Negative feedback regulation of UV-B–induced photomorphogenesis and stress acclimation in Arabidopsis

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Plants respond to low levels of UV-B radiation with a coordinated photomorphogenic response that allows acclimation to this environmental stress factor. The key players in this UV-B response are COP1 (an E3 ubiquitin ligase), UVR8 (a β-propeller protein), and HY5 (a bZIP transcription factor). We have shown previously that an elevated UV-B–specific response is associated with dwarf growth, indicating the importance of balancing UV-B–specific signaling. Negative regulators of this pathway are not known, however. Here, we describe two highly related WD40-repeat proteins, REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 (RUP1) and RUP2, that interact directly with UVR8 as potent repressors of UV-B signaling. Both genes were transcriptionally activated by UV-B in a COP1-, UVR8-, and HY5-dependent manner. rup1 rup2 double mutants showed an enhanced response to UV-B and elevated UV-B tolerance after acclimation. Overexpression of RUP2 resulted in reduced UV-B–induced photomorphogenesis and impaired acclimation, leading to hypersensitivity to UV-B stress. These results are consistent with an important regulatory role for RUP1 and RUP2, which act downstream of UVR8–COP1 in a negative feedback loop impinging on UVR8 function, balancing UV-B defense measures and plant growth.


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V-B (280–315 nm) radiation of wavelengths exceeding ~295 nm as an integral part of the sunlight reaching the surface of the Earth induces a broad range of physiological responses. UV-B stress induces mostly unspecific damage responses in living organisms (1, 2); however, plants demonstrate UV-B–specific photoreulatory responses regulated by an as-yet molecularly unidentified UV-B receptor that is different from photoreceptors responding to the visible part of the light spectrum (3–5). This pathway is characterized molecularly by the involvement of the UVR8 (UV RESISTANCE LOCUS 8) protein, which was recently shown to enhance survival under simulated sunlight with realistic UV-B levels (6). In contrast, no difference in the performance of uvr8 mutants and WT was seen when the UV radiation was filtered out (6).

UVR8 is a β-propeller protein with a sequence similarity to the eukaryotic guanine nucleotide exchange factor RCC1 (7). Although UVR8 has little in vitro exchange activity, it interacts with histones and is associated with chromatin of the ELONGATED HYPOCOTYL 5 (HY5) promoter region (8). Moreover, UV-B radiation stimulates the rapid nuclear accumulation of UVR8, which is necessary but not sufficient for its function (9). Recent data show that UVR8 interacts with the multifunctional E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) in a UV-B–dependent manner (6).

The COP1 protein comprises an N-terminal RING-finger domain, a coiled-coil region, and a C-terminal WD40-repeat domain (10). The protein–protein interaction with UVR8 depends on the WD40-repeat domain in COP1 (6). Both COP1 and UVR8 then impinge on the transcriptional activation of the HY5 gene, which encodes a bZIP transcription factor with a central function in the UV-B signaling pathway (6, 8, 11, 12).

In addition to the transcriptional activation, COP1-mediated degradation of HY5 protein is inhibited under UV-B, probably due to the interaction of UVR8 with COP1 (6, 12). Despite the recent identification of important positive players and pathways, the “brakes” in UV-B–specific signaling are not well known. The recently described ROOT UVB SENSITIVE 1 (RUS1) protein seems to negatively regulate a postulated UV-B response pathway that is restricted to roots and thus differs from the COP1/ UVR8 pathway (13). However, the UV-B–resistant but dwarfed phenotype of Arabidopsis lines overexpressing UVR8 clearly points to the need for tight control of the UV-B response in the latter pathway (6).

In response to visible light, the action of positive signaling factors downstream of the phytochrome (red/far-red) and cryptochrome (blue/UV-A) photoreceptors is counterbalanced by an important set of repressor proteins, including the four members of the SUPPRESSOR OF PHYA-105 (SPA) gene family and COP1, which interact and form complexes in vivo (14, 15). These proteins are repressors of light signaling in both dark-grown and light-grown seedlings, and their absence in mutant plants leads to marked dwarfism or seedling lethality (10, 15). In contrast, the COP1 protein positively regulates the UV-B–specific response independent of the SPA proteins (12).

Repressors of the COP1/UVR8-mediated UV-B–specific pathway were unknown until now. Here we describe two redundant UVR8-interacting WD40-repeat proteins, RUP1 and RUP2, that are important repressors of UV-B–induced photomorphogenesis and UV-B acclimation. These proteins play a crucial negative feedback regulatory role balancing UV-B–specific responses and ensuring normal plant growth.

Results

RUP1 and RUP2 Transcripts Are Rapidly and Transiently Induced by UV-B in a COP1–, UVR8–, and HY5-Dependent Manner. We previously analyzed specific responses to UV-B at the level of transcriptomic change (6, 11) and confirmed the transcriptional activation of several genes using the luciferase reporter (including At5g52250; see below) (16). We selected two genes induced early in response to narrowband UV-B irradiance encoding highly similar WD40-repeat proteins for detailed analysis. We
named these genes REDPRESSOR OF UV-B PHOTOMORPHOGENESIS (RUP) 1 and 2 (At5g2250 and At5g23730). Quantitative RT-PCR confirmed their early responsiveness to supplementary narrowband UV-B radiation (Fig. 1 A and B). Moreover, the UV-B-mediated up-regulation of both genes was found to depend on the presence of functional HY5, COP1, and UVR8 proteins (Fig. 1 A and B), showing these to be potential effectors of the main players.

The RUP1 (385 aa) and RUP2 (368 aa) proteins are highly homologous, with 63% identity in an overlap of 349 amino acids (Fig. S1). Both proteins consist of seven WD40-repeats with apparently no additional domains. In transgenic Arabidopsis lines that constitutively express RUP1-YFP and RUP2-YFP under control of the CaMV 35S-promoter, both RUP-YFP fusion proteins localized to the nucleus and the cytoplasm (Fig. S2A). Their subcellular localization was similar in continuous darkness, white light, and white light supplemented with UV-B radiation (Fig. S24). In agreement, RUP2-GFP protein expressed under its own promoter was detected in both cytosolic and nuclear fractions (Fig. S2). However, the very low expression levels of RUP2-GFP in this line prevented microscopic analysis of its subcellular localization. Thus, RUP gene expression is induced by UV-B downstream of the UVR8-COP1 pathway, and the constitutively overexpressed RUP-YFP fusion proteins localize to both nucleus and cytoplasm, independent of the light conditions.

RUP Proteins Interact with UVR8. Interestingly, the closest relatives of the RUP proteins, based on sequence conservation of WD40-repeat domains, are the structurally related COP1 and SPA proteins (Fig. 2A and Fig. S3). The SPA proteins are repressors of photomorphogenesis with no role in UV-B signaling, whereas the COP1 protein represses photomorphogenesis but promotes UV-B-specific signaling (12). Our previous results demonstrated that the UV-B-dependent interaction of UVR8 with COP1 depends on the WD40 domain of COP1 (6). This prompted us to investigate whether RUP proteins also interact directly with UVR8, using the bimolecular fluorescent complementation (BiFC) assay (17) in transiently transformed mustard hypocotyl cells (6). Reconstitution of a functional YFP signal from the complementary “split YFP” parts attached to the UVR8 and either RUP1 or RUP2 proteins was clearly identified (Fig. 2B). No YFP signal was seen when YC-RUP1 and YC-RUP2 were used in combination with empty vector controls, YN-COP1, or YN-RUP2 and YN-RUP1, respectively, indicating that the interaction was specific (Fig. 2B and Fig. S4). In contrast to the UV-B-dependent interaction of UVR8 with COP1 (6), the interaction with RUP1 and RUP2 occurred to the same extent under conditions devoid of UV-B radiation (Fig. 2B).

To further investigate RUP-UVR8 interaction in planta, we performed communoprecipitation experiments using transgenic
lines constitutively expressing YFP-tagged RUP2 in rup2-1 mutants. In agreement with the BiFC data, endogenous UVR8 protein was coimmunoprecipitated with RUP2-YFP from rup2-1/Pro_35S::RUP2-YFP, independent of UV-B radiation (Fig. 2C). In contrast, no coimmunoprecipitation of UVR8 was found for control plants (Col), and YFP-COP1 coimmunoprecipitated UVR8 only under supplemental UV-B (Fig. 2C), as described previously (6). In agreement with the transcriptional activation of the RUP2 gene by UV-B (Fig. 1B), a clear increase in RUP2-GFP protein level was detected in response to supplemental UV-B when RUP2-GFP was expressed under its own promoter (Pro_35S::RUP2-GFP, Fig. 2D). Accordingly, in this line, UVR8 coimmunoprecipitated RUP2-GFP only under supplemental UV-B when the latter was expressed (Fig. 2E). Thus, the RUP proteins are UV-B–induced UVR8-interacting proteins that likely play a role in UV-B signaling.

**rup1 rup2 Double Mutants Are Hypersensitive to Photomorphogenic UV-B Radiation.** To analyze the involvement of the two RUP proteins in UVR8-mediated UV-B signaling, we isolated homozygous knockout mutants for the *RUP1* and *RUP2* genes. RUP1 and RUP2 are encoded by intronless genes, and we identified T-DNA insertional mutants with impaired functionality of both proteins (Fig. S5A). Indeed, in both mutants, the T-DNA insertion led to the absence of the respective mRNAs, as determined by RNA gel blot analysis (Fig. S5B). Because RUP1 and RUP2 probably are functionally redundant, as suggested by their high sequence conservation, we also generated a double-null mutant. As expected, both transcripts were missing from the *rup1 rup2* mutant (Fig. S5B).

Interestingly, *rup1 rup2* double mutants showed a strong hypersensitivity to supplemental narrowband UV-B radiation at the level of hypocotyl growth inhibition (Fig. 3A), and flavonoid (Fig. 3B) and anthocyanin accumulation (Fig. 3D and Fig. S5C). The *rup1* single mutant was similar to WT, whereas the *rup2* single mutant already showed weak UV-B hypersensitivity (Fig. 3B). The UV-B hypersensitivity of the *rup1 rup2* double mutant also was reflected in the molecular analysis, which showed more strongly induced UV-B–responsive *HY5* and *CHS* gene expression than in the WT (Fig. S5D and E). The gene expression data also were reflected in the differences in *HY5* and *CHS* protein levels under UV-B (Fig. 3C). There was no major difference in UVR8 protein level between the *rup1 rup2* double mutant and WT, indicating that *rup1 rup2* hypersensitivity is not due to elevated UVR8 levels (Fig. 3C).

It should be noted that the *RUP1* and *RUP2* genes are also transcriptionally activated by red, far-red, and blue light, indicating a more general role in light responses (Fig. S6A). However, our initial analysis found no involvement of RUP1 and RUP2 in the response to these wavelengths at the level of hypocotyl growth inhibition and expression of the *HY5* and *CHS* marker genes (Fig. S6B–F). Thus, we conclude that RUP1 and RUP2 proteins have a major negative regulatory function in the UV-B photoregulatory response of *Arabidopsis*.

**UV-B Hypersensitivity of rup1 rup2 Depends on Functional UVR8 and HY5 Proteins.** To examine whether the UV-B hypersensitivity of the *rup1 rup2* mutant requires *HY5* and UVR8 proteins, we generated *rup1 rup2 hy5* and *rup1 rup2 uvr8* triple mutants and analyzed their UV-B responses. Measurement of UV-B–induced anthocyanin accumulation as well as *HY5* and *CHS* gene expression showed that both *uvr8* and *hy5* suppress the *rup1 rup2* mutant phenotype and thus are epistatic to *rup1 rup2* (Fig. 3D–F). Thus, both UVR8 and HY5 proteins are required for UV-B hypersensitivity in *rup1 rup2* double mutants.

**RUP2 Overexpression Results in Blockage of UV-B–Specific Signaling.** Because the genetic loss-of-function data strongly indicated a redundant function for RUP1 and RUP2 as crucial repressors of UV-B–induced photomorphogenesis, we generated CaMV 35S promoter–driven *RUP2* overexpression lines. *RUP2* overexpression resulted in a strong block of UV-B–induced expression of the *HY5* and *CHS* marker genes (Fig. 4A and B), strongly supporting RUP2’s role as a repressor of UV-B signaling. Protein gel blot analysis confirmed the repressive effect on CHS and HY5 at the protein level and demonstrated that the hypersensitivity is not due to down-regulation of UVR8 protein in the *RUP2* overexpression lines (Fig. 4C).

**rup1 rup2 Acclimatizes Better to UV-B than WT, and RUP2 Overexpression Lines Are Impaired in UV-B Tolerance.** Taken together, our data indicate an important role of the RUP proteins as...
repressors of the UVR8/COP1-mediated UV-B photomorphogenic pathway. We previously showed that UV-B acclimation is absent in uvr8 mutants and enhanced in UVR8 overexpression lines (6). Because our data indicated similar phenotypes for RUP2 overexpression and the uvr8 mutation and for UVR8 overexpression and the rup1 rup2 double mutation, we directly tested the importance of RUP proteins for UV-B acclimation by combining weak, narrowband UV-B exposure with subsequent broadband UV-B stress. As described previously (6), exposure of WT seedlings for 7 d to narrowband UV-B that activated photomorphogenic responses resulted in enhanced tolerance to a subsequent broadband UV-B stress treatment (Fig. 5A). This acclimation effect was absent in RUP2 overexpression lines and enhanced in rup1 rup2 double mutants (Fig. 5A and B).

To examine the importance of the RUP proteins under more realistic conditions, we grew plants in sun simulators with a natural spectral balance throughout the UV-to-infrared spectrum (18). Under these realistic conditions, rup1 rup2 mutant plants were clearly tolerant to UV-B radiation but were dwarfed and dark green (Fig. 5C), very similar to the UVR8 overexpressor phenotype described previously (6). The UV-B response mediated by the COP1/UVR8 pathway is associated with the accumulation of flavonol glycosides; thus, we quantified the relative levels of the flavonol quercetin under the sun simulator growth conditions. In WT Col, we found an ~5-fold UV-B–mediated increase in quercetin level, similar to that in rup1 and rup2 single mutants (Fig. 5D). In contrast, the UV-B response at the level of quercetin accumulation was increased by ~11-fold in the rup1 rup2 double mutant (Fig. 5D). It also should be noted that although the RUP2 overexpression lines demonstrated no dramatic difference from WT in overall growth phenotype, they did exhibit a reduced UV-B response in terms of quercetin accumulation (Fig. 5D). Thus, we conclude that the RUP proteins influence the important balance between two connected products of UV-B–specific signaling, namely growth inhibition and the mounting of UV-B defense measures.

Fig. 4. Overexpression of RUP2 represses the UV-B response. (A and B) Quantitative RT-PCR analysis of HY5 (A) and CHS (B) gene activation in response to UV-B in two independent RUP2 overexpression lines compared with WT Col and the rup2-1 single mutant. Error bars represent the SD of technical triplicates. (C) Immunoblot analysis of UVR8, HY5, CHS, and actin (loading control) protein levels. In A–C, 4-d-old seedlings were irradiated with UV-B for the indicated times before harvesting. RUP2Ox#3/#5=ProUVR8::RUP2 in rup2-1, lines 3 and 5.

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Discussion

The survival of sessile plants in sunlight is ensured by UV-protective responses that are largely regulated by the UV-B-specific UVR8–COP1 pathway. Activation of these responses must be well balanced; a reduced response results in UV-B damage and cellular death (as in the uvr8 mutant), whereas an exaggerated response results in impaired growth and dwarfism (as produced by UVR8 overexpression) (6). Here we show that the Arabidopsis RUP1 and RUP2 proteins are crucial repressors of UV-B–induced photomorphogenesis that result in an adequate and balanced UVR8/COP1-mediated UV-B response.

Previous work has shown that specific perception of UV-B radiation by a postulated UV-B receptor results in rapid UVR8–COP1 interaction (6). This allows the COP1/UVR8-mediated activation of numerous genes, including HY5, which confers UV acclimation and protection (6, 8, 12), and RUP1 and RUP2, which provide negative feedback regulation through direct interaction with UVR8 (Fig. 2 D and E; see also the model shown in Fig. S7). The latter two genes encode β-propeller proteins belonging to the very diverse superfamily of WD40-repeat regulatory proteins, which comprises 237 potential proteins in Arabidopsis containing four or more copies of the WD40 motif (19). The common defining feature of these proteins is an ∼40-aa stretch typically ending in Trp-Asp (WD), but there is only limited amino acid sequence conservation otherwise. In many instances, repeated WD40 motifs act as sites for protein–protein interaction, and many proteins containing WD40 repeats are known to serve as platforms for the assembly of protein complexes (19). The two RUP proteins show significant sequence conservation in their seven WD40-repeat domains with the COP1 and SPA1–SPA4 proteins, including a conserved 16-aa DWD (DDBD-binding WD40) motif (20, 21). Members of the DWD motif–containing subset of WD40 proteins were shown to act as substrate receptors for DDDB1CU14-ROC1-based E3 ubiquitin ligases (22). Such a function remains to be described for the RUP proteins, however. Nevertheless, a phylogenetic analysis based on the WD40-repeat region indicated that the most closely related sequences to RUP1 and RUP2 encoded in the Arabidopsis genome are the SPA proteins and COP1, with which they share about 33% and 37% identity in the WD40 domain, respectively. COP1 and SPA protein family members function as repressors under visible light devoid of UV-B (10, 15), a light environment prevalent in laboratory experiments, which neglects the influence of the UV-B radiation intrinsic to sunlight. A detailed understanding of the regulatory role of UV-B is needed to understand the control by light of plant growth and development, however. Recent experiments using sun simulator conditions to analyze the performance of the uvr8 mutant and UVR8 overexpression lines demonstrated the importance of the UV-B–specific photoregulatory pathway (6). The related phenotypes of RUP2 overexpression and rup1 rup2 double mutants described here demonstrate the importance of these UV-B–specific repressors under UV-B radiation, similar to the COP1 and SPA proteins in conditions devoid of UV-B. Indeed, the phenotype of the rup1 rup2 double mutant under supplemental UV-B radiation (e.g., short hypocotyl, dwarfism, high anthocyanin) is very reminiscent of the cop1 and combinatorial spo mutants grown under white light without UV-B.

The SPA–COP1 E3 ligase complexes are a point of convergence downstream of multiple light signals and constitute a central repressor of photomorphogenesis that is inactivated by visible light in an as-yet unknown molecular manner (14). Interestingly, the coiled-coil domain and the WD40-repeat domain of SPA1 are sufficient for its function, and the kinase-related domain apparently is not required (23, 24). Arabidopsis COP1 interacts with the four members of the SPA protein family (SPA1–SPA4) with their coiled-coil domains (25). In contrast, RUP proteins have no coiled-coil domain. In agreement with this, we found no interaction with COP1. However, similar to the activation of RUP1 and RUP2 by UV-B, the levels of SPA1, SPA3, and SPA4 transcripts were increased by red, far-red, and blue light, consistent with a negative feedback role in light-grown seedlings (23).

In contrast to RUP1 and RUP2, expression of the other main factors (COP1 and UVR8) responsible for the UV-B response in Arabidopsis is constitutive and not regulated by UV-B. Nevertheless, a change in the abundance of interacting proteins, such as the RUP1 and RUP2 (or SPA) proteins, in response to an exogenous signal could alter the activity and/or specificity of the complex as a whole. Thus, the regulation of RUP1 and RUP2 expression seems crucial to the adjustment of plant growth and development to changes in the light environment. Our data suggest that a rapid increase in RUP1 and RUP2 abundance is necessary to prevent overstimulation when seedlings are exposed to UV-B.

Interestingly, RNAi of a gene encoding a RUP-related protein in tomato, LeCOP1LIKE, results in field-grown plants with exaggerated photomorphogenesis, dark-green leaves, and elevated fruit carotenoid levels (26). This finding led to the conclusion that LeCOP1LIKE is involved in light signal transduction and functions as a negative regulator of fruit pigmentation. However, it should be noted that the closest homolog of LeCOP1LIKE in Arabidopsis is RUP1, and thus the enhanced photomorphogenesis described for the LeCOP1LIKE-RNAi lines under natural conditions in the field might be linked instead to its potential function as a repressor of UV-B signaling in tomato. Thus, manipulating UV-B signaling might provide another way to modify the nutrient quality of plants (27).

In summary, we have shown that the UV-B–specific response impinging on plant growth is precisely balanced by the UV-B–activated and UVR8-interacting RUP1 and RUP2 proteins. RUP1 and RUP2 are early responsive genes that function as negative regulators of the UV-B response in Arabidopsis through direct interaction with the UVR8 protein. This negative feedback loop prevents an exaggerated photomorphogenic UV-B response that would strongly affect plant growth and development.

Materials and Methods

Plant Material and UV-B Irradiation. cop1–1, hy5–215, uvr8–6, rup1–1 (SALK_060638), and rup2–1 (SALK_108845) are in the Columbia ecotype (Col) (6, 28–30). Plants were grown and irradiated exactly as described previously (6, 12). The condition of the treatments in the sun simulator was a 14-h day period with mean photosynthetically active radiation (400–700 nm) of 730 μmol m−2 s−1 and 12 h of UV-B irradiance with a biologically effective dose of 500 mW m−2 [weighted by the generalized plant action spectrum (31), normalized at 300 nm]. Controls were grown excluding the entire UV radiation spectrum. Spectroradiometric measurements were performed using a double-monochromator system (model DTM-300; Bentham) and are shown in Fig. S8. The temperature was maintained at 23 °C during the day and 18 °C at night. The relative humidity was kept constant at 60%.

RNA Extraction and Analysis by Real-Time PCR. Arabidopsis RNA was isolated with the Plant RNeasy Kit (Qiagen) according to the manufacturer’s instructions. Quantitative real-time PCR was performed in a 96-well format using the 7300 Real-Time PCR System and TaqMan probes (Applied Biosystems). cDNA was synthesized from 50 ng of RNA with random hexamers using the TaqMan Reverse-Transcription Reagent Kit (Applied Biosystems). Quantitative PCR reactions were performed using the Absolute QPCR Rox Mix Kit (Abgene), following the manufacturer’s instructions. The gene-specific probes and primers were as follows: RUP1 (AT5g52250), probe 6-FAM-CGCATCCACCGGACAGCGTT-TGTTTTC-3′ and RUP1–rev (5′-TCCGTTAGGTCGACGTAGT-3′); RUP2 (AT5g23-730), probe 6-FAM-TGCTACCGGGGCTATTCCAGA-TAMRA with RUP2–for (5′-TATTGACATCCATGATACAAG-3′) and RUP2–rev (5′-AGGGACGGCTAAGAAGAAGA-3′) and CHS (At5g11260) and HY5 (At5g11260) as described previously (5). cDNA concentrations were normalized to a standard of 18S rRNA transcript levels using the Eukaryotic 18S rRNA Kit (Applied Biosystems). Expression was determined in triplicate.
Immunoprecipitation Assays and Protein Gel Blot Analysis. Immunoprecipitation of YFP-COP1 and RUP2-YFP using monoclonal anti-GFP antibodies (Invitrogen) and protein A-agarose (Roche Applied Science) was performed as described previously (6). For protein gel blot analysis, total cellular proteins (10 μg) or immunoprecipitates were separated by electrophoresis in 10% SDS-polyacrylamide gels and electrophoretically transferred to PVDf membranes according to the manufacturer’s instructions (Bio-Rad). Polyclonal anti-UVR8 (6), anti-HYS (12), anti-actin (Sigma-Aldrich), anti-CHS (Santa Cruz Biotechnology), and monoclonal anti-GFP (BABC0) were used as primary antibodies, with HRP-conjugated protein A (Pierce) or anti-rabbit, anti-goat, and anti-mouse immunoglobulins (DAKo) used as secondary antibodies. Signal detection was performed using the ECL Plus Western Detection Kit (GE Healthcare).

Extraction and Measurement of Flavonoids and Anthocyanins. Anthocyanins were extracted and quantified as described previously (32). Flavonoid measurements were analyzed as described previously (12). Experiments were carried out at least in triplicate. Analysis of the soluble phenolic compound quercetin from f1oc12 was performed by reversed-phase-HPLC as described previously (33).

Bimolecular Fluorescence Complementation. The RUP1 and RUP2 coding sequences were transferred into Gateway-compatible BiFC vectors, pE-SPYNE-GW and pE-SPYCE-GW (kindly provided by W. Drögel-Laser, University of Göttingen). Transient transformation of mustard seedlings using the Biolistic PDS-1000/He System (Bio-Rad) and BiFC assays were carried out as described previously (34).

Epifluorescence and Light Microscopy. For epifluorescence and light microscopy, the seedlings were transferred to glass slides and examined with a Zeiss Axioskop II microscope. Excitation and detection of YFP and CFP were performed with standard YFP and CFP filter sets, respectively (AHF analysetechnik). Documentation of representative cells was done by photography using a Zeiss Axiocam digital camera system.

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hy5-215 (3): HY5_for3 (5′-TAA GAA AAA TGG AGC AAC-3′) + HY5_rev4 (5′-CTC ATC GCT TTT AAC CCT-3′) = 0.34kb WT; HY5_for3 + hy5-215 rev (5′-CTC ATC GCT TTT AAC CCT-3′) = 0.34kb for hy5-215.

rup1-1 (SALK_060638): At5g52250_for1 (5′-CCG GGC AAA CTT AGT AGT C-3′) + S_108846_RP (5′-CTT GAA GAA GGT CAT TCC CA-3′) = 1kb WT; S_108846_RP + LBa1 = 0.69kb for rup1-1.

rup2-1 (SALK_108846): S_108846_LP (5′-CCG GGC AAA CTT AGT AGT C-3′) + S_108846_RP (5′-CTT GAA GAA GGT CAT TCC CA-3′) = 1kb WT; S_108846_RP + LBa1 = 0.69kb for rup2-1.

uvr8-6 (SALK_033468) (4): UVR8_for5 (5′-AGG AGT GAT ATG CAT TC-3′) + UVR8_rev6 (5′-TCC CAA ACT AGA CAG C-3′) = 1.26kb WT; UVR8_for5 + LBa1 = 0.67kb for uvr8-6.

Cloning of RUP1 and RUP2 Genes and Generation of Transgenic Arabidopsis Lines. The WT RUP2 genomic fragment and RUP1 and RUP2 ORFs were cloned into pDONR207 and sequenced to check the integrity of the cloned fragment; the primers are described below. Gateway-based cloning was then used to insert the ORF into the binary destination vectors pB2GW7 and pB7WGY2 (5) and insert the RUP2 genomic clone into pMDC107 (6). The constructs were verified by sequencing, and Arabidopsis plants were transformed using Agrobacterium. The resulting transgenic lines described in this work have the transgene integrated at a single locus.

The following primers were used to amplify and clone RUP1 and RUP2 coding sequences into the pDONR207 vector (Invitrogen):

RUP1 full-length –STOP (1,145 bp): RUP1-BP_for (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC AAT GGA GGC TTT GTT CTT C-3′) and RUP1-BP_rev (5′-GGG GAC CAC TTT GTA CAA AAA AGC TGG GTC TGT TGG TTT GCC GAA-3′)

RUP1 full-length +STOP (1,148 bp): RUP1-BP_for (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC AAT GGA GGC TTT GTT CTT C-3′) and RUP1-BPMD_rev + Stop (5′-GGG GAC CAC TTT GTA CAA AAA AGC TGG GTC TTA GCT TTT TGG GCC GAA-3′)

RUP2 full-length –STOP (1,102 bp): RUP2-BP_for (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC AAT GGA CAC TCT TCA TCC T-3′) and RUP2-BP_rev (5′-GGG GAC CAC TTT GTA CAA AAA AGC TGG GTC TGT TTT TCC GCC CAC-3′)

RUP2 full-length +STOP (1,105 bp): RUP2-BP_for (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC AAT GGA CAC TCT TCA TCC T-3′) and RUP2-BP_rev + Stop (5′-GGG GAC CAC TTT GTA CAA AAA AGC TGG GTC TTA GCT TTT TCC GCC CAC-3′)

The following primers were used to amplify and clone the RUP2 genomic fragment:

RUP2 genomic –STOP (2,671 bp): RUP2gen-BP_for (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC AAT GGA CAC TCT TCA TCC T-3′) and RUP2gen-BP_rev (5′-GGG GAC CAC TTT GTA CAA AAA AGC TGG GTC TTA GCT TTT TCC GCC CAC GAA-3′)

Nuclear Protein Extraction. Total protein was extracted from 12-d-old seedlings after a 6-h exposure to supplemental narrowband UV-B in extraction buffer [20 mM MOPS (pH 7), 0.5 M hexylene glycol, 10 mM MgCl2, 5 mM β-mercaptoethanol, and one Complete Mini Protease Inhibitor Mixture Tablet (Roche) added per 10 mL of buffer]. The homogenate was passed through four layers of Miracloth (Calbiochem), after which Triton X-100 was added until a 0.5% final concentration was achieved. The total extract was centrifuged for 10 min at 1,000 × g at 4 °C. The supernatant was collected as cytosolic fraction and the pellet as nuclear fraction. The nuclear fraction was washed three times with extraction buffer. The protein concentrations of the total, nuclear, and cytosolic fractions were determined by the Amido black method, and equal amounts of protein were loaded onto an 8% SDS/PAGE gel.

Phylogeny. The evolutionary history based on the WD40-repeats of the 85 Arabidopsis DWD proteins was inferred using the neighbor-joining method (7). The optimal tree with the sum of branch length 66.52398388 is shown in Fig. S3. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and represent the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 492 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (8).

Fig. S1. Amino acid sequence alignment of Arabidopsis RUP1 and RUP2 proteins. Identical and similar amino acids are highlighted in black and gray, respectively. Dashes indicate gaps in the sequence to optimize the alignment.

Fig. S2. Fluorescent protein–tagged RUP1 and RUP2 proteins show nuclear and cytosolic localization. (A) Subcellular localization of RUP1-YFP and RUP2-YFP in stably transformed Arabidopsis plants. Representative cells of 4-d-old seedlings grown in darkness (D), continuous white light (−UV), and continuous white light with supplementary narrowband UV-B (+UV) are shown. (Scale bar: 10 μm.) (B) Protein gel blot of total protein, and cytosolic (cyt.) and nuclear (nuc.) fractions of rup1 rup2/ProRUP2-GFP plants grown in white light supplemented with narrowband UV-B probed with anti-GFP, anti-UGPase (cytosolic control), and anti-histone H3 (nuclear control) antibodies.
Fig. S3. A neighbor-joining phylogenetic tree constructed from 85 *Arabidopsis* DWD proteins containing WD40-repeats (according to ref. 1).

Fig. S4. Additional controls for BiFC assays with empty vectors YC-RUP1 and YC-RUP2.

Fig. S5. (A) Structure of RUP1 and RUP2, and the location of T-DNA insertions in rup1-1 and rup2-1. (B) RNA gel blot analysis of WT (Col), rup1-1 and rup2-1 single mutants, and rup1 rup2 double mutants. Total RNA was isolated from 4-d-old seedlings irradiated with UV-B for the indicated times before harvesting. Blots were sequentially hybridized with specific probes for RUP1 and RUP2. Ethidium bromide–stained rRNA is shown as a loading control. (C) Seedlings were grown in darkness, under white light (−UV-B), or under white light supplemented with UV-B (+UV-B). The extracts used for anthocyanin measurements are shown below the pictures of the seedlings. (D and E) Quantitative RT-PCR analysis of HY5 (D) and CHS (E) gene activation in response to UV-B in rup1 rup2 double mutants compared with WT Col. Four-day-old seedlings were irradiated with UV-B for the indicated times before harvesting. Error bars represent the SD of three biological repetitions.
Fig. S6. \textit{RUP1} and \textit{RUP2} genes are red light (R)-, far-red light (FR)-, and blue light (B)-inducible, but \textit{rup1 rup2} double mutants are not affected in hypocotyl growth inhibition under these light qualities. (A) Quantitative RT-PCR analysis of \textit{RUP1} and \textit{RUP2} gene activation in response to R, FR, and B light by WT Col. Four-day-old etiolated seedlings grown in darkness were irradiated for 1 and 6 h with the indicated light qualities and compared with the dark control. (B–D) Hypocotyl length measurements of WT and \textit{rup1 rup2} double mutants grown for 4 d under different FR (B), R (C), and B (D) photon fluence rates compared with \textit{phyA}, \textit{phyB}, and \textit{cry1 cry2} mutants, respectively (\(n = 30\)). (E) Quantitative RT-PCR of \textit{HY5} gene activation in response to 1 h of B, R, and FR compared with control samples that were kept in the dark (D). (F) Quantitative RT-PCR of \textit{CHS} gene activation in response to 6 h of B, R, and FR. In E and F, 4-d-old etiolated seedlings of WT, \textit{rup1 rup2} double mutant, and \textit{rup2-1/Pro}\textsubscript{35S}:\textit{RUP2} overexpression lines were irradiated with B (38.5 \(\mu\text{mol m}^{-2} \text{s}^{-1}\)), R (38.9 \(\mu\text{mol m}^{-2} \text{s}^{-1}\)), and FR (20.0 \(\mu\text{mol m}^{-2} \text{s}^{-1}\)) for the indicated time. Data from control seedlings kept in the dark (D) were set at 1. Error bars represent SD of technical triplicates.

Fig. S7. Model of the \textit{RUP}-mediated feedback that represses the UV-B photoregulatory pathway. Under supplementary UV-B (WL + UVB), the specific perception by a UV-B photoreceptor results in rapid UVR8–COP1 interaction. UV-B perception results in stabilization of the HY5 bZIP transcription factor and the COP1/UVR8-mediated activation of \textit{HY5} gene expression. Activation of \textit{RUP1} and \textit{RUP2} gene expression in response to UV-B requires UVR8, COP1, and HY5 proteins. \textit{RUP1} and \textit{RUP2} protein accumulation then provides a negative feedback regulation of the UV-B photoregulatory pathway through direct interaction with UVR8.
Fig. S8. Spectral irradiances of the study in the sun simulator for control and UV treatment. (Insert) UV range of 270–400 nm in logarithmic scale.