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Research

Evolutionary conservation of structure and function of the UVR8 photoreceptor from the liverwort *Marchantia polymorpha* and the moss *Physcomitrella patens*

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Summary

- The ultraviolet-B (UV-B) photoreceptor UV RESISTANCE LOCUS 8 (UVR8) mediates photomorphogenic responses to UV-B in Arabidopsis through differential gene expression, but little is known about UVR8 in other species. Bryophyte lineages were the earliest diverging embryophytes, thus being the first plants facing the UV-B regime typical of land. We therefore examined whether liverwort and moss species have functional UVR8 proteins and whether they are regulated similarly to Arabidopsis UVR8.
- We examined the expression, dimer/monomer status, cellular localisation and function of *Marchantia polymorpha* and *Physcomitrella patens* UVR8 in experiments with bryophyte tissue and expression of green fluorescent protein (GFP)–UVR8 fusions in *Nicotiana* leaves and transgenic Arabidopsis.
- *P. patens* expresses two *UVR8* genes that encode functional proteins, whereas the single *M. polymorpha UVR8* gene expresses two transcripts by alternative splicing that encode functional UVR8 variants. *P. patens* UVR8 proteins form dimers that monomerise and accumulate in the nucleus following UV-B exposure, similar to Arabidopsis UVR8, but *M. polymorpha* UVR8 has weaker dimers and the proteins appear more constitutively nuclear.
- We conclude that liverwort and moss species produce functional UVR8 proteins. Although there are differences in expression and regulation of *P. patens* and *M. polymorpha* UVR8, the mechanism of UVR8 action is strongly conserved in evolution.

Introduction

Ultraviolet-B (UV-B) wavelengths are the most energetic component of the daylight spectrum and they impact diverse organisms and ecosystems (Klein, 1978; Jordan, 1996; Rozema *et al.*, 1997). It is well established that UV-B is a key regulator of plant processes, influencing metabolism and morphogenesis through differential gene expression (Brosché & Strid, 2003; Ulm & Nagy, 2005; Jenkins, 2009). Exposure of plants to low doses of UV-B initiates an acclimation response that establishes protection against potentially damaging levels of UV-B (Jansen *et al.*, 1998; Frohnmeyer & Staiger, 2003; Jenkins, 2009). This response includes the production of UV-absorbing compounds that minimise penetration of UV-B into plant tissues (Jordan, 1996; Rozema *et al.*, 1997; Ryan *et al.*, 2001) and the stimulation of DNA repair and antioxidant activities within cells (Hideg *et al.*, 2013; Li *et al.*, 2015). In addition, low doses of UV-B promote morphogenic responses such as the suppression of extension growth (Jansen, 2002; Frohnmeyer & Staiger, 2003; Robson *et al.*, 2015). Although exposure of nonacclimated plants to high, above ambient levels of UV-B can activate stress signalling pathways that are not specific to UV-B and cause tissue necrosis (Brosché & Strid, 2003; Jenkins, 2009), plants growing in ambient levels of UV-B rarely exhibit signs of UV stress, showing that acclimation to UV-B is usually sufficient to provide tolerance to natural fluctuations in UV-B levels. In bryophytes, UV-B radia tion mostly causes similar effects to those found in vascular plants, including morphogenic changes and induction of UV-absorbing compounds (Wolf *et al.*, 2010; Martínez-Abaigar & Núñez-Olivera, 2011; Fabón *et al.*, 2012a,b). In particular, Wolf *et al.* (2010) concluded that the moss *Physcomitrella patens* is more tolerant than Arabidopsis to UV-B stress.

Low fluence rates of UV-B, which initiate acclimation, are perceived by the UV-B photoreceptor UV RESISTANCE LOCUS 8 (UVR8) (Tilbrook *et al.*, 2013; Jenkins, 2014). UVR8 is a seven-bladed β-propeller protein that exists as a homodimer before UV-B exposure (Rizzini *et al.*, 2011; Christie *et al.*, 2012; Wu *et al.*, 2012). The dimer structure is maintained by salt-bridge interactions between charged amino acids of adjacent monomers. In contrast to other photoreceptors that use a bound

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chromophore to absorb light, UVR8 uses specific tryptophan amino acids in its primary sequence for UV-B absorption (Rizzini *et al.*, 2011; Christie *et al.*, 2012; Wu *et al.*, 2012). UV-B photoreception leads to rapid dissociation of the dimer into monomers. In turn, monomeric UVR8 initiates signal transduction by binding to the CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) protein (Favory *et al.*, 2009; Rizzini *et al.*, 2011). This interaction leads to the stimulation of transcription of a set of genes responsible for the range of UVR8-mediated responses, including those encoding the key regulatory transcription factor ELONGATED HYPOCOTYL 5 (HY5) and the enzyme CHALCONE SYNTHASE (CHS), which catalyses the first committed step in the flavonoid biosynthesis pathway (Brown *et al.*, 2005; Favory *et al.*, 2009).

Studies of UVR8 structure, regulation and function have focused on *Arabidopsis thaliana* because of its suitability for molecular genetic experimentation. However, it is evident that UVR8 is highly conserved in plant evolution. UVR8 genes are present in all plant taxa examined to date, including green algae, and the encoded amino acid sequences are very similar to those in angiosperms (Rizzini *et al.*, 2011; Wu *et al.*, 2011; Fernandez *et al.*, 2016). It has been suggested that UVR8 evolved to assist early photosynthetic plants survive during exposure to sunlight. A recent study showed that UVR8 from *Chlamydomonas reinhardtii* is able to functionally complement Arabidopsis *uvr8* mutant plants, indicating conservation of the molecular mechanism of action (Tilbrook *et al.*, 2016). In addition, evidence was presented that UVR8 could regulate gene expression in *Chlamydomonas* cells in response to UV-B.

Apart from the study with *Chlamydomonas*, information on the function of UVR8 in diverse plant species is lacking. Of particular interest are the bryophytes, which comprise the liverworts, mosses and hornworts. These taxa occupy a critical position in the evolution of plants because they were the earliest diverging embryophyte lineages and thus were important in plant land colonisation. Yet, the fossil record and molecular phylogenies have not definitively clarified the evolutionary sequence of the three bryophyte lineages (Qiu *et al.*, 2007; Liu *et al.*, 2014; Wickett *et al.*, 2014; Bowman *et al.*, 2016). Although plants growing in surface layers of aquatic environments would have been exposed to UV-B wavelengths, growth on land presented the challenge of sustained exposure to increased levels of UV-B. While the first land plants required effective protection against potentially damaging UV-B radiation, the presence of UV-B also provided a cue to regulate metabolic and developmental processes. Hence an ability to detect and respond to UV-B radiation would have provided a potential fitness benefit. In this sense, the comparison between extant bryophytes and extant flowering plants can help to infer the role played by UV-B detection systems in early land plant evolution. At present there is little information on the molecular basis of bryophyte responses to UV-B. In this respect, Wolf *et al.* (2010) showed that UV-B induces substantial changes in gene expression in *P. patens*. However, there is no information on UVR8 action in the bryophytes. Thus, our aim was to study the structure and function of UVR8 from the moss *P. patens* and the liverwort *Marchantia polymorpha*. The rationale for studying UVR8 in these two species is that: they represent two early diverging evolutionary lineages of land plants that were important for land colonisation, a process in which UV-B radiation could play a role as an evolutionary pressure; liverworts and mosses may represent two different modalities of bryophytes in their responses to UV-B radiation (Fabón *et al.*, 2012b); responses of bryophytes to UV-B radiation depend on the species used (Boelen *et al.*, 2006; Martínez-Abaigar & Núñez-Olivera, 2011), so that using two different species can allow, to a certain extent, assessment of the variability of UVR8 behaviour in bryophytes; and the two species studied are increasingly important as model species in plant biology.

Materials and Methods

Plant material

M. polymorpha L. subsp. *ruderalis* Bischl. & Boisselier Tak-1 was provided by Professor Takayuki Kohchi (Kyoto University). *P. patens* (Hedw.) Bruch & Schimp. strain Gransden 2004 was provided by Professor Begoña Benito (Universidad Politécnica de Madrid). Seeds of wild-type *A. thaliana* ecotype Landsberg *erecta* (L*er*) were obtained from the Nottingham Arabidopsis Stock Centre. Seeds of the *uvr8-1* mutant (L*er* background; Kliebenstein *et al.*, 2002) were originally provided by Dr Dan Kliebenstein (University of California, Davis). The transgenic Arabidopsis line expressing *UVR8$_{pro}$: green fluorescent protein (GFP)-UVR8* in the *uvr8-1* background (line 6-2) was described by Kaiserli & Jenkins (2007).

Experiments with *Marchantia* and *Physcomitrella*

Marchantia plants were grown in sterile culture on agar containing half-strength Gamborg B5 basal medium (Sigma Aldrich) under continuous 80 μmol m^{-2} s^{-1} white light (warm white light-emitting diodes) in a growth cabinet at 20°C. *Physcomitrella* was grown under the same conditions but on agar containing BCD medium supplemented with 1 mM $CaCl_2$ (Nishiyama *et al.*, 2000).

For protein assays, plants were placed in darkness for 16 h and then exposed to either 20 μmol m^{-2} s^{-1} white light as above or 3 μmol m^{-2} s^{-1} narrowband UV-B (Philips TL20W/01RS, λ_{max} 312 nm; spectrum shown in Cloix *et al.*, 2012) for 30 min. Tissue was harvested into liquid nitrogen and protein extracted as described by Kaiserli & Jenkins (2007), but without the freeze/thaw steps. UVR8 dimer/monomer status was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with nonboiled samples (Heilmann & Jenkins, 2013; Findlay & Jenkins, 2016). Western blots were incubated with a rabbit polyclonal antibody raised against purified wild-type UVR8 in both the dimeric and the monomeric forms; the specificity of this antibody is shown in experiments with wild-type and *uvr8-1* mutant plants (see Fig. 3). The immunoblots

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were stained with Ponceau S to reveal the Rubisco large subunit (rbcL), which was used as a loading control.

For *Marchantia* transcript assays, plants were placed in darkness for 16 h and then exposed to either 20 μmol m^{-2} s^{-1} white light or 3 μmol m^{-2} s^{-1} narrowband UV-B for 3 h. RNA was isolated using the RNeasy plant mini kit (Qiagen) and cDNA was synthesised using SuperScript reverse transcriptase (RT; Thermo Fisher Scientific, Waltham, MA, USA). *MpUVR8.1 and MpUVR8.2* transcript levels in cDNA samples were measured by quantitative real-time PCR using an Applied Biosystems StepOne Plus instrument and specific primers for each transcript (see Fig. 2c; Table 1). Transcript levels were quantified relative to those of control *ACTIN7* transcripts assayed in the same cDNA samples, using primers described by Saint-Marcoux *et al.* (2015; Table 1).

Cloning of cDNA

To obtain *Marchantia* UVR8 sequences, cDNA synthesised from *Marchantia* RNA was used as a template for PCR using degenerate primers designed to conserved regions of the UVR8 sequences of Arabidopsis, *P. patens* and the lycopodiophyte *Selaginella moellendorffii* (Table 1). The cDNA products were used for 3′ and 5′ rapid amplification of cDNA ends (RACE) using commercial kits (Thermo Fisher Scientific) and primers designed from the initial cDNA products (Table 1), following the manufacturer's instructions to obtain full-length cDNAs.

To prepare constructs for transient expression and stable transformation, cDNA encoding each complete UVR8 coding sequence was subcloned into the pEZR(K)L-C vector downstream of enhanced GFP (eGFP) and the cauliflower mosaic virus (CaMV) *35S* promoter (Brown *et al.*, 2005; Cloix *et al.*, 2012). The fusions were sequenced to check that they had been made correctly.

Transient expression in *Nicotiana benthamiana*

Agrobacterium cells transformed with the appropriate plasmid were infiltrated into the lower side of *N. benthamiana* leaves using a syringe, as described by Heilmann *et al.* (2016). The infiltrated plants were placed in a growth room at 28°C and left for 2–3 d before treatment. Plants were transferred to darkness for 16 h and then exposed to either 20 μmol m^{-2} s^{-1} white light or 3 μmol m^{-2} s^{-1} narrowband UV-B for 3 h. UVR8 dimer/monomer status was examined as described above following protein extraction according to Heilmann *et al.* (2016) and incubation of western blots with an anti-GFP antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). The cellular localisation of GFP fluorescence was examined using a Leica TCS SP8 (SMD FSU) confocal microscope.

Production and analysis of transgenic plants

Marchantia and *Physcomitrella* GFP-UVR8 fusion proteins were expressed in *uvr8-1* by *Agrobacterium*-mediated transformation using the pEZR(K)L-C vector constructs described above. T_1 progeny were used to produce at least two independent homozygous T_3 lines for each of the two *UVR8* sequences in each species. The level of transgene expression in each line was examined by immunodetection of UVR8 on a western blot.

To measure hypocotyl length, seedlings were grown for 4 d on agar plates containing half-strength Murashige and Skoog (MS) salts in 1.5 μmol m^{-2} s^{-1} white light supplemented, or not in controls, with 1.5 μmol m^{-2} s^{-1} narrowband UV-B (Cloix *et al.*, 2012). CHS protein abundance was assayed in seedlings grown in the same conditions for 7 d (Heilmann *et al.*, 2016). Protein extracts were prepared for electrophoresis on 7.5% SDS-PAGE gels. Western blots were probed with an anti-CHS antibody

Table 1 Primers used in this study

Primer	Sequence	Usage
UVR8-F:	AGATTGCTTGTGGYGAYAGTCA	Amplification of MpUVR8 fragment
UVR8-R:	ARYTGYCCATAYTTGCTCCA	Amplification of MpUVR8 fragment
MpUVR8/RACE-F:	GTTTGGCAATCACCACACAG	RACE-PCR to obtain MpUVR8 CDS
MpUVR8/RACE-Fn:	TGCATTCGAGGGCATAGCTG	RACE-PCR to obtain MpUVR8 CDS
MpUVR8/RACE-R:	GGTGTGTCGCCAGCCACACGCAACAA	RACE-PCR to obtain MpUVR8 CDS
MpUVR8/RACE-Rn:	GCCCCTGCAGCCAACATCTTGACAGC	RACE-PCR to obtain MpUVR8 CDS)
MpUVR8 Full length-F:	ATGGAAGACTCGAAGATGTC	Amplification of MpUVR8 full length CDS
MpUVR8 Full length-R:	TTAAAAGCCCGTCCGTAGCC	Amplification of MpUVR8 full length CDS
MpUVR8.1-F:	ATGGTGATGCAGTGGACAGA	Amplification of MpUVR8.1 transcript: qPCR
MpUVR8.1-R:	ATATTCCTGGAAAGCGATCTCA	Amplification of MpUVR8.1 transcript: qPCR
MpUVR8.2-F:	GGGGAAATGTGTTTTCATGG	Amplification of MpUVR8.2 transcript: qPCR
MpUVR8.2-R:	ATTATCTCCAACCGTTTCAACC	Amplification of MpUVR8.2 transcript: qPCR
MpACTIN-F:	AGGCATCTGGTATCCACGAG	Reference gene: qPCR
MpACTIN-R:	ACATGGTCGTTCCTCCAGAC	Reference gene: qPCR
AtHY5-F:	GGCTGAAGAGGTTGTTGAGGAAC	qPCR
AtHY5-R:	AGCATCTGGTTCTCGTTCTGAAGA	qPCR
AtACTIN-F:	CTCTCCCGCTATGTATGTCG	Reference gene: qPCR
AtACTIN-R:	TCCATCTCCTGCTCGTAGTC	Reference gene: qPCR

At, Arabidopsis wild-type; CDS, coding sequence; Mp, *Marchantia polymorpha*; qPCR, quantitative PCR, RACE, rapid amplification of cDNA ends.

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(Santa Cruz, Santa Cruz, CA, USA). The blots were stained for the rbcL loading control, as above.

HY5 transcript levels in RNA samples were measured using quantitative real-time RT-PCR as described previously (Heilmann *et al.*, 2016). Plants grown on agar plates containing half-strength MS salts for 10 d in 100 μmol m^{-2} s^{-1} constant white light as above at 20°C were transferred to 20 μmol m^{-2} s^{-1} constant white light for 4 d and then additionally exposed, or not in controls, to 1.5 μmol m^{-2} s^{-1} narrowband UV-B for 4 h. *HY5* transcript levels were measured relative to control *ACTIN2* transcripts in the same cDNA samples.

For dimer/monomer status and localisation assays, plants grown for 7 d as above were transferred to 20 μmol m^{-2} s^{-1} constant white light for 4 d and then exposed, or not in controls, to 3 μmol m^{-2} s^{-1} narrowband UV-B for 30 min. GFP-UVR8 dimer/monomer status and localisation were examined as in the transient expression experiments, but using the anti-UVR8 polyclonal antibody.

In the above analyses, similar results were obtained with independent transgenic lines expressing each fusion and the data presented are representative of at least three separate experiments. The transgenic lines used in the experiments shown are as follows: GFP–AtUVR8 6-2; GFP–MpUVR8.1 7-a and 21-2; GFP–MpUVR8.2 10-2 and 11-1; GFP–PpUVR8.1 20-4; GFP–PpUVR8.2 2-5.

Results

Physcomitrella expresses two *UVR8* genes and *Marchantia* two alternative transcripts from a single *UVR8* gene

The *P. patens* genome (Rensing *et al.*, 2008; Zimmer *et al.*, 2013) contains two *UVR8* genes. The UVR8 amino acid sequences designated here as PpUVR8.1 and PpUVR8.2 (Fig. 1) are encoded by the *P. patens* genes Pp3c10_2230 and Pp3c3_15300, respectively (*P. patens* genome v3.3; https://phytozome.jgi.doe.gov/pz/portal.html). PpUVR8.1 and PpUVR8.2 are 78% identical to each other and 21 and 17 amino acids longer, respectively, than Arabidopsis UVR8. The additional amino acids are located in the C-terminal region of the protein (Fig. 1). The full-length coding sequences were amplified by PCR from cDNA synthesised from RNA from *Physcomitrella* gametophytes, demonstrating that both genomic sequences are expressed.

Before the availability of a published genome sequence, full-length *UVR8* cDNA sequences were obtained from *M. polymorpha* by PCR-RACE. RNA from *Marchantia* thallus was used to produce cDNA, which was then used as a template for PCR using degenerate primers corresponding to highly conserved regions of the UVR8 amino acid sequence. The resulting partial cDNAs were used for RACE amplification to obtain full-length cDNA sequences. Two cDNAs of slightly different length

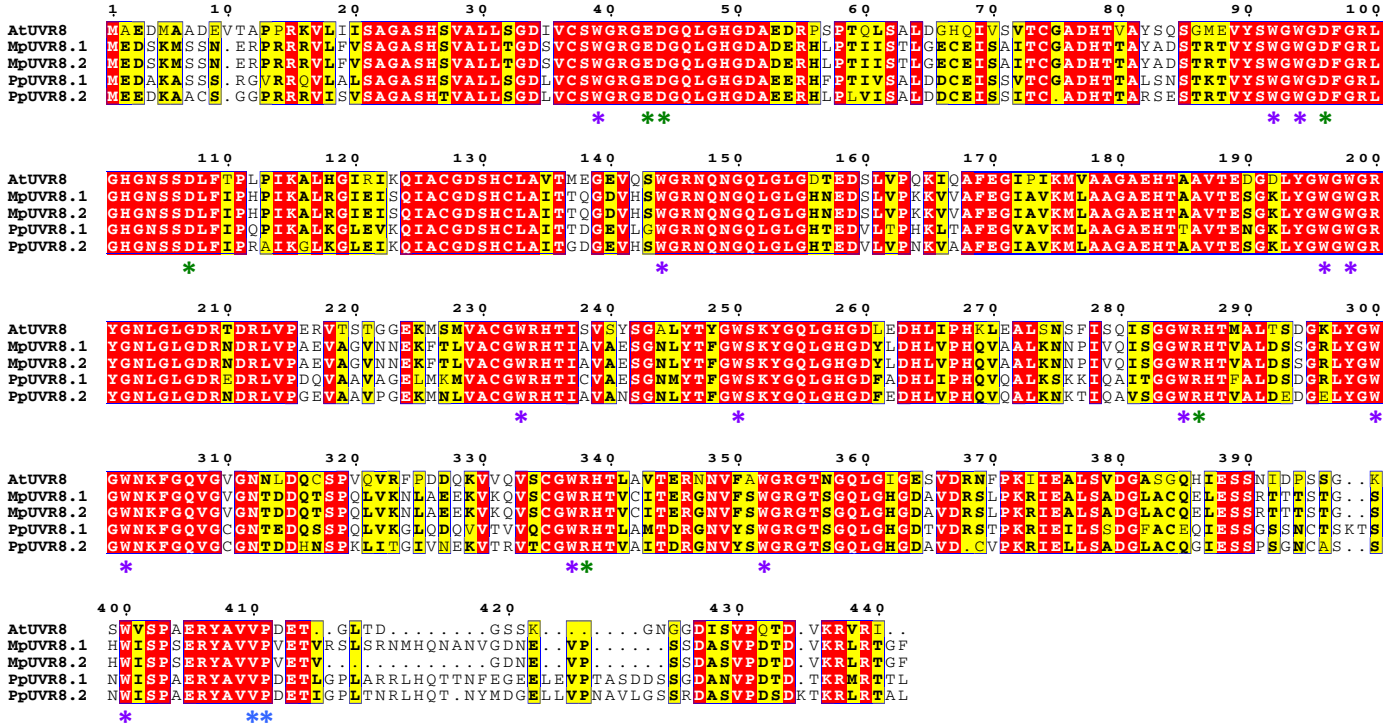


Fig. 1 Alignment of UV RESISTANCE LOCUS 8 (UVR8) amino acid sequences from *Marchantia polymorpha* (MpUVR8.1 and MpUVR8.2) and *Physcomitrella patens* (PpUVR8.1 and PpUVR8.2) with the *Arabidopsis thaliana* UVR8 sequence (AtUVR8). Red and yellow boxes show identical and similar amino acids, respectively. Asterisks indicate conserved amino acids that are particularly important in UVR8 structure and function: tryptophans (purple); charged amino acids key to dimer formation (green); amino acids required for interaction with CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) and REPRESSOR OF UV-B PHOTOMORPHOGENESIS (RUP) proteins (blue). The alignment was produced using MultAlin (Corpet, 1988) and the figure prepared using ESPript 3.0 (Robert & Gouet, 2014).

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were obtained (Fig. 2a). The *Marchantia* UVR8 sequences encoded by the cDNAs (designated as MpUVR8.1 and MpUVR8.2) are identical except for the presence of an additional 13 amino acids in MpUVR8.1 within the C-terminal region (Fig. 1). However, existing genome sequence data for *M. polymorpha* (Honkanen *et al.*, 2016) indicate that only one *UVR8* gene is present. Comparison of the cDNA and genomic sequences, together with the encoded amino acid sequences (Fig. 2b), indicates that alternative splicing of an intron gives rise to transcripts of different lengths. In the *M. polymorpha* genome annotation (https://phytozome.jgi.doe.gov/pz/portal.html) the alternative *MpUVR8.1* and *MpUVR8.2* transcripts are designated as Mapoly0023s0125.1 and Mapoly0023s0125.2, respectively.

Experiments were therefore undertaken to examine expression of the *UVR8* transcripts in *Marchantia*. Primers were designed to amplify specifically transcripts corresponding to the two cDNA sequences (Fig. 2c). Quantitative RT-PCR assays with one-way ANOVA (Fig. 2d) indicated that there was no significant effect of treatment (darkness, white light or exposure to UV-B) on relative expression of the two transcripts.

The UVR8 amino acid sequences from both bryophytes are very similar to UVR8 from Arabidopsis, which has only one *UVR8* gene (PpUVR8.1 and PpUVR8.2 are 64% and 65% identical, and MpUVR8.1 and MpUVR8.2 are 66% and 68% identical, respectively, to Arabidopsis UVR8). Notably, amino acids that are key to UVR8 structure and function are highly conserved (see Fig. 1). In the absence of UV-B, Arabidopsis UVR8 forms a homo-dimer maintained by interactions between charged amino acids across the dimer interface. In this respect, the interactions of R286 with D96/D107 and of R338 with E43/D44 are particularly important (Christie *et al.*, 2012; Wu *et al.*, 2012; Heilmann *et al.*, 2016), and the equivalent residues are present in the bryophyte sequences. Arabidopsis UVR8 has 14 tryptophans, which have roles in maintaining structural integrity and, importantly, in photoreception (Rizzini *et al.*, 2011; Christie *et al.*, 2012; O'Hara & Jenkins, 2012; Wu *et al.*, 2012), and the number and positions of these tryptophans are totally conserved in *Physcomitrella* and *Marchantia* UVR8 sequences. Within the C-terminal region of Arabidopsis UVR8 there is a 27 amino acid region (C27: amino acids 397–423) that is important in interaction with COP1 and the REPRESSOR OF UV-B PHOTOMORPHOGENESIS (RUP) proteins (Cloix *et al.*, 2012; Yin *et al.*, 2015). There is strong conservation over the first 18 amino acids of this region in the bryophyte UVR8 sequences, including V410 and P411, which are required for interaction with COP1 and the RUPs. The above findings indicate that the *Physcomitrella* and *Marchantia* sequences encode functional UVR8 photoreceptors.

Physcomitrella and *Marchantia* UVR8 differ in dimer/monomer status

In Arabidopsis plants, UVR8 is predominantly present as a homo-dimer in the absence of UV-B and as a monomer following UV-B exposure (Rizzini *et al.*, 2011; O'Hara & Jenkins, 2012; Huang *et al.*, 2013). The different forms are resolved on SDS-PAGE provided that the samples are not boiled before electrophoresis (Fig. 3); boiled samples contain only monomeric UVR8. The dimer/monomer status of UVR8 in protein extracts from *Physcomitrella* and *Marchantia* was examined using the above method and immunodetection with a polyclonal antibody raised against purified Arabidopsis UVR8. As shown in Fig. 3,

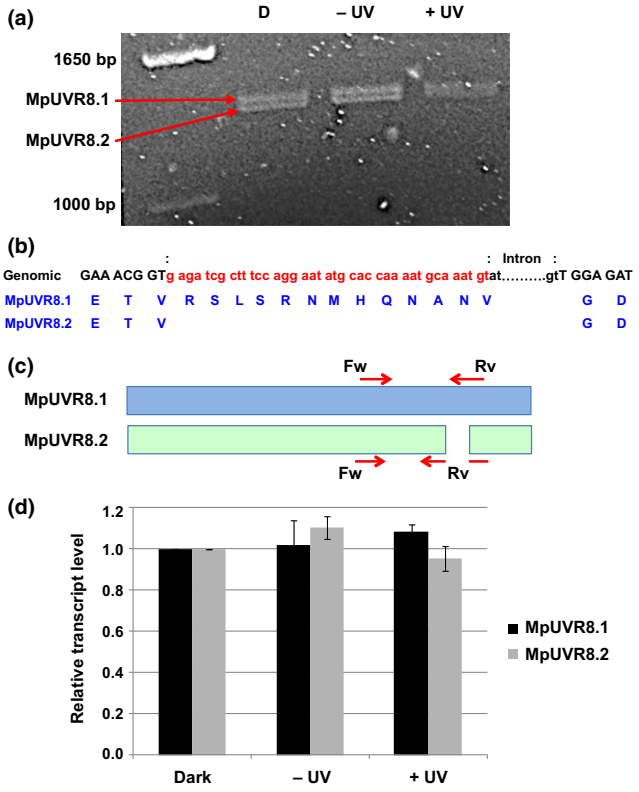


Fig. 2 Alternative transcripts of *Marchantia polymorpha UV RESISTANCE LOCUS 8* (*UVR8*) gene *MpUVR8*. (a) PCR products obtained from *M. polymorpha* cDNA using primers that amplify the complete coding sequence. The two bands are derived from alternative transcripts of the single *UVR8* gene. The first lane shows the size markers indicated. Plants placed in darkness for 16 h (D) were exposed to 20 μmol m^{-2} s^{-1} white light (−UV) or 3 μmol m^{-2} s^{-1} UV-B (+UV) for 3 h. (b) Part of the genomic sequence of the *M. polymorpha UVR8* gene is shown together with the corresponding coding sequences (in blue) of MpUVR8.1 (E412 to D429) and MpUVR8.2 (E412 to D416). The red sequence is present in the cDNA encoding MpUVR8.1 but not MpUVR8.2. Above the genomic sequence, colons indicate putative intron splice junctions. The intron between the second and third colons is 177 bp. Alternative splicing at either the first or the second splice junction would produce the transcripts encoding MpUVR8.2 and MpUVR8.1, respectively. (c) Location of the forward (Fw) and reverse (Rv) primers used to assay specifically the *MpUVR8.1* and *MpUVR8.2* transcripts. The reverse primer for *MpUVR8.1* anneals to sequence that is not present in *MpUVR8.2* and the reverse primer for *MpUVR8.2* anneals to sequences flanking the additional sequence present in *MpUVR8.1*. (d) Relative expression of *MpUVR8.1* and *MpUVR8.2* transcripts assayed by quantitative reverse transcriptase PCR using the primers in (c), normalised to the level in darkness. Plants were treated as in (a). Error bars show ± SE for three biological replicates each with three technical replicates. The global effects of treatment (darkness, white light and UV-B) for each transcript expressed were not significant (one-way ANOVA).

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Physcomitrella UVR8 behaves similarly to Arabidopsis UVR8 in that the dimeric protein is present in samples from tissue not exposed to UV-B and although the monomer is also present, the monomer increases in abundance following UV-B exposure. However, in *Marchantia* dimeric UVR8 is barely detectable and the protein appears monomeric both before and after UV-B exposure (Fig. 3).

To counter the possibility that the antibody aberrantly failed to recognise dimeric UVR8 in *Marchantia*, and to examine any potential differences between MpUVR8.1 and MpUVR8.2, experiments were undertaken to transiently express the proteins in *N. benthamiana* leaves as GFP fusions. The dimer/monomer status of the expressed fusion proteins was monitored using an anti-GFP antibody. As shown in Fig. 4(a), transiently expressed

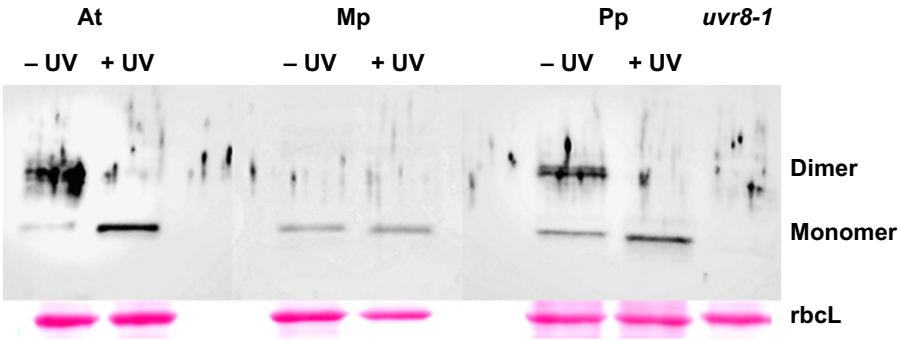
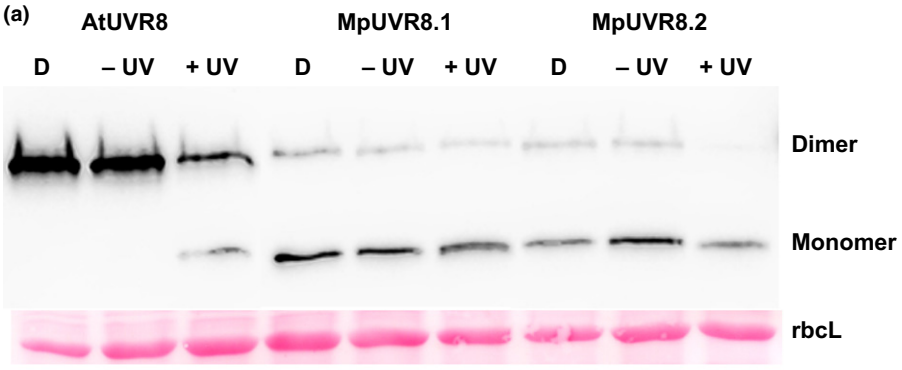


Fig. 3 Dimer/monomer status of UV RESISTANCE LOCUS 8 (UVR8) in *Marchantia polymorpha* and *Physcomitrella patens*. Plants placed in darkness for 16 h were exposed to 20 μmol m^{-2} s^{-1} white light (−UV) or 3 μmol m^{-2} s^{-1} UV-B (+UV) for 30 min. Protein extracts from *Marchantia* (Mp), *Physcomitrella* (Pp) and Arabidopsis wild-type (At) and *uvr8-1* mutant were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis with nonboiled samples and immunoblots were incubated with an anti-UVR8 antibody. Stained Rubisco large subunit (rbcL) is shown as a loading control. UVR8 dimer and monomer are indicated.



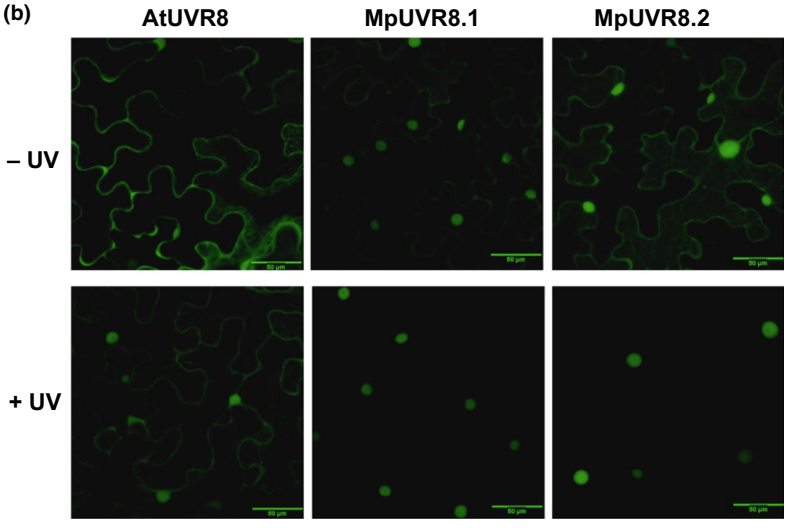


Fig. 4 Transient expression of green fluorescent protein (GFP)-AtUVR8, GFP-MpUVR8.1 and GFP-MpUVR8.2 fusion proteins in *Nicotiana benthamiana* leaves by infiltration with *Agrobacterium*. (a) Plants placed in darkness for 16 h (D) were exposed to 20 μmol m^{-2} s^{-1} white light (−UV) or 3 μmol m^{-2} s^{-1} UV-B (+UV) for 3 h. Protein extracts were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis with nonboiled samples and immunoblots were incubated with an anti-GFP antibody. Stained Rubisco large subunit (rbcL) is shown as a loading control. GFP-UVR8 dimer and monomer are indicated. (b) Cellular localisation of GFP-UVR8 fusions revealed by confocal microscopy. Plants were treated as in (a). Bars, 50 μm.

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Arabidopsis GFP-UVR8 appears as a dimer in the absence of UV-B, and UV-B exposure initiates monomerisation. By contrast, GFP fusions of MpUVR8.1 and MpUVR8.2 were present predominantly in the monomeric form, regardless of whether the leaves were exposed to UV-B. Together, the experiments with *Marchantia* protein extracts and *N. benthamiana* transient expression indicate that both MpUVR8.1 and MpUVR8.2 form weaker dimers than Arabidopsis UVR8 *in vivo*.

In Arabidopsis, UV-B exposure stimulates the accumulation of UVR8 in the nucleus (Kaiserli & Jenkins, 2007; Yin *et al.*, 2016). Transiently expressed Arabidopsis GFP-UVR8 is present mainly in the cytosol in tissue not exposed to UV-B, but localisation in the nucleus increases following UV-B exposure (Fig. 4b), consistent with previous observations in transgenic plants (Kaiserli & Jenkins, 2007). By contrast, both *Marchantia* GFP-UVR8 fusion proteins are clearly present in the nucleus in the absence of UV-B and little if any difference in localisation is evident following UV-B exposure.

Physcomitrella and *Marchantia* UVR8 are functional in Arabidopsis

To test whether the bryophyte UVR8s are functional, each of the cDNAs was expressed as a GFP fusion in the Arabidopsis *uvr8-1* mutant. T_1 transgenic plants were selected and used to produce homozygous T_3 lines. The level of expression of GFP-UVR8 in each line was assayed by immunodetection on western blots of protein extracts (Fig. 5) and at least two independent lines were selected for each bryophyte GFP-UVR8 fusion for functional assays.

Quantitative RT-PCR assays were used to assay UV-B induction of *HY5* transcripts, which is a key gene expression response mediated by UVR8 (Brown *et al.*, 2005; Favory *et al.*, 2009). Transgenic lines expressing each of the two *Marchantia* and *Physcomitrella* UVR8 sequences showed significant UV-B induction of *HY5* transcript levels, as did Arabidopsis GFP-UVR8, whereas the *uvr8-1* control lacked the response (Fig. 5). In addition, each GFP-UVR8 fusion was active in promoting accumulation of the flavonoid biosynthesis enzyme CHS in response to UV-B in the transgenic seedlings, similar to Arabidopsis GFP-UVR8, but in contrast to *uvr8-1* plants (Fig. 5). As reported previously, and shown in Fig. 6, UV-B suppresses hypocotyl growth in wild-type Arabidopsis plants whereas *uvr8-1* is impaired in the response; expression of wild-type UVR8 fused to GFP or yellow fluorescent protein (YFP) in *uvr8-1* plants functionally complements the mutant phenotype (Favory *et al.*, 2009; O'Hara & Jenkins, 2012; Huang *et al.*, 2014). Transgenic *uvr8-1* plants expressing each of the

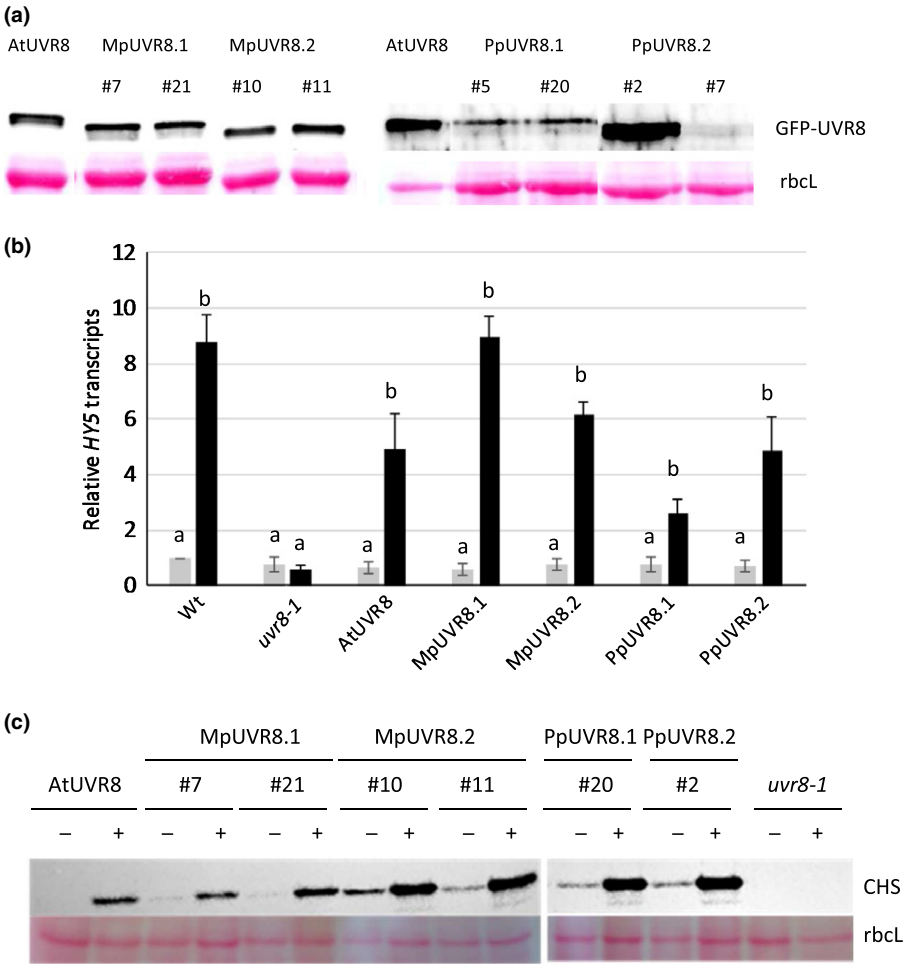


Fig. 5 *Physcomitrella* and *Marchantia* UV RESISTANCE LOCUS 8 (UVR8) are functional in regulating gene expression in transgenic Arabidopsis. (a) Transgenic lines of Arabidopsis *uvr8-1* expressing green fluorescent protein (GFP)-AtUVR8, GFP-MpUVR8.1, GFP-MpUVR8.2, GFP-PpUVR8.1 and GFP-PpUVR8.2. Protein extracts were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis with boiled samples and the immunoblot was incubated with an anti-UVR8 antibody; stained Rubisco large subunit (rbcL) is shown as a loading control. (b) *ELONGATED HYPOCOTYL 5* (*HY5*) transcript levels were assayed in 10-d-old seedlings exposed to 20 μmol m^{-2} s^{-1} white light (grey bars) or 1.5 μmol m^{-2} s^{-1} UV-B for 4 h (black bars). *HY5* transcript levels were measured by quantitative reverse transcriptase PCR relative to control *ACTIN2* transcripts and normalised to the value for GFP-AtUVR8 minus UV-B. Error bars show SE for three biological replicates each with three technical replicates. Different letters show significant differences between treatments (at least $P < 0.05$, Student's *t*-test). (c) CHALCONE SYNTHASE (CHS) protein abundance in 7-d-old seedlings grown in 1.5 μmol m^{-2} s^{-1} white light with (+UV) or without (−UV) 1.5 μmol m^{-2} s^{-1} UV-B. Proteins were analysed as in (a) except using an anti-CHS antibody.

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Marchantia and *Physcomitrella* UVR8 sequences showed hypocotyl growth suppression by UV-B, similar to wild-type Arabidopsis (Fig. 6).

The dimer/monomer status and localisation of the GFP fusions was examined in transgenic plants. Arabidopsis GFP-UVR8 shows conversion of the dimer to the monomer in response to UV-B exposure, as described previously (Cloix *et al.*, 2012; O'Hara & Jenkins, 2012; Fig. 7a). With the PpUVR8.1 and PpUVR8.2 GFP fusions, both dimer and monomer are present in the absence of UV-B, and UV-B exposure stimulates an increase in the relative level of monomer. The MpUVR8.1 and MpUVR8.2 GFP fusions appear monomeric before UV-B exposure, with dimer below the limit of detection, and again only monomer is detectable following UV-B treatment (Fig. 7a). Arabidopsis GFP-UVR8 shows increased nuclear localisation following UV-B treatment of plants (Kaiserli & Jenkins, 2007; Fig. 7b). Both *Physcomitrella* GFP-UVR8 fusion proteins showed increased accumulation in the nucleus relative to the cytoplasm following UV-B exposure of the transgenic plants, but there was a higher level of nuclear localisation in the absence of UV-B at least for PpUVR8.2 compared to Arabidopsis GFP-UVR8 (Fig. 7b). The *Marchantia* GFP-UVR8 fusions were strongly localised in the nucleus in both the presence and the absence of UV-B, although there was evidence of slightly more cytoplasmic localisation before UV-B exposure. These findings are largely consistent with those obtained in bryophyte protein extracts (Fig. 3) and transient expression (Fig. 4).

Discussion

This study shows that both *P. patens* and *M. polymorpha* express functional UVR8 photoreceptor proteins, similar to previous reports for *A. thaliana* (Kliebenstein *et al.*, 2002; Brown *et al.*, 2005; Favory *et al.*, 2009) and *C. reinhardtii* (Tilbrook *et al.*, 2016). Whereas Arabidopsis expresses a single UVR8 protein, both *Physcomitrella* and *Marchantia* are able to produce two distinct *UVR8* transcripts and therefore potentially two UVR8 variants. This is not uncommon, as examination of genome sequence data indicates that several species contain at least two *UVR8* genes (Fernandez *et al.*, 2016), including the spike moss (lycopodiophyte) *Selaginella moellendorffii*, the dicot *Brassica oleracea* and the monocot *Hordeum vulgare*. The possession of multiple genes provides the potential for differential regulation of expression and, moreover, the expression of protein variants that might differ in activity or regulation. Differential expression of multiple *UVR8* genes has been reported in litchi fruits (Zhang *et al.*, 2016), but to date there is no evidence of functional differences between UVR8 proteins within any species. In each of the bryophyte species studied here, both *UVR8* transcripts are expressed. Although there was no indication of significant differential expression of the *M. polymorpha* genes, at least under the conditions examined here, RNAseq data (https://phytozome.jgi.doe.gov/pz/portal.html) indicate that *PpUVR8.1* is expressed at a much higher level (*c.* 340-fold higher) than *PpUVR8.2* in gametophytes under various conditions. In Arabidopsis, *UVR8* is

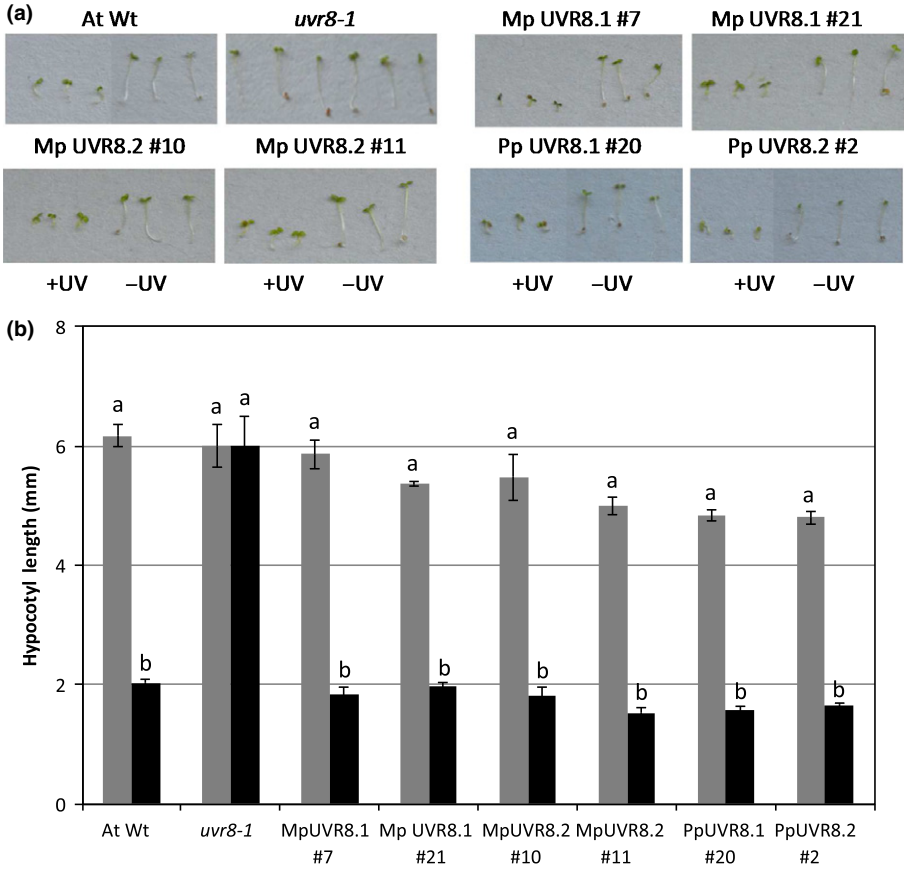


Fig. 6 *Physcomitrella* and *Marchantia* UV RESISTANCE LOCUS 8 (UVR8) are functional in regulating hypocotyl growth in transgenic Arabidopsis. (a) Four-day-old seedlings grown in 1.5 μmol m^{-2} s^{-1} white light with (+UV) or without (−UV) 1.5 μmol m^{-2} s^{-1} UV-B. (b) Hypocotyl lengths of seedlings as in (a); grey bars: −UV-B, black bars: +UV-B. Error bars are ± SE, $n = 15$. Different letters show significant differences between treatments (at least $P < 0.001$, Student's *t*-test).

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constitutively expressed (Kliebenstein *et al.*, 2002; Kaiserli & Jenkins, 2007; Rizzini *et al.*, 2011), but changes in *UVR8* transcript expression in response to various developmental and environmental factors have been reported in other species (Liu *et al.*, 2015; Loyola *et al.*, 2016; Wu *et al.*, 2016; Zhao *et al.*, 2016). Hence the present study provides a basis for future research to explore *UVR8* expression and function in bryophytes.

M. polymorpha is the only species known to date in which alternative splicing generates transcripts encoding UVR8 variants. The presence or absence of the additional 13 amino acids in the C-terminal region does not alter the dimer/monomer status, cellular localisation or function of the *Marchantia* UVR8 proteins, at least when expressed in Arabidopsis. The alternative transcripts are present at similar levels in *Marchantia*, judging by the abundance of products amplified from cDNA (Fig. 2a), and quantitative RT-PCR data suggests no difference in expression in darkness, white light and UV-B. It is not known whether both MpUVR8.1 and MpUVR8.2 proteins accumulate in *Marchantia* even though their transcripts are present, but this seems highly likely unless there is translational control or differential protein stability; and both proteins are stable when expressed in *Nicotiana* and *Arabidopsis*. Thus, at present it is not clear whether the differential splicing has any functional significance in *Marchantia*.

Amino acids with key functional roles in *Arabidopsis* UVR8, including the principal salt-bridge amino acids and the 14 tryptophans, are totally conserved in the *Physcomitrella* and *Marchantia* UVR8 sequences. The differences between the *Physcomitrella* and *Marchantia* UVR8 sequences, and also between the bryophyte and Arabidopsis sequences, are mainly in the C-terminal region. These differences evidently do not impair the function of the proteins when expressed in Arabidopsis. Notably, the first 18 amino acids of the C27 region, including the critical VP residues, are strongly conserved, consistent with the key role of this region in interactions with the COP1 and RUP proteins. Such interactions may be important for UVR8 activity in the bryophytes since both the *Physcomitrella* and the *Marchantia* genomes contain putative *COP1* and *RUP* sequences.

The *Physcomitrella* UVR8 proteins are similar to Arabidopsis UVR8 in that they form dimers in the absence of UV-B and monomerise following UV-B exposure, both in *Physcomitrella* protein extracts and when the proteins are expressed in Arabidopsis; nevertheless, an increased level of monomer was observed in the absence of UV-B in transgenic plants. GFP fusions of the *Physcomitrella* UVR8 proteins show UV-B stimulation of nuclear localisation in transgenic Arabidopsis, although there is a little more *Physcomitrella* GFP-UVR8 in the nucleus in the absence of UV-B than for Arabidopsis GFP-UVR8.

The *Marchantia* UVR8 proteins behave differently from Arabidopsis and *Physcomitrella* UVR8 in that the dimer is less stable. *Marchantia* UVR8 appears principally monomeric in thallus protein extracts and also when both MpUVR8.1 and MpUVR8.2 are expressed as GFP fusions either transiently in *Nicotiana* or stably in *Arabidopsis*. However, the nonboiled gel method used to visualise the dimer and monomer very sensitively detects even slight weakening of the UVR8 dimer (Heilmann *et al.*, 2016), so it is possible that *Marchantia* UVR8 does form a dimer *in vivo*, but nonetheless a weaker dimer than in *Physcomitrella* and Arabidopsis. It is not clear why *Marchantia* UVR8 has a less stable dimer, because the charged amino acids that maintain the dimeric structure are conserved. Perhaps other differences in sequence (Fig. 1), although apparently minor, cause subtle changes in charge distribution at the dimer interface that influence dimer stability. *Marchantia* UVR8 also differs in regulation of its localisation. Whereas UV-B stimulates nuclear accumulation of Arabidopsis GFP-UVR8, the *Marchantia* GFP fusion is more strongly nuclear localised in the absence of UV-B and exposure to UV-B has little if any effect on its distribution, both in transient and in stable expression. The correlation between increased nuclear accumulation and increased levels of monomer

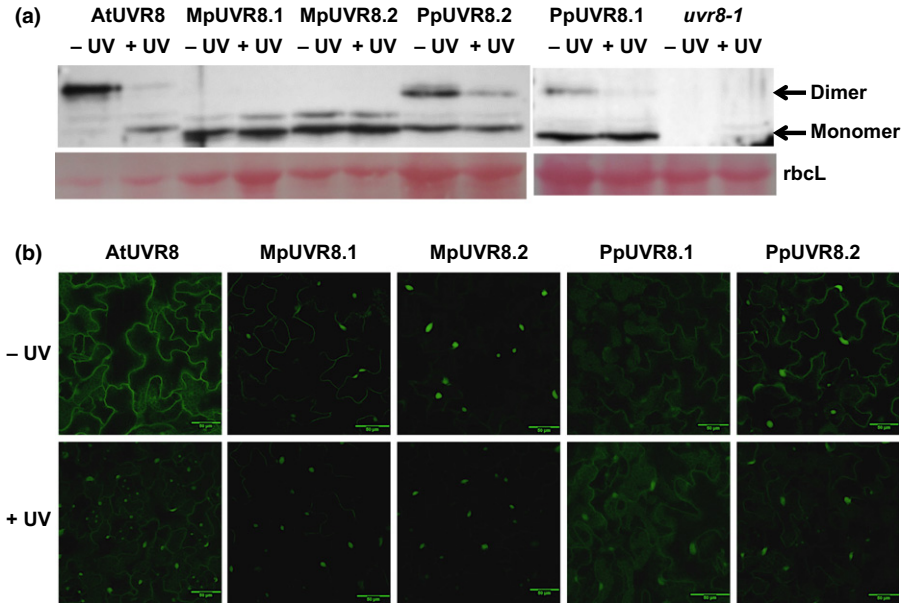


Fig. 7 Dimer/monomer status and localisation of green fluorescent protein (GFP)-UV RESISTANCE LOCUS 8 (UVR8) fusions in transgenic Arabidopsis. (a) Plants were exposed to 20 μmol m^{-2} s^{-1} white light (−UV) or 3 μmol m^{-2} s^{-1} UV-B for 30 min (+UV). Protein extracts were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis with nonboiled samples and immunoblots were incubated with an anti-UVR8 polyclonal antibody. Stained Rubisco large subunit (rbcL) is shown as a loading control. GFP-UVR8 dimer and monomer are indicated. (b) Cellular localisation of GFP-UVR8 fusions revealed by confocal microscopy. Plants were treated as in (a), except for 3 h. Bars, 50 μm.

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in the absence of UV-B supports the hypothesis that monomeric UVR8 accumulates in the nucleus (Qian *et al.*, 2016). The greater difference in dimer/monomer status and localisation observed between *Marchantia* and Arabidopsis UVR8 than between *Physcomitrella* and Arabidopsis UVR8 supports the hypothesis that vascular plants are more phylogenetically distant from liverworts than from mosses (Qiu *et al.*, 2007; Bowman *et al.*, 2016), although the phylogenetic relationships between land plant lineages are not yet completely clear (Wickett *et al.*, 2014). In addition, it is unknown at present if differences between *Marchantia* and *Physcomitrella* UVR8 determine in some way the different modalities of UV-B responses typical of liverworts and mosses regarding, for example, the accumulation of UV-absorbing compounds (Fabón *et al.*, 2012b).

The experiments with transgenic Arabidopsis provide unequivocal evidence that each of the bryophyte UVR8 proteins is functional. Both of the *Physcomitrella* UVR8 proteins and both of the *Marchantia* UVR8 variants complement the impaired UV-B response of the *uvr8-1* mutant with respect to hypocotyl growth suppression. In addition, the bryophyte UVR8 proteins mediate *HY5* transcript expression and CHS protein accumulation in response to UV-B. There is no indication that any of the bryophyte UVR8 proteins are more or less functional than the others under the experimental conditions employed, which is not surprising given the strong conservation of the amino acid sequences. It is interesting that the *Marchantia* UVR8 proteins are functional even though they have weaker dimers and appear to form increased levels of monomer *in vivo* in the absence of UV-B. Recent research indicates that monomeric UVR8 can be active in photoreception, both *in vitro* (Heilmann *et al.*, 2014; Mathes *et al.*, 2015) and *in vivo* (Heilmann *et al.*, 2016), and so functionality is not necessarily directly correlated with UV-B-induced dimer-to-monomer conversion.

In conclusion, this study demonstrates that the liverwort *M. polymorpha* and moss *P. patens* express functional UVR8 proteins. Although interesting differences in the mechanism of *UVR8* expression and in the dimer/monomer status and localisation of UVR8 protein are observed between the two species, fundamentally the research highlights how strongly the molecular mechanism of UVR8 photoreceptor action has been conserved over millions of years of evolution. Thus, it is reasonable to speculate that UVR8 functions in bryophytes to mediate responses to UV-B through the regulation of gene expression, as it does in *Arabidopsis* and *Chlamydomonas*. Wolf *et al.* (2010) reported that UV-B exposure causes differential expression of *c.* 400 genes in *Physcomitrella*, and hence it is likely that UVR8 mediates at least some of these expression changes. Moreover, UV-B affects broadly similar processes in bryophytes and flowering plants, including the synthesis of UV-absorbing phenolic compounds and developmental changes (Wolf *et al.*, 2010; Martínez-Abaigar & Núñez-Olivera, 2011; Fabón *et al.*, 2012a,b) and UVR8 may be involved in such responses. If UVR8 functioned in ancient bryophytes in the same way as proposed here for extant species, it is likely that it played a key role in enabling the bryophytes to cope with the continual exposure to potentially damaging levels of UV-B associated with terrestrial environments. Furthermore, the availability of UVR8 would enable plants to exploit the presence of UV-B as an environmental cue to regulate morphogenesis and development. It will now be fascinating to discover which processes UVR8 mediates in the mosses and liverworts, and that will require the production and characterisation of mutant plants. Hence, the present study provides a molecular basis to further understand UVR8 function in the bryophytes.

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Author contributions

G.I.J., J.M-A. and E.N-O. planned and designed the research. G.S., C.C. and M.H. performed experiments. All authors analysed the data. G.I.J. wrote the manuscript with input from the other authors.

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