\(_2\)SO\(_4\) and 0.1 M HEPES pH 7.4 or 0.1 M Tris pH 8.5. Crystals were harvested and cryoprotected in mother liquor supplemented with 25% glycerol and frozen under liquid nitrogen.

Crystals of complexes of HYH\(^{27–34}\)–COP1\(^{349–675}\), HFR1\(^{57–64}\)–COP1\(^{349–675}\), STO\(^{240–247}\)–COP1\(^{349–675}\), and CRY1\(^{544–552}\)–COP1\(^{349–675}\) were grown in sitting drops and appeared after several days at 20°C when 5 mg/ml of COP1 supplemented with three- to 10-fold molar excess in peptide was mixed with twofold (v/v) more mother liquor (1:2 ratio; protein:buffer) containing 1.25 M sodium malonate pH 7.5. Crystals were harvested and cryoprotected in mother liquor supplemented with 25% glycerol and frozen under liquid nitrogen.

All datasets were collected at beam line PX-III of the Swiss Light Source, Villigen, Switzerland. Native datasets were collected with \( \lambda = 1.03 \, \text{Å} \). All datasets were processed with XDS (Kabsch, 1993) and scaled with AIMLESS as implemented in the CCP4 suite (Winn et al, 2011).

**Crystallographic structure solution and refinement**

The structures of all the peptide-COP1 WD40 complexes were solved by molecular replacement as implemented in the program
Phaser (McCoy et al., 2007), using PDB-ID 5IGO as the initial search model. The final structures were determined after iterative rounds of model-building in COOT (Emsley & Cowtan, 2004), followed by refinement in REFMAC5 (Murshudov et al., 2011) as implemented in CCP4 and phenix.refine (Adams et al., 2010). Polder omit maps were generated for the UVR8\textsuperscript{404–413}–COP1 structure by omitting residue Tyr407 of the bound peptide as implemented in phenix.polder. Final statistics were generated as implemented in phenix.table_one. All figures were rendered in UCSF Chimera (Petterson et al., 2004).

**Plant transformation**

To generate the cop1-5/Pro\textsubscript{35S}:YFP-COP1 line, COP1 cloned into pENTR207C was introduced into the Gateway-compatible binary vector pB7WGY2 (Karimi et al., 2002). COP1 mutated versions were generated by PCR-based site-directed mutagenesis, cloned into pDONR207, and then introduced in pB7WGY2 (Karimi et al., 2002). The wild type version of the construct contains an additional Gateway-cloning-related 14 amino acids’ linker sequence between the YFP and COP1. cop1-5 heterozygous plants (kan\textsuperscript{5}) were transformed using the floral dip method (Clough & Bent, 1998). Lines homozygous for the cop1-5 mutation and for single locus insertions of the Pro\textsubscript{35S}:YFP-COP1 transgene were selected.

To generate lines expressing chimeric UVR8 receptors, the HYS and TRIB1 sequences were introduced by PCR to the UVR8 coding sequences as indicated, and the chimeras were cloned into the Gateway-compatible binary vector pB2GW7 (Karimi et al., 2002) for transformation into the uvr8-7 mutant background. Lines homozygous with single genetic locus transgene insertions were selected.

To generate a co mutant in the Ws background, designated co-11, plants were transformed with the CRISPR/Cas9 binary vector pHHE401E (Wang et al., 2015) in which an sgRNA specific to the CO cds was inserted (see Appendix Table S1). A plant was isolated in T2 and propagated, harboring a 1 base-pair insertion after the codon for residue Asp137 leading to a frameshift and a premature stop codon after four altered amino acids (DPGR\textsuperscript{5}; D representing Asp137 in CO, * representing the premature stop).

**Plant growth conditions**

For experiments at seedling stage, *Arabidopsis* seeds were surface-sterilized and sown on half-strength MS medium (Duchefa), stratified in the dark at 21°C for 48 h, and grown under aseptic conditions in controlled light conditions at 21°C. For hypocotyl length and anthocyanin measurements, the MS medium was supplemented with 1% sucrose (AppliChem). For flowering experiments, *Arabidopsis* plants were grown on soil in long-day (16 h/8 h; light/dark cycles) growth chambers at 21°C.

UV-B treatments were performed as described before, using Osram L18W/30 tubes, supplemented with narrowband UV-B from Philips TL20W/01RS tubes (Oravecz et al., 2006; Favory et al., 2009).

**Hypocotyl length assays**

For hypocotyl length measurements, at least 60 seedlings were randomly chosen, aligned, and scanned. Measurements were performed using the NeuronJ plugin of ImageJ (Meijering et al., 2004). Violin and box plots were generated using the ggplot2 library in R (Wickham, 2009).

**Anthocyanin quantification**

Accumulation of anthocyanin pigments was assayed as described previously (Yin et al., 2012). In brief, 40–60 mg of seedlings were harvested, frozen, and grinded before adding 250 µl acidic methanol (1% HCl). Samples were incubated on a rotary shaker for 1 h, the supernatant was collected, and absorbances at 530 and 655 nm were recorded using a spectrophotometer. Anthocyanin concentration was calculated as \( (A_{530} - 2.5 \ast A_{655})/mg \), where mg is the fresh weight of the sample.

**Protein extraction and immunoblotting**

For total protein extraction, plant material was grinded and incubated with an extraction buffer composed of 50 mM Na-phosphate pH 7.4, 150 mM NaCl, 10% (v/v) glycerol, 5 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM DTT, 2 mM Na\textsubscript{3}VO\textsubscript{4}, 2 mM NaF, 1% (v/v) Protease Inhibitor Cocktail (Sigma), and 50 µM MG132, as previously described (Arongaus et al., 2018).

Proteins were separated by electrophoresis in 8% (w/v) SDS-polyacrylamide gels and transferred to PVDF membranes (Roth) according to the manufacturer’s instructions (iBlot dry blotting system, Thermo Fisher Scientific), except for CRY2 immunoblots, which were transferred on nitrocellulose membranes (Bio-Rad). For protein gel blot analyses, anti-UVR8\textsuperscript{426–440} (Favory et al., 2009), anti-UVR8\textsuperscript{410–424} (Heijde & Ulm, 2013), anti-GFP (Living Colors® A.V. monoclonal Antibody, JL-8; Clontech), anti-actin (A0480; Sigma-Aldrich), and anti-CRY2\textsuperscript{608–602} (Eurogentec, raised against the peptide N\textsuperscript{-}CEGKNL- GIQDSSDIQ-C and affinity purified) were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulins (Dako) were used as secondary antibodies. Signal detection was performed using the ECL Select Western Blotting Detection Reagent (GE Healthcare) and an Amersham Imager 680 camera system (GE Healthcare).

**Quantitative real-time PCR**

RNA was extracted from seedlings using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions. RNA samples were treated for 20 min with RNA-free DNase (Qiagen) followed by addition of DEPC-treated EDTA for inactivation at 65°C for 10 min. Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems), using a 1:1 mixture of oligo-dT and random hexamer primers. Quantitative real-time PCR was performed on a QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific) using PowerUp SYBR Green Master Mix reagents (Applied Biosystems). Gene-specific primers for CHS, COP1, ELIP2, HY5, RUP2, and UVR8 were described before (Favory et al., 2009; Gruber et al., 2010; Heijde et al., 2013); 18S expression was used as reference gene (Vandenbussche et al., 2014); and expression values were calculated using the ΔΔC\textsubscript{T} method (Livak & Schmittgen, 2001) and normalized to the wild type. Each reaction was performed in technical triplicates; data shown are from three biological repetitions.
Flowering time assays

For quantitative flowering time measurements, the number of days to flowering was determined at bolting, and rosette and cauline leaf numbers were counted when the inflorescence reached approximately 1 cm in length (Möller-Steinbach et al., 2010).

Yeast 2-hybrid and 3-hybrid assays

For yeast 2-hybrid assay, COP1 and its mutated variants were introduced into pGADT7-GW (Marrocco et al., 2006; Yin et al., 2015) and HY5, UVR8, and UVR8C44 were introduced into pBTM116-D9-GW (Stelzl et al., 2005; Yin et al., 2015; Binkert et al., 2016). Vectors were co-transformed into the L40 strain (Vojtek & Hollenberg, 1995) using the lithium acetate-based transformation protocol (Gietz, 2014). Transformants were selected and grown on SD/-Trp/-Leu medium (Formedium). For the analysis of β-galactosidase activity, enzymatic assays using chlorophenol red-β-D-galactopyranoside (Roche Applied Science) as substrate were performed as described (Yeast Protocols Handbook, Clontech). For repression of ProMet25:UVR8 expression, SD/-Trp/-Leu/-Met medium was supplemented with 1mL-methionine (Fisher Scientific).

For assays, yeast cells were grown for 2 days at 30°C in darkness or under narrow-band UV-B (Philips TL20W/01RS; 1.5 μmol/m²/s), as indicated.

Quantification and statistical analysis

Data of ITC and GCI binding assays are reported with errors as indicated in their figure legends.

Data availability

The atomic coordinates of complexes have been deposited with the following Protein Data Bank (PDB) accession codes: HY559–58–COP1349–675: 6QTO (https://www.rcsb.org/structure/6qto), UVR8406–413–COP1349–675: 6QTQ (https://www.rcsb.org/structure/6qtq), HY5–COP1349–675-Lys422Ala: 6QTR (https://www.rcsb.org/structure/6qtr), UVR8–COP1349–675-Lys422Ala: 6QTS (https://www.rcsb.org/structure/6qts), HYH77–34–COP1349–675: 6OTT (https://www.rcsb.org/structure/6ott), STO240–247–COP1349–675: 6QTU (https://www.rcsb.org/structure/6qtu), HFR157–64–COP1349–675: 6QTV (https://www.rcsb.org/structure/6qtv), CRY1544–552–COP1349–675: 6QTW (https://www.rcsb.org/structure/6qtw), and COL3287–294–COP1349–675: 6QTX (https://www.rcsb.org/structure/6qtx).

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Author contributions

KL, RP, RU, and MH designed experiments and wrote the article. KL performed all protein expression and purification, ITC, GCI and X-ray crystallography experiments and their analyses. RP with the help of RC generated all plant lines, and performed all in vivo plant and yeast experiments and their analyses.

Conflict of interest

The authors declare that they have no conflict of interest.

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