Dimer/monomer status and in vivo function of salt-bridge mutants of the plant UV-B photoreceptor UVR8

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SUMMARY

UV RESISTANCE LOCUS8 (UVR8) is a photoreceptor for ultraviolet-B (UV-B) light that initiates photomorphogenic responses in plants. UV-B photoreception causes rapid dissociation of dimeric UVR8 into monomers that interact with CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) to initiate signal transduction. Experiments with purified UVR8 show that the dimer is maintained by salt-bridge interactions between specific charged amino acids across the dimer interface. However, little is known about the importance of these charged amino acids in determining dimer/monomer status and UVR8 function in plants. Here we evaluate the use of different methods to examine dimer/monomer status of UVR8 and show that mutations of several salt-bridge amino acids affect dimer/monomer status, interaction with COP1 and photoreceptor function of UVR8 in vivo. In particular, the salt-bridges formed between arginine 286 and aspartates 96 and 107 are key to dimer formation. Mutation of arginine 286 to alanine impairs dimer formation, interaction with COP1 and photoreceptor function of UVR8 in vivo. Notably, a UVR8 mutant in which aspartates 96 and 107 are conservatively mutated to asparagine is strongly impaired in dimer formation but mediates UV-B responses in vivo with a similar dose–response relationship to wild-type. The UV-B responsiveness of this mutant does not correlate with dimer formation and monomeration, indicating that monomeric UVR8 has the potential for UV-B photoreception, initiating signal transduction and responses in plants.

Keywords: UV-B, UVR8, photoreceptor, photomorphogenesis, Arabidopsis thaliana.

INTRODUCTION

Ultraviolet-B (UV-B) wavelengths (280–315 nm) in sunlight have a major impact on the biosphere. The high energy of UV-B radiation has the potential to damage molecules such as DNA, impair cellular activities and cause cell death. However, plants have evolved effective mechanisms to protect themselves from damage by UV-B, which enable them to survive constant exposure to sunlight. In particular, plants synthesize UV-absorbing sunscreen compounds that are deposited in the outer tissues, and they employ efficient cellular systems for repairing damage by UV-B (Frohnmeyer and Staiger, 2003; Ulm and Nagy, 2005; Jenkins, 2009). The exposure of plants to low doses of UV-B stimulates transcriptional responses that underpin UV-protection (Ulm et al., 2004; Brown et al., 2005; Favory et al., 2009). Furthermore, UV-B acts as a regulatory stimulus for other responses in plants, including the suppression of extension growth (Favory et al., 2009; Hayes et al., 2014), entrainment of the circadian clock (Feher et al., 2011) and defence against insect herbivory (Ballaré et al., 2012).

Regulatory responses to UV-B are mediated by the photoreceptor UV RESISTANCE LOCUS8 (UVR8; Brown et al., 2005; Favory et al., 2009; Rizzini et al., 2011; Tilbrook et al., 2013; Jenkins, 2014a). Arabidopsis uvr8 mutant plants are defective in photomorphogenic responses to UV-B and are highly susceptible to damage by UV-B because they are unable to stimulate expression of genes concerned with UV-protection (Kliebenstein et al., 2002; Brown et al., 2005; Favory et al., 2009). UVR8 is a 7-bladed β-propeller protein that is present as a homo-dimer in plants (Rizzini et al., 2011; Christie et al., 2012; O’Hara and Jenkins, 2012; Wu et al., 2012; Jenkins, 2014b). Unlike other photoreceptors, UVR8 does not use a prosthetic chromophore for light
sensing, instead specific tryptophan amino acids of UVR8 act as chromophores for UV-B detection (Rizzini et al., 2011; Christie et al., 2012; O’Hara and Jenkins, 2012; Wu et al., 2012; Zeng et al., 2015). Photoreception induces rapid dissociation of the dimer into monomers (Rizzini et al., 2011; Christie et al., 2012; Wu et al., 2012), which then interact with the CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) protein to initiate signaling and hence regulate transcription of target genes involved in UVR8-mediated responses (Favory et al., 2009; Rizzini et al., 2011; Cloix et al., 2012). One of the genes most rapidly induced by UV-B following UVR8 photoreception encodes the transcription factor ELODANCED HYPOCOTYL5 (HY5). This transcription factor, sometimes acting with the related HY5 HOMOLOG (HYH) is a key effector of transcriptional responses regulated by UVR8 (Brown et al., 2005; Oravecz et al., 2006; Brown and Jenkins, 2008; Favory et al., 2009; Huang et al., 2013). In addition, UVR8 photoreception stimulates expression of genes encoding the REPRESSOR OFboiled (Rizzini et al., 2012). Hydrophobic interactions between the monomers are negligible. The dimerisation surface of each monomer is rich in basic amino acids, notably arginine, and acidic aspartate and glutamate residues. These amino acids are distributed such that patches of complementary electrostatic potential are aligned opposite each other across the dimer interface. The salt-bridge interactions are sufficiently strong that the dimer is maintained even in the presence of SDS, as long as the protein sample is not boiled (Rizzini et al., 2011; Christie et al., 2012; Wu et al., 2012). However, monomerisation occurs when salt-bridges are neutralised by low pH and high ionic strength (Christie et al., 2012; Wu et al., 2012). Moreover, studies with purified UVR8 expressed in Escherichia coli show that mutation of particular salt-bridging amino acids prevents dimer formation (Christie et al., 2012; Wu et al., 2012).

Inevitably the molecular environment of UVR8 in cells will differ from that in vitro. It is therefore essential to establish whether findings with the purified protein extend to UVR8 in plants. Hence, the aim of the present study was to determine whether particular salt-bridge amino acids are important in maintaining UVR8 structure in plants and to assess the consequences of altered dimer/monomer status on UVR8 function in vivo. We show that specific arginine and aspartate amino acids at the UVR8 dimerisation surface are required for dimer formation in transgenic Arabidopsis plants. However, although mutants in some of these amino acids are impaired in dimer formation both in vitro and in vivo, they are nevertheless able to functionally complement Arabidopsis uvr8-1 plants. This study highlights the methodological difficulty of establishing the dimer/monomer status of a UVR8 mutant protein in plants, and provides evidence that UVR8 can perceive UV-B and initiate signaling even in its monomeric form.

RESULTS

Dimer/monomer status of selected UVR8 salt-bridge mutant proteins

We wished to study the effects of mutations of several charged amino acids at the dimer interface on UVR8 dimer/monomer status in vivo. The selection of amino acids and mutations was based on examination of the UVR8 crystal structure and on biochemical studies of purified UVR8 mutant proteins, which identified amino acids that are critical for dimer formation in vitro (Christie et al., 2012; Wu et al., 2012). A key residue is arginine R286, which is adjacent to the principal chromophore tryptophan, W285, and forms double and single H-bonded salt-bridges, respectively, with aspartates D107 and D96 (Figure 1a). Size exclusion chromatography (SEC) shows that purified wild-type UVR8 is a dimer when not exposed to UV-B and a monomer following UV-B exposure, whereas mutation of either R286 to alanine (UVR8R286A) or D96 and D107 to asparagine (UVR8D96N,D107N) causes UVR8 to become constitutively monomeric in vitro (Figure 1b; Christie et al., 2012; Wu et al., 2012). In contrast, UVR8R286K, in which R286 is conservatively mutated to positively charged lysine, appears dimeric and monomerises in response to UV-B (Figure 1b). The SEC elution volume of the UVR8R286K dimer differs from that of wild-type UVR8, most likely because of a change in the hydrodynamic radius (shape) of the protein; this difference is not evident when the salt concentration is increased (Figure S1). Similar to R286, R146 forms a double H-bonded salt-bridge, in this case with E182 (Figure S2a). However, in contrast to UVR8R286A, UVR8R146A is a dimer in vitro that monomerises in response to UV-B (Christie et al., 2012; Wu et al., 2012; Figure S2b). R234, which is adjacent to UV-B chromophore W233, forms an intra-molecular salt-bridge with E182 (Figure S2a). UVR8R234A adopts a conformations in vitro that is non-responsive to UV-B (Figure S2c), most likely because the mutation could disrupt the spatial arrangement of the chromophore tryptophans, impairing UV-B photoreception. R338 is adjacent to the triad tryptophan W337 and forms a single hydrogen-bonded salt-bridge with D44 and a non hydrogen-bonded ionic interaction with E43, as well as a water mediated hydrogen bond with its backbone carbonyl (Figure S3a). The UVR8R338A mutant is reported to be constitutively monomeric in vitro (Wu et al., 2012). However, the dimer/
monomer status of UVR8R338A in vitro is dependent on the salt concentration; it is monomeric in 500 mM NaCl but appears to be in equilibrium between dimer and monomer in low salt concentrations (Figure S3b).

**Mutation of key UVR8 salt-bridge amino acids impairs dimer formation in plants**

The mutant UVR8 proteins described above were expressed as GFP fusions in the null uvr8-1 mutant. Several transgenic lines were obtained for each mutant and compared with a control GFP-UVR8 fusion that was shown previously to functionally complement uvr8-1 (Brown et al., 2005; Kaiserli and Jenkins, 2007; Figure S4). The dimer/monomer status of the mutant UVR8 proteins was examined initially using SDS–PAGE with non-boiled samples. This assay shows that wild-type GFP-UVR8 is a dimer that monomerises after UV-B exposure, but in contrast each mutant protein appears constitutively monomeric (Figure 2a). However, this method very sensitively detects even slight weakening of the dimer and does not rigorously determine dimer/monomer status; for instance, purified UVR8 mutant proteins, such as UVR8R146A and UVR8R286K, which appear dimeric in the absence of UV-B when examined by SEC (Figures 1b and S2b), appear constitutively monomeric in the SDS–PAGE assay (Figure S5). We therefore used additional methods to examine dimer/monomer status of the mutant proteins.

We expressed each GFP-tagged UVR8 mutant protein transiently in Nicotiana leaves, immunoprecipitated the protein from an extract and used SEC to determine its dimer/monomer status. In this assay, wild-type GFP-UVR8 is a dimer that monomerises after UV-B exposure of the protein extracts to UV-B (Figure 2b). In contrast, each of the mutant proteins is constitutively monomeric in this assay. However, the conditions used to obtain immunoprecipitated UVR8 could promote monomerisation of mutants with weak dimers and so the results may not reflect the dimer/monomer status of the mutant proteins in planta.

We therefore used cross-linking with dithiobis(succinimidylpropionate) (DSP), to establish whether UVR8 mutant proteins are dimeric or monomeric in plants. This method has been used previously to show that wild-type UVR8 forms a dimer that dissociates into monomers following UV-B exposure (Rizzini et al., 2011). In the experiment shown in Figure 2(c), the cross-linking agent was added to protein extracts of plants not exposed to UV-B. Wild-type GFP-UVR8 is dimeric in this assay, as is GFP-UVR8R146A, GFP-UVR8R286K appears as a dimer with some monomer present, consistent with SEC of the purified protein at elevated salt concentrations (Figure S1). However, for both GFP-UVR8R286A and GFP-UVR8D96N,D107N, only monomeric protein is detectable.

**Some, but not all, salt-bridge mutants interact constitutively with COP1**

UV-B photoreception stimulates monomerisation and interaction of UVR8 with COP1 to initiate signaling (Rizzini et al., 2011). COP1 interacts with a 27 amino acid region near the C-terminus of UVR8 and also with the β-propeller core of the protein (Cloix et al., 2012; Yin et al., 2015). It is proposed that the C-terminus becomes accessible to COP1 following UV-B exposure of UVR8 (Cloix et al., 2012). However, some tryptophan mutants of UVR8 bind COP1 in the absence of UV-B (O’Hara and Jenkins, 2012; Heijde et al., 2013; Huang et al., 2013), suggesting that these mutations expose the C-terminus. Because several of the salt-bridge mutants have weakened dimers, we examined their interaction with COP1, which impacts on their potential ability to function.

We examined whether the salt-bridge mutants were able to interact with COP1 using a co-immunoprecipitation assay. As shown in Figure 3, GFP-UVR8 interacted with COP1 in the presence but not the absence of UV-B, as
Some salt-bridge mutants are functional in plants

To test whether the UVR8 mutants are functional in initiating photomorphogenic responses to UV-B, we examined both the suppression of hypocotyl extension and the induction of gene expression, assaying specifically HY5 and CHS transcript levels and CHS protein accumulation.

As shown in Figure 4(a), hypocotyl growth is suppressed by a low fluence rate of narrowband UV-B in wild-type plants and in the control GFP-UVR8 transgenic line, but the response is impaired in uvr8-1 plants (Favory et al., 2009; Cloix et al., 2012). The GFP-UVR8R286A, GFP-UVR8R338A and GFP-UVR8R146A mutants do not show growth suppression and are of similar length to uvr8-1 under UV-B. In contrast, GFP-UVR8R286K, GFP-UVR8R146A and GFP-UVR8D96N,D107N show very similar hypocotyl growth suppression to wild-type and GFP-UVR8 plants, and are evidently functional in the UV-B response. Very similar results were obtained for the induction of gene expression. HY5 and CHS transcript accumulation are induced by UV-B in two independent lines of the GFP-UVR8R286K and GFP-UVR8D96N,D107N mutants, but the GFP-UVR8R286A mutant fails to show induction (Figure 4b). Equivalent results were obtained for CHS protein accumulation in these mutants (Figure 4c). GFP-UVR8R146A also shows UV-B induction of CHS, whereas GFP-UVR8R338A has little if any response, consistent with the hypocotyl suppression data. GFP-UVR8R146A shows a small response to UV-B in CHS induction that is not apparent in hypocotyl growth suppression. The molecular and UV-B response phenotypes of the UVR8 mutants are summarised in Table S1.

To further test whether the putatively monomeric GFP-UVR8D96N,D107N mutant is similarly responsive to UV-B as wild-type GFP-UVR8, we examined the dose-response relationship for HY5 transcript accumulation. We previously reported that this UV-B response shows reciprocity between exposure duration and fluence rate (Brown et al., 2009), and used this information to select treatment conditions for the present study. The results (Figure 5) show that the GFP-UVR8D96N,D107N mutant and GFP-UVR8 both respond to UV-B with linear increases in HY5 transcript levels over the same fluence range. The mutant has a lower fold-induction of HY5 transcripts, but this is likely because the plants used for these experiments expressed lower amounts of photoreceptor protein than the GFP-UVR8 control (Figure 5b). It is known that the magnitude of response mediated by UVR8 is related to its level of expression (Favory et al., 2009).

Because the putatively monomeric GFP-UVR8D96N,D107N mutant mediated responses to UV-B similarly to wild-type GFP-UVR8, we further examined whether the mutant fails to form dimers in vivo. We used a sensitive bimolecular fluorescence complementation (BiFC) assay (Walter et al., 2004) in which the protein is transiently expressed in... reported previously (Favory et al., 2009; Cloix et al., 2012). However, GFP-UVR8R286A did not interact with COP1 at all. In contrast, each of the other mutants tested interacted with COP1 in both the presence and absence of UV-B. For GFP-UVR8R286K and GFP-UVR8R146A, which have weakened dimers that monomerise in response to UV-B, and monomeric GFP-UVR8D96N,D107N, more COP1 was consistently co-immunoprecipitated from UV-B-exposed plants.

Figure 2. Dimer/monomer status of UVR8 salt-bridge mutants expressed in plants.

(a) Western blot of whole cell extracts from uvr8-1 plants expressing either GFP-UVR8 or GFP-UVR8 salt-bridge mutants exposed (+) or not (−) to 4 μmol m⁻² sec⁻¹ narrowband UV-B for 30 min. SDS-loading buffer was added and samples were run on a 7.5% SDS-PAGE gel without boiling. An immunoblot was probed with anti-UVR8 antibody. Ponceau staining of Rubisco large subunit (rbcL) is shown as a loading control. The GFP-UVR8 immunoblot was probed with anti-UVR8 antibody. © 2016 The Authors. The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd., The Plant Journal, (2016), 88, 71–81

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Nicotiana leaves as a fusion with either the N- or C-terminal region of YFP (Figure 6a). Dimer formation is expected to permit reconstitution of YFP and hence generate a fluorescence signal, whereas no signal should be seen if the mutant protein is constitutively monomeric. Expression of either the N- or C-terminal fusion protein together with the complementary expression vector containing no fusion protein is used as a control. As shown in Figure 6(b), a fluorescence signal is detected for UVR8D96N,D107N predominantly in the nucleus under both minus and plus UV-B conditions, whereas no fluorescence is seen with the empty-vector controls (Figure 6c). Similar results were obtained for UVR8D96N,D107N in six independent experiments.

DISCUSSION

Mutations of UVR8 salt-bridge amino acids affect dimer stability to varying extents. However, the method used to assess dimer/monomer status is critical. SDS–PAGE with non-boiled samples is convenient for monitoring the dimer/monomer status of wild-type UVR8 in response to different treatments (Rizzini et al., 2011; Christie et al., 2012; Heijde and Ulm, 2013; Heilmann and Jenkins, 2013; Huang et al., 2014), but it has limited value for characterising mutant proteins because it very sensitively detects any reduction in affinity in the dimer. Thus, all salt-bridge mutants examined to date appear constitutively monomeric when analysed by this method, either as purified proteins (Christie et al., 2012; Wu et al., 2012; Figure S5) or in plant extracts (Figure 2a). In contrast, SEC with purified proteins shows that some salt-bridge mutants (UVR8R286K, UVR8R146A) are dimeric (Figures 1b and S2b) and are converted to monomers upon UV-B illumination. Nevertheless, caution is required with the SEC analysis because some salt-bridge mutations affect the hydrodynamic radius of the protein, and hence the elution volume (e.g. UVR8R286K, Figure 1b), most likely by altering the position of the flexible C-terminal region. Moreover, the conformation of some mutants (e.g. UVR8R338A; Figure S3b) is significantly affected by ionic strength. It would be valuable to develop a quantitative method to evaluate the relative strength of the UVR8 dimer in different mutants by measuring a dissociation constant, but this has not yet been done for purified protein and extending the method to plant extracts would be difficult.

To assess the dimer/monomer status of salt-bridge mutants in vivo, we attempted to employ SEC with immunoprecipitated, transiently expressed proteins. Using this method, wild-type UVR8 appears as a dimer that monomerises after UV-B exposure, as expected. In contrast, the salt-bridge mutants appear constitutively monomeric. While this observation could suggest that the molecular environment in vivo impairs dimerisation of salt-bridge mutants, it is more likely that the high pH used to elute the immunoprecipitated proteins causes dissociation of the weakened mutant dimers. A more reliable method to assess dimer/monomer status in vivo is cross-linking of proteins, as it does not employ a chemical treatment that will promote monomerisation. The results obtained using cross-linking are consistent with the SEC data for purified mutant proteins. In the absence of UV-B, GFP-UVR8R146A is predominantly a dimer, whereas GFP-
UVR8\textsuperscript{R286K} is a mixture of dimer and monomer. In contrast, GFP-UVR8\textsuperscript{R286A} and GFP-UVR8\textsuperscript{D96N,D107N} are monomeric with no detectable dimer, again in agreement with the \textit{in vitro} data. Nonetheless, although the data obtained with cross-linking concur with those obtained for purified proteins, the assay is performed with protein extracts rather than intact cells, and it is important to determine the dimer/monomer status of the protein where it functions in cells.

The BiFC analysis of UVR8\textsuperscript{D96N,D107N} (Figure 6) contradicts the cross-linking data in that dimers are detected in the nucleus. A possible explanation is that the amount of dimer formed by the mutant protein is below the limit of detection of the cross-linking assay; nuclear UVR8 is estimated to be approximately 10% of the total UVR8 in wild-type cells (Kaiserli and Jenkins, 2007), and probably an even smaller fraction of the UVR8\textsuperscript{D96N,D107N} protein in the nucleus is in the dimeric form. On the other hand, BiFC is very sensitive and the YFP interaction is irreversible, so the fluorescence observed may represent trapping of short-lived, weak interaction between monomers of the mutant proteins. Thus, although BiFC shows that the UVR8\textsuperscript{D96N,D107N} mutant is capable of being trapped in a YFP linked state \textit{in vivo}, the method may over-state the extent and stability of dimer formation. The other methods employed all indicate that UVR8\textsuperscript{D96N,D107N} is constitutively monomeric, within their limits of detection, and it is therefore likely that this mutant forms very little dimer \textit{in vivo}, and the dimers present are likely to be unstable and short lived.

We therefore conclude that mutations that affect the ability of purified UVR8 to form a dimer have an equivalent effect \textit{in vivo} despite evident differences in the cellular environment, not least in ionic composition and the presence of proteins that could potentially influence dimer/monomer status. Furthermore, the \textit{in vivo} experiments highlight the importance of specific charged amino acids in maintaining the dimer structure. In particular, the R286-D96/D107 salt-bridges are crucial for maintaining the dimer both \textit{in vivo} and \textit{in vitro}. The UVR8\textsuperscript{D96N,D107N} and UVR8\textsuperscript{R286A} mutants are strongly impaired in dimer formation, whereas UVR8\textsuperscript{R286K} forms a weakened dimer, indicating that the positive charge of the arginine/lysine residue is critical for dimerisation.

Assays of hypocotyl growth suppression and gene expression show that some salt-bridge mutants are
expression responses to UV-B are very similar to those of wild-type UVR8. These mutations should have little impact on UV-B photoreception by UVR8. R146 is not close to the tryptophan triad and UVR8R286K is a conservative mutation. D96 and D107 are not adjacent to the chromophore tryptophans and are not part of the charge network that surrounds them (Christie et al., 2012; Wu et al., 2012), and because aspartate and asparagine are very similar in size the UVR8D96N,D107N mutations will not cause spatial disruption within the monomer.

Interaction of UVR8 with COP1 is necessary to initiate signalling and transcriptional responses. The GFP-UVR8R286A mutant is unable to bind COP1 and is non-functional, consistent with the findings of Huang et al. (2014). A possible explanation is that this mutation prevents UV-B-induced conformational changes that make the C-terminus, and potentially other regions of the protein, accessible for binding to COP1. In contrast, the constitutive binding of COP1 to GFP-UVR8D96N,D107N is likely caused by exposure of residues involved in binding. Similarly, the binding of COP1 to the weakened dimers of GFP-UVR8R146A and GFP-UVR8R286K is presumably the result of physical exposure of the region(s) interacting with COP1 in the non-illuminated photoreceptor as a result of the mutations. Although several of the mutants studied here bind COP1 constitutively, none initiate responses in the absence of UV-B, indicating that interaction with COP1 is not sufficient to initiate signalling. Similarly, the constitutive binding of COP1 to GFP-UVR8R234A and GFP-UVR8R338A is not sufficient for function. These results are consistent with those reported previously for alanine mutants of triad tryptophans (O’Hara and Jenkins, 2012). In contrast, strong over-expression of UVR8R286A gives a cop mutant phenotype, likely because of sequestration of COP1, and the plants show constitutive activation of UV-B signalling (Heijde et al., 2013). A similar phenotype is reported for UVR8R338A (Huang et al., 2014), in contrast to the data in Figure 4. A possible explanation of these different findings is that the lower level of transgenic expression employed in this study and previously (Kaiserli and Jenkins, 2007; O’Hara and Jenkins, 2012) is too low to promote obvious constitutive activation.

The finding that the in vivo UV-B responses of GFP-UVR8D96N,D107N are very similar to those of wild-type UVR8 is particularly interesting, because the mutant is evidently strongly impaired in dimer formation. The dose–response analysis (Figure 5) indicates that the mutant and wild-type photoreceptor proteins are similarly responsive to UV-B over a fluence range where the response is not saturated. The lower fold-induction of HY5 transcripts in GFP-UVR8D96N,D107N can be explained by the smaller amount of photoreceptor protein in the plants used for the experiments. Evidence from over-expression lines (Favory et al., 2009) indicates that an increased level of UVR8 protein gives an increased magnitude of response.
The established model of UVR8 action is that the dimer acts in UV-B photoreception, which causes monomerization, and then the monomer interacts with COP1 to initiate signaling and hence responses (Tilbrook et al., 2013; Jenkins, 2014a). This model is inadequate to explain the in vivo UV-B response of GFP-UVR8D96N,D107N. There is, at most, only a very low concentration of dimer present in GFP-UVR8D96N,D107N plants, whereas responsiveness to UV-B is similarly efficient to that of wild-type GFP-UVR8. Clearly, the physiological response of GFP-UVR8D96N,D107N does not correlate with monomer formation through UV-B-induced dimer dissociation. The simplest explanation is that the monomeric protein is competent in photoreception, which initiates signaling and UV-B responses in vivo. Thus, the molecular and physiological phenotype of GFP-UVR8D96N,D107N indicates that dimer formation is not essential for photoreception by UVR8. UV-B photoreception could activate monomeric UVR8D96N,D107N bound to COP1 by, for example, causing a conformational change to the protein that initiates signaling. Recent studies show that photochemical events associated with UV-B photoreception are detectable in both dimeric and monomeric UVR8 (Mathes et al., 2015), indicating that monomeric UVR8 is capable of UV-B photoreception, at least in vitro.

The findings with the GFP-UVR8D96N,D107N mutant raise the question of whether monomeric UVR8 could be active in photoreception in wild-type plants to initiate responses. Recent research shows that UVR8 does not behave as a simple dimer/monomer UV-B switch under photoperiodic conditions, but establishes a photo-equilibrium between the dimer and monomer forms (Findlay and Jenkins, 2016). Monomeric UVR8 is present in plants even at low ambient levels of UV-B and could potentially be active in photoreception to initiate responses. At present, dimer and monomer photoreception cannot be distinguished in plants by distinct biophysical signals or associated physiological responses, and therefore further research is needed to test the intriguing possibility that monomeric UVR8 is active in photoreception in wild-type plants.

**EXPERIMENTAL PROCEDURES**

**Experiments with purified proteins**

Site-directed mutagenesis was carried out using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer’s instructions and verified by sequencing. The primers used for site-directed mutagenesis are listed in Table S2. Proteins were expressed in E. coli and purified as described previously (Christie et al., 2012). UV-B exposure of proteins was undertaken with a narrowband UV-B source with maximal emission at 311 nm (Philips TL20W/01RS; spectrum shown in Cloix et al., 2012).
Purified proteins were exposed to 1.5 μmol m⁻² sec⁻¹ narrow-band UV-B on ice for 1 h. Protein samples were prepared for electrophoresis without boiling as described (Christie et al., 2012) and loaded on a 7.5% SDS–PAGE gel. Gels were stained with Coomassie Blue.

Analytical SEC was performed on a Superdex 200 HR10/30 column (GE Healthcare, Little Chalfont, UK) equilibrated with wash buffer containing 50 mM Tris pH 7.5, 150 mM NaCl (or 500 mM for high-salt samples), 1 mM β-mercaptoethanol (β-ME) and 0.02% sodium azide, and run at a flow rate of 0.5 ml min⁻¹ at 4°C on an AKTA FPLC system (Christie et al., 2012). Aldolase, albumin and ovalbumin were used as standards.

Plant material
Seeds of wild-type Arabidopsis thaliana ecotype Landsberg erecta (Ler) were obtained from the Nottingham Arabidopsis Stock Center. Seeds of the uvr8-1 mutant allele (Ler background; Kleibenstein et al., 2002) were obtained from Dr Dan Kleibenstein (University of California, Davis). The uvr8-1/UVR8ΔSTD-GFP–UVR8 transgenic line 6-2 was described by Kaiserli and Jenkins (2007).

Mutant UVR8 proteins were expressed in uvr7-1 using Agrobacterium-mediated transformation. Mutant UVR8 sequences were sub-cloned into the pEZR(K)L-C vector downstream of eGFP and the CaMV 35S promoter (Brown et al., 2005; Cloix et al., 2012). DNA sequencing confirmed that the fusions were made correctly. At least three independent transgenic lines were selected for each fusion with a level of transgene expression comparable to that of the control GFP–UVR8 fusion (Figure S4).

Arabidopsis experiments
Except where indicated below, plants were grown on agar plates containing half-strength Murashige and Skoog (MS) salts under constant white light so that all tissue was harvested 2 h after the start of illumination. Western blotting and immunodetection using anti-GFP (Clontech, Heidelberg, Germany) antibody. Immunoblots were probed with an anti-CHS (Santa Cruz Biotechnology) antibody. Immunoblots were stained with Ponceau S to reveal the Rubisco large subunit, which was used as a loading control.

For cross-linking of proteins, proteins were extracted in PBS containing protease inhibitor cocktail tablets (complete, Roche, Welwyn, UK). Samples were then centrifuged for 10 min at 16 000 g at 4°C and the supernatant transferred to a fresh tube. DSP (4 mM final concentration; Thermo Scientific, Waltham, MA, USA) was then added to the extract and incubated on ice for 30 min. Immediately afterwards, protein sample buffer without reducing agent (β-ME) or with β-ME (5% final concentration to reverse cross-linking) was added, and samples were boiled for 10 min before separation on a 10% SDS–PAGE gel and subsequent immunodetection using anti-UVR8 antibody.

Interaction of wild-type and mutant GFP–UVR8 fusions with COP1 was examined by co-immunoprecipitation (Cloix et al., 2012). Plants were grown on agar plates as described above and put in darkness for 16 h. The plants were treated for 3 h with 3 μmol m⁻² sec⁻¹ narrowband UV-B. Whole cell extracts were prepared as described in Kaiserli and Jenkins (2007) in the absence or presence of 3 μmol m⁻² sec⁻¹ narrowband UV-B. The co-immunoprecipitation assays were carried out in the same light conditions using anti-GFP microbeads (μMacs, 130-091-370, Miltenyi Biotec) as described previously (Cloix et al., 2012). The ‘input’ samples applied to the microbead columns and the immunoprecipitate eluates were analysed by SDS–PAGE followed by Western blotting and immunodetection using anti-GFP (Clontech) and anti-COP1 (kindly provided by Dr Nam-Hai Chua; Jang et al., 2010) antibodies.

The RT-PCR assays of HY5 and CHS transcript levels in plant RNA samples (Figure 4b) were measured as described previously (Cloix et al., 2012), with the primers stated in Brown and Jenkins (2008). Plants grown as above were exposed, or not in controls, to 3 μmol m⁻² sec⁻¹ broadband UV-B (Q-panel UV-B-313 fluorescent tubes covered with cellulose acetate; spectrum shown in Cloix et al., 2012) for 4 h. Transcript levels of ACTIN2 were assayed in the same cDNA samples as a control. For each gene, PCR was monitored over a range of cycle numbers to select optimal conditions for visualisation of the PCR product and quantification. Transcript levels in different RNA samples were compared using cycle numbers within the linear range of amplification.

For the dose-response experiments (Figure 5), plants were grown on agar plates as above in a 16 h light (60 μmol m⁻² sec⁻¹) 8 h dark cycle for 14 days, and transferred to darkness for 16 h prior to exposure to narrowband UV-B at different doses as described by Brown et al. (2009): 20 and 40 min treatments with 0.3, 0.6 and 0.9 μmol m⁻² sec⁻¹ followed by transfer to darkness so that all tissue was harvested 2 h after the start of illumination. qRT-PCR assays of HY5 transcripts were undertaken similarly to Brown et al. (2009), but using different primers (HY5: forward GGCCTGAGGAGTTGTTTAGGAAC; reverse AGCTACTGTTCTC GTTCTGAAG. ACTIN2: forward GTATTGTCGTGATCTCTGGTG; reverse GAGGTAAATCGAATGGTCAGC).

For measurements of hypocotyl length, seedlings were grown for 4 days on agar plates containing half-strength MS salts in 1.5 μmol m⁻² sec⁻¹ white light supplemented, or not in controls, with 1.5 μmol m⁻² sec⁻¹ narrowband UV-B (Cloix et al., 2012). For the analysis of CHS protein, seedlings were grown under the same conditions for 7 days. Whole cell extracts were made and protein samples boiled prior to electrophoresis on a 7.5% SDS–PAGE gel. Immunoblots were probed with an anti-CHS (Santa Cruz Biotechnology, Heidelberg, Germany) antibody. Immunoblots were stained with Ponceau S to reveal the Rubisco large subunit, which was used as a loading control.

Similar results for dimer/monomer status, COP1 interaction, hypocotyl growth suppression and gene expression were obtained for several lines expressing a particular fusion. Unless indicated otherwise, the transgenic lines used in the experiments shown are as follows: GFP–UVR8 6-2; GFP–UVR8ΔSTD 6-8; GFP–UVR8ΔSTD 2-3; GFP–UVR8ΔSTD 74-2 or 5-2 (for the dose–response experiments); GFP–UVR8ΔSTD 16-5; GFP–UVR8ΔSTD 9-3 and GFP–UVR8ΔSTD F1462 line 9. The data presented are representative of at least three independent experiments.

Transient expression in Nicotiana benthamiana for SEC
A single colony from Agrobacterium cells freshly transformed with the desired plasmid DNA was inoculated in 10 ml of LB medium with appropriate antibiotics and grown overnight at 28°C under constant shaking (200 rpm). When cultures had reached an OD600 of about 0.6–1.0, cells were pelleted by centrifugation at 2000 g for 10 min. The cells were then resuspended in 10 mM MgCl₂, 10 mM MES pH 6.5 and 200 μM acetoxyringerine at an OD600 of 0.2 and incubated at room temperature for 3 h. The Agrobacterium medium was infiltrated into the lower side of
N. benthamiana leaves using a syringe. The infiltrated plants were moved back into the growth room at 28°C and left for 2–3 days before examining gene expression by confocal microscopy and preparation of protein extracts.

For protein extraction, N. benthamiana leaf segments were frozen in liquid nitrogen and ground with a mortar and pestle. A spatula of polyvinyl-pyrolidone, an effective absorbent for phenolic compounds, was added as soon as the liquid nitrogen had evaporated. Once ground, the plant material was transferred to a microcentrifuge tube and approximately one volume of extraction buffer (1 mM EDTA, 10% glycerol, 5 mM DTT, 0.1% v/v Triton, 25 mM Tris-HCl, pH 7.5) was added and vortexed to mix. Samples were centrifuged at 16,000 g for 15 min at 4°C and the supernatant was transferred to a fresh tube. The transiently expressed GFP fusions were immunoprecipitated using anti-GFP microbeads (μMacs, 130-091-370, Miltenyi Biotec) as described previously (Cloix et al., 2012). The immunoprecipitated proteins were examined by SEC, using the same method as for the purified proteins, followed by standard SDS-PAGE and immunodetection using an anti-GFP antibody (Clontech).

**BiFC experiments**

The GFP-UVR8D96N/D107N fusion was cloned into the pSPYNE and pSPYCE vectors (Walter et al., 2004) containing the N- and C-terminal regions of YFP, respectively. Agrobacteria containing the plasmids were grown overnight as above, and resuspended together in 10 mM MgCl2, 10 mM MES pH 6.5 and 200 μM acetoxyringerine at an equivalent OD600 of 0.1 for each culture. The Agrobacterium suspension was incubated at room temperature for 2 h before infiltration into N. benthamiana leaves as described above. Plants were exposed to low fluence rate (1 μmol m−2 sec−1) narrowband UV-B in a growth chamber at 21°C for approximately 60 h; controls were kept under a UV-B cutoff filter. Leaves were examined for YFP fluorescence in at least three fields of view using a Zeiss LSM confocal microscope (Jena, Germany).

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Dimer/monomer status of UVR8R338A examined by SEC.

**Figure S2.** Dimer/monomer status of UVR8R326E and UVR8D96N/D107N examined by SEC.

**Figure S3.** Dimer/monomer status of UVR8R338A examined by SEC.

**Figure S4.** Expression levels of GFP-UVR8 mutants in transgenic lines.

**Figure S5.** Dimer/monomer status of purified mutant proteins examined by SDS-PAGE with non-boiled samples.

**Table S1.** Summary of phenotypes of UVR8 salt-bridge amino acid mutants.

**Table S2.** Primers used for site-directed mutagenesis.

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