The molecular and physiological responses of Physcomitrella patens to ultraviolet-B radiation

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Abstract

Ultraviolet-B (UV-B) radiation present in sunlight is an important trigger of photomorphogenic acclimation and stress responses in sessile land plants. Although numerous moss species grow in unshaded habitats, our understanding of their UV-B responses is very limited. The genome of the model moss Physcomitrella patens, which grows in sun-exposed open areas, encodes signaling and metabolic components that are implicated in the UV-B response in flowering plants. In this study, we describe the response of P. patens to UV-B radiation at the morphological and molecular levels. We find that P. patens is more capable of surviving UV-B stress than Arabidopsis (Arabidopsis thaliana) and describe the differential expression of approximately 400 moss genes in response to UV-B radiation. A comparative analysis of the UV-B response in P. patens and Arabidopsis reveals both distinct and conserved pathways.


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Ultraviolet-B (UV-B) radiation (280–315 nm) is intrinsic to sunlight reaching the surface of the earth. During the Phanerozoic period (within the last 545 million years), the levels of UV-B reaching the biosphere generally decreased (Rozema et al., 2009). In particular, the development of a stratospheric ozone layer filtering out all UV-C (less than 280 nm) and part of the UV-B radiation was likely a prerequisite for terrestrial plant life and accompanied by the development of phenolic sunscreens in plants (Rozema et al., 1997). During early land plant evolution, the divergence of the last common ancestor of bryophytes (comprising liverworts, true mosses, and hornworts) and vascular plants (comprising lycophytes, ferns, and seed plants) occurred shortly after the water-to-land-transition in the Ordovician period, at least 450 million years ago (Zimmer et al., 2007). If the level of UV-B reaching the ground was significantly higher at that time than today, it is to be expected that protective mechanisms and their corresponding signaling pathways evolved at that time and thus should be present in all land plants.

Today, ambient UV-B radiation still has a broad effect on plants relying on sunlight for photosynthesis, and its level varies strongly with season and time of day as well as with latitude and altitude (Paul and Gwyn-Jones, 2003). As an environmental stress factor, it may evoke diverse damage to a broad range of cellular constituents, including DNA (Britt, 2004). However, plants in nature are seldom visibly damaged by UV-B but are generally rather well acclimated and thus protected. This largely results from effective repair and protection mechanisms (Frohnmeyer and Staiger, 2003; Ulm and Nagy, 2005; Jenkins, 2009). A common protective measure against UV-B radiation is the synthesis of secondary metabolites acting as UV-absorbing compounds and accumulating in the vacuoles of epidermal cells. These compounds are mostly phenolic, including flavonoids and sinapate esters, and are at least partly induced by UV-B (Jenkins, 2009; Stracke et al., 2010). Next to this protective sunscreen, plants contain effective DNA repair pathways, particularly including photolyases as efficient and error-free DNA repair enzymes (Britt, 2004).

In seed plants, UV-B is known to have at least two distinct effects: one in response to the evoked damage and the other in response to the perception of UV-B by a postulated receptor, leading to UV-B-induced photomorphogenesis and thus acclimation (Frohnmeyer and Staiger, 2003; Ulm and Nagy, 2005; Jenkins, 2009). The response depends on the wavelength, fluence rate, and duration of the UV-B irradiation as well as the extent of adaptation. In general, UV-B radiation results in the activation of a diverse and distinct set of signaling responses leading to a
reprogramming in gene expression (for review, see Ulm and Nagy, 2005). The damage response pathway includes activation of more general stress responses (Ulm, 2003), whereas the UV-B-specific pathway involves the recently described UVR8-COP1-HY5 (for UV Resistance Locus8-Constitutive Photomorphogenically Elongated Hypocotyl5) pathway in Arabidopsis (Arabidopsis thaliana; Ulm et al., 2004; Brown et al., 2005; Oravec et al., 2006; Favory et al., 2009).

To date, relatively little is known about the molecular response of bryophytes to UV-B radiation, which might be due to the general view of bryophytes as being shade adapted. However, many mosses, like the model organism Physcomitrella patens, grow in unshaded habitats exposed to high light and UV-B radiation intensities (Rensing et al., 2009). Components mediating UV-B protection were found in Antarctic mosses, namely UV-absorbing pigments (probably comprising carotenoids and flavonoids) in Andreaea regularis (Newsham, 2003) and phenolic compounds in Ceratodon purpureus (Clarke and Robinson, 1965). The secondary plant metabolism leading from Phe to the synthesis of flavonoids is induced when exposed to high light and UV-B radiation intensities (Rensing et al., 2009). Components mediating UV-B protection were found in Antarctic mosses, namely UV-absorbing pigments (probably comprising carotenoids and flavonoids) in Andreaea regularis (Newsham, 2003) and phenolic compounds in Ceratodon purpureus (Clarke and Robinson, 1965). In the order Bryales, to which P. patens belongs, the presence of glycosylated quercetins was shown previously (Melchert and Alston, 1965). The secondary plant metabolism leading from Phe to the synthesis of flavonoids is induced under UV-B radiation in seed plants (Li et al., 1993; Caldwell et al., 1998; Jenkins, 2008; Stracke et al., 2010), and components of this as well as of the blue/UV light signaling pathway are present in P. patens (Jiang et al., 2006; Rensing et al., 2008). Also, homologs of the Arabidopsis E3 ubiquitin ligase COP1, the β-propeller protein UVR8, and the bZIP transcription factor HY5 are encoded by the P. patens genome; the P. patens COP1 homologs constitute a multigene family, and two homologs each of HY5 and UVR8 are present (Richardt et al., 2007; Rensing et al., 2008).

Here, we describe the molecular and physiological responses of the model bryophyte P. patens to UV-B radiation and compare them with the responses of the model flowering plant Arabidopsis. In addition, we hypothesize on key genes (involved in stress responses) that were instrumental in enabling plant life to conquer the terrestrial habitat.

RESULTS AND DISCUSSION

Morphogenic Response to UV-B Radiation

Changes in the morphogenic response of P. patens to UV-B radiation were detected and compared with their respective non-UV-B-treated controls using UV-B-absorbing cutoff filters (Supplemental Table S1). Five weeks of supplemental UV-B radiation caused changes in the morphology of gametophyte colonies grown from single gametophores (Fig. 1, A–D). The alterations also manifested as a statistically significant (P ≤ 0.05) decrease in gametophyte colony circumference under UV-B compared with the respective UV-B control (Fig. 1E). The colony circumference, however, was not affected by the more than 6-fold higher photon fluence rate in the white light field with supplemental broad-band UV-B (25.1 μmol m⁻² s⁻¹) as compared with supplemental narrow-band UV-B (3.6 μmol m⁻² s⁻¹; Fig. 1, A, C, and E), indicating specific regulation by UV-B. Morphological alterations due to varying light levels are manifested as different protonemal cell types developing under light conditions without supplemental UV-B irradiation. At lower fluence rates, mainly photosynthetically active caulonemata are formed to spread the colony, while higher fluence rates primarily evoke the formation of caulonemata, subsequently developing into gametophores, as described before (Thelander et al., 2005).

Besides differences in circumference between UV-B-treated and control colonies, differences in developed cell types were apparent as well. For example, a decrease in the juvenile gametophyte stage, the protonemata, was observed (Fig. 1, B and D). Within the white light field with supplemental narrow-band UV-B, protonemata developed only in a small strip around the gametophore, whereas under the white light field with supplemental broad-band UV-B, no protonema growth was observed at all (Fig. 1, B and D). Those findings are in agreement with an increase in gametophore fresh weight under supplemental broad-band UV-B (Supplemental Fig. S1A). Photomorphogenic changes due to UV-B irradiation promote UV-B protection in flowering plants like Arabidopsis (Favory et al., 2009; Jenkins, 2009). Similarly, the reduced surface area of gametophore colonies minimizes the contact surface toward damaging UV radiation. Additionally, apparently resistant gametophores were formed under supplemental broad-band UV-B (Fig. 1D; growth of chlorophyll was not observed).

Gametophores grown for 5 weeks showed a statistically significant (P ≤ 0.05) decrease in chlorophyll content if grown in the white light field with supplemental narrow-band UV-B (Supplemental Fig. S1B). Reduced chlorophyll content under supplemental UV-B irradiation has been reported for several other plant species as well (Kakani et al., 2003; Gao et al., 2004), which might be due to lowered biosynthesis or degradation of chlorophyll (Teramura, 1983).

Resistance to UV-B Radiation Stress

UV-B Irradiation Affects the Development of Protonema from Spores

The juvenile stages in the life cycle of P. patens were more affected by UV-B radiation than adult gametophores. In the white light field with supplemental broad-band UV-B, protonema colonies did not establish, yet gametophores seemed unaffected and were able to grow (Fig. 1D). Germination and subsequent filamentous growth from spores only occurred in suitable environmental conditions lacking harmful doses of UV-B (Fig. 2). While in the white light field with supplemental narrow-band UV-B reduced fila-
mentous growth still occurred (Fig. 2A), spores failed to germinate under supplemental broad-band UV-B (Fig. 2B). Differences in growth between the non-UV-B-irradiated controls in the different light fields resulted from differences in light fluence rates and an apparent influence on spore germination (compare 8 d –UV-B in Fig. 2, A and B). *P. patens* gametophores are the adult stage of the gametophyte, which are exposed to sunlight in their natural habitat analogously to the sporophyte of flowering plants. This implies that gametophores need to be more protected with regard to UV irradiation in comparison with the short-lived, juvenile protonemata.

**Higher UV-B Resistance of P. patens in Comparison with Arabidopsis**

*P. patens* irradiated under harmful levels of UV-B radiation sustains damage in terms of necrotic lesions in an exposure-time-dependent manner (Fig. 3, A–D). Twelve hours of broad-band UV-B irradiation caused selective bleaching of gametophores, but the damage itself is not instantly visible. Starting 2 weeks after the irradiation procedure, necrotic lesions appear to their whole extent (Fig. 3, C and D). Yet, even after 16 h of irradiation with UV-B, *P. patens* gametophores are not entirely affected and still exhibit green parts, unlike Arabidopsis, which appears completely bleached after 12 h of irradiation and 3 weeks of subsequent incubation (Fig. 3E).

**Regeneration from UV-B-Damaged Gametophores Results in the Initiation of Filamentous Growth**

High-level broad-band UV-B irradiation with 24 h of exposure resulted in gametophore colonies with a glazed appearance 11 d after irradiation (Fig. 3F). Within 28 d after irradiation, regreening of individual cells occurred, demonstrating a high regeneration capability of damaged gametophores. The regenerated cells arose from the bottom of the gametophyte colony and/or from the gametophore stem itself. At this time, the formation of protonema filaments was observed (Fig. 3H). Subsequently, gametophores developed from those protonema filaments and were detectable several weeks later (Fig. 3I). The high regeneration capability of *P. patens* has been reported before (e.g. small quantities of gametophytic or sporophytic tissue are able to regenerate; Cove, 2005), and growth of protonemata from isolated gametophore leaflets is used as a system to study reembryonalization (Hasebe, 2007; Mosquina et al., 2009). The observation that at first only filamentous growth occurs indicates a reprogramming of differentiated gametophore cells into apical stem cells, generating protonemata that subsequently develop gametophores.

**Molecular Response to Broad-Band UV-B Radiation**

*Low-Level UV-B Does Not Activate Differential Gene Expression in P. patens*

Induction of early UV-B-responsive genes after 1 h of irradiation at relatively low narrow-band UV-B levels

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**Figure 1.** Gametophyte growth phenotype under supplemental UV-B radiation. A to D, Morphology of gametophytes grown for 5 weeks without (–UV-B: WG370; A and C) and with (+UV-B: WG303; B and D) supplemental UV-B irradiation. Tissue types are shown by arrows (prot = protonema and gam = gametophore). Gametophyte colonies were grown in a white light field with supplemental narrow-band UV-B (A and B) or in a white light field with supplemental broad-band UV-B (C and D). Bars = 1 mm. E, Average circumference of gametophyte colonies after 5 weeks. Error bars represent SD (n = 3 biological replicates, each with more than 10 colonies).
did not result in differential gene expression in *P. patens*, based on genome-wide microarray expression analysis. In *P. patens*, only a single gene (Phypa_216829, a putative ortholog of the Arabidopsis mitochondrial substrate carrier family protein AT2G17270) was identified as differentially expressed, while in Arabidopsis, several hundred genes are induced under identical UV-B conditions (Favory et al., 2009). These findings indicate

**Figure 2.** Filamentous growth from spores under UV-B. Development of filamentous protonema from *P. patens* spores under supplemental UV-B in the white light field with supplemental narrow-band (A) and broad-band (B) UV-B. Representative images were taken 8 d, 3 weeks, and 7 weeks after start of irradiation. Filters utilized were WG345 as nontreated control (−UV-B) and WG303 (+UV-B irradiation). Bars = 0.5 mm.

**Figure 3.** Bleaching of *P. patens* and Arabidopsis and regeneration of gametophytes after UV-B stress treatment. A to E, Bleached *P. patens* gametophores (A–D) and Arabidopsis seedlings (E) after UV irradiation. Images were taken 1 d (A), 3 d (B), 14 d (C), and 21 d (D and E) after irradiation. F to I, Regeneration of gametophore colonies after 24 h of irradiation under WG303 in the broad-band UV-B field. Images were taken 11 d (F), 28 d (G and H), and 6 months (I) after irradiation. Bars = 1 mm.
that *P. patens* shows a reduced sensitivity to low-level narrow-band UV-B radiation in comparison with Arabidopsis. This might result from a higher general level of resistance, as has been argued for other abiotic stresses before (Richardt et al., 2010).

**Genome-Wide Analysis of the UV-B Response in *P. patens* Displays Similarities to Arabidopsis**

The number of differentially expressed genes observed under broad-band UV-B irradiation was strikingly similar between *P. patens* (1 h of irradiation) and the Arabidopsis accessions Landsberg erecta (*Ler*) and Columbia (*Col*; 15 min of irradiation plus 45 min under standard conditions before harvesting). Many of the categories found to be significantly enhanced or suppressed ($q \leq 0.01$ for *P. patens*, $q \leq 0.05$ for Arabidopsis, Fisher’s exact test) were identical (Table I; genes found to be differentially expressed are shown in Supplemental Tables S2 and S3). Among 400 induced genes in *P. patens*, 72 putative orthologs were detected in Arabidopsis. Out of 401 (*Ler*) and 396 (*Col*) up-regulated genes, 117 were identified as putative orthologs in the transcriptome of *P. patens*. In total, 12 (8 + 2 + 2) of these genes (17%; Table II) were found to be induced in both *P. patens* and at least one of the Arabidopsis ecotypes (Fig. 4). The functional conservation of these putative orthologs within the UV-B response has thus been evolutionarily conserved for more than 400 million years. Some of the genes have not been functionally characterized in Arabidopsis so far, and several others encode metabolic functions (Table II). The Regulator of Chromosome Condensation (RCC1) family protein is especially interesting, as it contains the PFAM RCC1 domain (00415), which is also found in the Arabidopsis UVR8 protein (Kliebenstein et al., 2002). This particular ortholog with its single RCC1 domain might constitute an ancient signaling mediator that acts as a nucleotide-exchange factor in a Ran-related GTPase pathway.

**Conservation of Transcriptional Regulation**

The Gene Ontology molecular function classification outlines enhancement of transcription factor and transcriptional regulator (together: transcription-associated proteins [TAPs]) activity in *P. patens* as well as in Arabidopsis under broad-band UV-B radiation (Table I). Induction of several TAP genes was verified by quantitative real-time PCR (Fig. 5), confirming the microarray results. The number of TAPs per family induced under UV-B is shown in Supplemental Figure.

### Table I. Categories of Gene Ontology biological processes, cellular components, and molecular functions enhanced or suppressed in *P. patens* among the differentially expressed genes under broad-band UV-B in comparison with Arabidopsis ecotypes

Gene Ontology categories appearing as statistically significantly enhanced (+) or suppressed (−) among the differentially expressed genes in comparison with all genes under broad-band UV-B *P. patens*. Categories enhanced or suppressed in all differentially expressed genes in Arabidopsis are also given.

<table>
<thead>
<tr>
<th>Gene Ontology</th>
<th><em>P. patens</em></th>
<th>Arabidopsis Col</th>
<th>Arabidopsis Ler</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological process</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Aromatic compound metabolic process</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cation transport</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>Protein ubiquitination</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>Metal ion transport</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Translation</td>
<td>−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolic process</td>
<td>−</td>
<td></td>
<td></td>
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<tr>
<td>Macromolecule biosynthetic process</td>
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<td>−</td>
<td>−</td>
</tr>
<tr>
<td>RNA metabolic process</td>
<td>−</td>
<td></td>
<td></td>
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<tr>
<td>Protein metabolic process</td>
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<tr>
<td>Cellular protein metabolic process</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Biosynthetic process</td>
<td>−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular component</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ubiquitin ligase complex</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>Mitochondrial envelope</td>
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<td>Mitochondrial inner membrane</td>
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<tr>
<td>Organelle inner membrane</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Ribosome</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Ribonucleoprotein complex</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Molecular function</td>
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<td></td>
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</tr>
<tr>
<td>Transcription factor activity</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Calcium ion binding</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>Transferase activity, transferring glycosyl groups</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Transferase activity, transferring hexosyl groups</td>
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<tr>
<td>Transcription regulator activity</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Metal ion binding</td>
<td>−</td>
<td>−</td>
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</tr>
<tr>
<td>RNA binding</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Structural constituent of ribosome</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Structural molecule activity</td>
<td>−</td>
<td>−</td>
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</tbody>
</table>
The two transcription factor families with statistically significant enhancement ($P \leq 0.01$) of members under UV-B in *P. patens* are the AP2/EREBP and Zinc finger (AN1 and A20 type) families (Supplemental Fig. S2). For example, the AP2/EREBP domain-containing transcription factor Phypa_166576, the closest homolog of which in Arabidopsis belongs to the DREB (for drought-responsive element-binding protein) subfamily (DREB1C; AT4G25470), is up-regulated under broad-band UV-B irradiation in *P. patens*. Among 14 identified induced AP2/EREBP domain-containing gene models, three gene models were annotated as putative TINY transcription factors, thought to connect biotic and abiotic stress signaling in Arabidopsis (Sun et al., 2008). While 14 of 38 detected TAPs in induced gene models under broad-band UV-B belong to the AP2/EREBP transcription factors, none of the transcription factors identified in the gene set of repressed gene models belongs to this family (Supplemental Tables S2 and S3).

Among the UV-B-induced genes, there is a member of the MYB transcription factor family, namely gene model Phypa_184923. In Arabidopsis, UV induction of MYB transcription factors could also be shown (Ulm et al., 2004), especially MYB12, involved in the regulation of flavonoid biosynthesis (Mehrtens et al., 2005; Stracke et al., 2010). This transcription factor has recently been shown to represent one of the targets of the bZIP transcription factor HY5 in the light and UV-B response (Stracke et al., 2010). As can be seen from the phylogeny of the MYB family (Supplemental Fig. S3), there seems to be no putative ortholog of Arabidopsis MYB12 in *P. patens*. However, the induced *P. patens* gene clusters in a subclade containing the Arabidopsis MYB20 gene (AT1G66230) that is also induced under UV-B irradiation. The disjunct pattern of regulated MYBs within the tree suggests that the involvement of individual genes in the UV-B response evolved comparatively late within the different lineages.

### Specificity and Cross Talk of the Transcriptional Regulation

Comparison of all genes differentially regulated under UV-B with previously published data on *P. patens* TAP regulation upon abscisic acid (ABA) treatment and salt stress (Richardt et al., 2010) reveals that only six genes are differentially regulated by drought/salt stress as well as under UV-B. This suggests that the majority of TAP genes found to be regulated in this study might be specific for the UV-B response. It should be noted, however, that genome-wide expression profiling data for *P. patens* are scarce yet and further stresses need to be examined in order to conclude on gene sets specific to certain stress responses. However, there is some apparent cross talk: all six genes mentioned above (induced by UV-B) were previously found to be repressed by ABA.

### Table II. RBHs of *P. patens* gene models coinduced under UV-B in at least one of the Arabidopsis ecotypes

<table>
<thead>
<tr>
<th>Phypa Gene Model</th>
<th>BLAST Hit-Derived Annotation</th>
<th>RBH in Arabidopsis Ler</th>
<th>RBH in Arabidopsis Col</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phypa_34090</td>
<td>$\beta$-OHASE 1 ((\beta)-hydroxylase 1); carotene $\beta$-ring hydroxylase</td>
<td>AT4G25700</td>
<td>AT4G25700</td>
</tr>
<tr>
<td>Phypa_233417</td>
<td>C2 domain-containing protein (calcium binding)</td>
<td>AT4G34150</td>
<td>AT4G34150</td>
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<tr>
<td>Phypa_216740</td>
<td>Oxidoreductase, acting on the CH-CH group of donors</td>
<td>AT1G73650</td>
<td>AT1G73650</td>
</tr>
<tr>
<td>Phypa_182711</td>
<td>n.a.</td>
<td>AT1G19020</td>
<td>AT1G19020</td>
</tr>
<tr>
<td>Phypa_171650</td>
<td>RCC1 family protein</td>
<td>AT3G15430</td>
<td></td>
</tr>
<tr>
<td>Phypa_140413</td>
<td>4CL3; 4-coumarate-CoA ligase</td>
<td>AT1G65060</td>
<td>AT1G65060</td>
</tr>
<tr>
<td>Phypa_131728</td>
<td>Hydrolase, a $\beta$-fold family protein</td>
<td>AT4G24160</td>
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<tr>
<td>Phypa_120495</td>
<td>n.a.</td>
<td>AT4G24380</td>
<td>AT4G24380</td>
</tr>
<tr>
<td>Phypa_117986</td>
<td>HSPRO2 (Arabidopsis ortholog of sugar beet [Beta vulgaris] HS1 PRO-1 2)</td>
<td>AT2G40000</td>
<td></td>
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<tr>
<td>Phypa_108925</td>
<td>Mitochondrial substrate carrier family protein</td>
<td>AT4G24570</td>
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<tr>
<td>Phypa_181590</td>
<td>Harpin-induced protein-related/HIN1-related/harpin-responsive protein-related</td>
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<td>Phypa_167257</td>
<td>Glutaredoxin family protein</td>
<td>AT1G64500</td>
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</table>

**Figure 4.** Number of putative orthologs in induced gene models under broad-band UV-B. The intersection includes eight gene models, for which induction under UV-B could be confirmed between both Arabidopsis accessions and *P. patens* and vice versa. Gene models with RBHs within the set of UV-B-induced genes in only one ecotype of Arabidopsis are underlined. The total numbers of all gene models induced under broad-band UV-B in *P. patens* and the Arabidopsis accessions are shown in gray.
of fold changes obtained from quantitative real-time PCR, the expression level over all microarray experiments. Fold changes in (Phypa_222528), which was selected due to its high and constant values. Ct values of target genes in real-time PCR were normalized to Plant Physiol. Vol. 153, 2010 1129

induced by osmotic stress in et al., 2007; Richardt et al., 2010). The AP2/EREBPs were shown for members of the family (Cho et al., 2007; Liu et al., 2005). In algae, response to metal ions, cold, osmotic stress, and phytohormones has been demonstrated for the family (Riechmann and Meyerowitz, 1998), and inducibility by ethylene has been shown for one family member (Phypa_29828/PpACP1) in P. patens as well (Cho et al., 2007). More recently, AP2/EREBPs were shown to act in ABA and abiotic stress responses as well (Song et al., 2005). In P. patens, response to metal ions, cold, osmotic, stress, and phytohormones has been demonstrated for members of the family (Cho et al., 2007; Liu et al., 2007; Richardt et al., 2010). The AP2/EREBPs induced by osmotic stress in P. patens belong to ancient orthologous groups that cluster within the DREB or ERF (for ethylene response factor) subfamily of flowering plant AP2/EREBPs (Richardt et al., 2010). The distinct clusters within the family were already present in the last common ancestor of land plants and algae and later diversified independently in the different lineages (Shigyo et al., 2006; Richardt et al., 2010). Taken together, these data suggest that members of the diverse AP2/EREBP family are involved in both responses to specific stresses and general stress response pathways. As has been argued before (Richardt et al., 2010), cross talk between different response pathways through AP2/EREBP TAPs, but also their specific response to UV-B stress, might have been a key factor in the water-to-land transition of plants.

UV-B Induces Flavonoid Biosynthesis in P. patens

Microarray analyses under broad-band UV-B showed induction of P. patens genes annotated as coding for enzymes of the general phenylpropanoid and flavonoid biosynthesis pathway. Genes encoding five PAL (for Phe ammonia lyase), one 4CL (for 4-coumarate: CoA ligase [Phypa_140413; Pp4CL4]; Silber et al., 2008), one CHI (for chalcone isomerase; Phypa_167842), and two CHS (for chalcone synthase) enzymes were up-regulated under 1 h of broad-band UV-B radiation, which was confirmed by real-time PCR (Fig. 5). For two out of the four enzyme families with UV-B-activated members involved in these metabolic pathways (PAL and CHS), an expansion (in comparison with Arabidopsis) has been detected. The P. patens PAL gene family consists of 14 members in comparison with the PAL gene family in Arabidopsis, with only four members (Supplemental Fig. S4). For five of the P. patens gene models, induction could be shown under UV-B in microarray experiments and was confirmed by real-time PCR (Supplemental Fig. S4).

The CHS family in P. patens is even more dramatically expanded, with 19 putative CHS-encoding genes (out of 23 homologs) as compared with a single functional CHS in Arabidopsis (out of four homologs; Fig. 6). In a recent study, a general unresponsiveness of the P. patens CHS-like genes to changes in the light regime could be demonstrated (Koduri et al., 2009), except for two gene models, PpCHS01 (Phypa_104998) and PpCHS2c (Phypa_110814), the former being induced under UV-B irradiation as well (Fig. 6). In comparison with that study, we expanded the phylogenetic reconstruction by adding five homologous gene models, Phypa_126819, Phypa_72618, Phypa_68833, Phypa_129458, and Phypa_155379. The activation of CHS transcripts was suggested by the microarray data (Fig. 6), but due to the high sequence identity of the majority of this gene family, the UV-B responsiveness could not be pinned down to individual genes. However, by phylogeny-aided design of real-time PCR primers, three loci could be confirmed to be induced by UV-B (Fig. 5); in the case of [Phypa_201011; Phypa_100508], specific primers for gene model Phypa_201011 did not show induction, whereas oligonucleotides binding both gene models indicated up-regulation, suggesting induction of Phypa_100508 upon UV-B.

Up-regulation of putative CHS genes and other genes encoding putative enzymes of the phenylpro-
panoid pathway in *P. patens* indicated that derived metabolites might accumulate in response to UV-B. In fact, induction of flavonols, probably functioning as UV-B “sunscreens,” were detected in *P. patens* on the metabolic level (Fig. 7). However, the flavonol pattern present in *P. patens* gametophores substantially differs from the pattern found in Arabidopsis seedlings (Fig. 7A; Stracke et al., 2010). The overall levels were very low, and only one quercetin derivative was enhanced in *P. patens* grown under white light with supplemental broad-band UV-B (Fig. 7A, orange arrow). In addition, a currently unknown metabolite seems to increase, but to a low extent, under broad-band UV-B (Fig. 7A, blue arrow). In this regard, it should be noted that unspecified cell wall-bound phenolics have been reported to be extractable from Antarctic mosses (Clarke and Robinson, 2008). This fraction, if present in *P. patens*, would not be represented in the methanolic extracts analyzed in our study. UV-B irradiation also resulted in an increase of anthocyanins in Arabidopsis, with the white light field with broad-band UV-B being more effective (Fig. 7B). Anthocyanins, which were not detectable in other bryophytes before (Stafford, 1991; Koes et al., 1994; Rausher, 2006), could also not be found in *P. patens* methanolic extracts separated by high-performance thin-layer chromatography (Fig. 7B). Although several gene models in *P. patens* show high similarity to the key enzymes of anthocyanin synthesis in Arabidopsis, dihydroflavonol 4-reductase and leucoanthocyanidin dioxygenase, the metabolic pathway for the synthesis of anthocyanidins apparently evolved only later in the angiosperm lineage (Koes et al., 1994). The existence of UV-B-dependent induction of flavonol biosynthesis in *P. patens* supports the hypothesis that enzymes involved in the early flavonoid secondary metabolism and corresponding signal transduction pathways evolved with the water-to-land transition due to higher UV-B radiation exposure, as extant algal species are not able to synthesize flavonoids (Koes et al., 1994; Rausher, 2006). It is intriguing that *P. patens*, despite its apparently lower content in UV-induced flavonols, exhibits a higher tolerance to UV-B than Arabidopsis.

**Genome Evolutionary Considerations**

Among the 19 putative CHS genes that cluster with the Arabidopsis CHS, five are putatively derived from

![Figure 6. Outgroup rooted Bayesian phylogenetic tree of CHS proteins. The tree is based on an amino acid multiple sequence alignment of sequences from *P. patens*, Arabidopsis, and other plants. Posterior probabilities are indicated by the width of the branches. Taxon color code shows *P. patens* sequences in green; induced gene models [Phypa_201011; Phypa_100508] are highlighted in cyan. The single functional CH5 of Arabidopsis that shows induction under broad-band UV-B is colored in red. Gene models found to be induced under broad-band UV-B in real-time PCR are marked with cyan asterisks. CHS paralogs Phypa_155379 and Phypa_101257 are arranged as tandemly arrayed genes (symbolized by a right bracket) in the *P. patens* genome. Genes probably derived from the whole genome duplication are denoted by orange dots.](image)

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the whole genome duplication (Fig. 6), which is in line with the finding that genes involved in metabolic actions were preferentially retained after this event (Rensing et al., 2007). While the majority of the CHS genes have a lower rate of synonymous than non-synonymous substitutions (i.e. they are probably evolving under negative selection), some CHS genes exhibit a higher rate of synonymous substitutions (i.e. they probably evolve under positive Darwinian selection). An example of the latter is gene model Phypa_109184, which is induced under UV-B and is a remnant of the genome duplication. The CHS genes Phypa_101257 and Phypa_155379 are located as a tandem array (Rensing et al., 2008) on genomic scaffold 463 (Supplemental Fig. 6A). Closer inspection of the locus (http://www.cosmoss.org/cgi/gbrowse/physcome/) shows that this tandem array is adjacent to another one consisting of two genes encoding chlorophyll a/b-binding proteins (CAB; Phypa_155384 and Phypa_173457). On the 3’ border of the latter tandem array, another CHS gene (Phypa_101260) is located (Supplemental Fig. 6A). While the CHS Phypa_155379 (part of the CHS tandem) is represented by a uniquely matching microarray probe set, the other genes, due to their high sequence identity, are united in the probe sets [Phypa_101257; Phypa_101260; Phypa_110814] (CHS) and [Phypa_155384; Phypa_158424; Phypa_173457] (CAB). It is intriguing that the two additional genes (contained in the probe sets but not in close proximity in the genome), the CHS Phypa_110814 and the CAB Phypa_158424, are located next to each other on genomic scaffold 1 (Supplemental Fig. 6B). In both genomic regions, there is ample evidence of retrotransposon insertion, suggesting that these loci might have been subject to segmental duplication and transposition events in addition to retention after the genome duplication (in the case of Phypa_155379). None of the four probe sets appeared in the list of differentially regulated genes that were rigorously filtered using a false discovery rate correction. However, individual t tests of the three sets demonstrate that an approximately 3-fold induction of the CHS genes \( (P = 0.033 \) for the three-gene probe set and \( P = 0.08 \) for Phypa_155379) and an approximately 2-fold repression of the CAB genes \( (P = 0.03) \) might be observed (Supplemental Fig. 6C). The repression of the CAB genes would also match the UV-B-induced reduction in chlorophyll content mentioned above. In summary, these data implicate a high plasticity of the way genes involved in tolerance to UV stress are encoded in the *P. patens* genome. In addition, genomic proximity and common transposition of genes might be an indicator of involvement in common metabolic and regulatory pathways.

**CONCLUSION**

In this study, we describe the physiological and early transcriptional responses of the model moss *P. patens* to UV-B radiation. Under UV-B radiation, *P. patens* generates more gametophores present in colonies of smaller size, resulting in a reduced surface area that might represent an avoidance response, possibly also due to shading of underlying tissue. Spore germination is highly susceptible to UV-B irradiation; protonemata

![Figure 7](image-url)
(the juvenile growth form) are more susceptible than gametophores (the adult growth form). In terms of resistance to harmful levels of UV-B radiation, we find that Arabidopsis seedlings are more affected than

\textit{P. patens} gametophores. Moreover, the latter are able to regenerate, after UV-B irradiation, into protonemata and ultimately into gametophores, even after apparently complete bleaching of the tissue.

In terms of the genome-wide molecular response, we find that approximately 400 genes are induced upon strong UV-B in both plant species. The relationship between early UV-B-induced \textit{P. patens} genes and subsequent phenotypic changes remains to be determined by follow-up genetic studies. Interestingly, however, Gene Ontology categories of the set of UV-B-regulated genes are highly similar in the moss and the flowering plant. In terms of gene conservation, 12 putative orthologs involved in the UV-B response could be detected, including a gene that harbors a RCC1 domain. Transcription factors of the AP2/EREBP family were found to be overrepresented in the \textit{P. patens} response to UV-B. Among the AP2/EREBPs are putative DREB and TINY homologs and six genes that were found to be involved in other abiotic stress responses as well.

The \textit{CHS} and \textit{PAL} gene families are expanded in \textit{P. patens} in comparison with Arabidopsis, and several members of each family are induced upon UV-B radiation. The \textit{CHS} family shows evidence of a highly dynamic gene family evolution, including positive Darwinian selection and paralog retention after small-scale as well as large-scale duplication and transposition events. While no anthocyanins were found to be produced in \textit{P. patens}, a quercetin derivative was found to accumulate in response to UV-B radiation, possibly representing a component of the UV sunscreen.

Our analysis provides an entry point for a further detailed understanding of UV-B response, function, and regulation in \textit{P. patens} and will be helpful to unravel the evolutionary aspects of UV-B tolerance and acclimation that laid the ground for plants to conquer land. In particular, it is evident that potential key genes regulating the response to UV-B (such as those encoding AP2/EREBP transcription factors) or mediating molecular protection (e.g. \textit{PAL} and \textit{CHS}) have been conserved since the water-to-land transition. Several \textit{P. patens} genes found to be UV-B responsive may now be further connected to UV-B signaling in this species. Taking advantage of effective gene targeting by homologous recombination in \textit{P. patens} will allow a functional genetic analysis in order to pinpoint the underlying players.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

\textit{Physcomitrella patens} strain Gransden 2004 (Rensing et al., 2008) was cultivated on solidified (1% [w/v] agar) mineral medium [250 mg L \(^{-1}\) \text{KH}_2\text{PO}_4, 250 mg L \(^{-1}\) \text{MgSO}_4\cdot7\text{H}_2\text{O}, 250 mg L \(^{-1}\) KCl, 1,000 mg L \(^{-1}\) \text{Ca(NO}_3\text{)}_2\cdot4\text{H}_2\text{O}, and 125 mg L \(^{-1}\) Fe\text{SO}_4\cdot7\text{H}_2\text{O}, pH 5.8, with KOH on 0-cm petri dishes enclosed by laboratory film at 22°C with a 16-h-light/8-h-dark regime under 70 \text{mW m}^{-2} \text{s}^{-1} white light (long-day conditions). The \textit{Arabidopsis} (\textit{Arabi-

dopsis thaliana}) wild type used was Col. \textit{Arabidopsis} seeds were surface sterilized by 70% ethanol, stratified at 4°C for at least 2 d, and aseptically grown on Murashige and Skoog medium (Duchefa Biochemie) with 1% Suc and 0.8% agar in a standard growth chamber (Ulm et al., 2004).

**UV-B Irradiation**

Photomorphogenic changes, growth from spores, flavonoid induction, as well as changes in gene expression were detected using a white light field (Osram L18W/30 tubes) supplemented with Philips TL20W/01RS narrow-band UV-B tubes (Oravecz et al., 2006), termed white light field with supplemental narrow-band UV-B. Photosynthetically active radiation (PAR) of 4.2 \text{mW m}^{-2} \text{s}^{-1} was measured with a LI-250 Light Meter (LI-COR Biosciences), and an average UV-B energy fluence rate was determined as 0.09 \text{mW cm}^{-2} using a VLL-3W Ultraviolet Light Meter with a CX-312 sensor (Vilber Lourmat).

A white light field (two Philips TLD 36W/18 Blue and two OSRAM L 36W/73 Blacklight Blue tubes) supplemented with two Vilber-Lourmat T40M UV-B broadband-tubes, as described previously (Kucera et al., 2003; Stracke et al., 2010), was employed for the examination of photomorphogenic responses, spore sensitivity assays, and flavonoid induction, described as white light field with supplemental broad-band UV-B, with an average PAR of 28.5 \text{mW m}^{-2} \text{s}^{-1} and a UV-B fluence rate of 0.26 \text{mW cm}^{-2}. For damage responses resulting in necrotic lesions with subsequent regeneration and UV-B molecular response, a broad-band UV-B field (PAR of 19.1 \text{mW m}^{-2} \text{s}^{-1} and UV-B fluence rate of 2.0 \text{mW cm}^{-2}) consisting of six Philips TL 40W/12 UV fluorescent tubes (Ulm et al., 2004) was applied. Transmission cutoff filters of the WG series (Schott) with half-maximum transmission at the indicated wavelength were used. If not indicated differently, WG345 was used as a UV-B control and WG303 was used as +UV-B. For long-term experiments (longer than 1 week), lids were left on the petri dishes to avoid desiccation and to keep the cultures axenic. These experiments included gametophyte growth phenotype, chlorophyll content, gametophyte colony circumference measurements, and germination of spores under supplemental UV-B. The resulting reduction in irradiation levels under the respective light fields and transmission filters are described in Supplemental Table S1.

For analyses of gene induction under the respective fields, plants were adapted to low-light conditions as present in the narrow-band UV field without supplemental UV irradiation for 24 h prior to irradiation. The broadband and narrow-band fields were used for expression analyses of early UV-B-responsive genes in \textit{P. patens}. Material was harvested in the middle of the light period.

**Phylogenetic and Other Computational Analyses**

Protein sequences derived from BLAST searches with at least 30% sequence identity over an alignment length of 80 amino acids were selected using \textit{P. patens} and Arabidopsis queries. In the case of the \textit{MYB} family tree (Supplemental Fig. S3), all sequences containing two or more PFAM “Lz” Myb, DNA-binding domains and no ARID, response_reg, G2_like, and triblixis domains, respectively, were selected using HMMER 2.3.2 (http://www.hmmer.janelia.org/) from the genomes of \textit{P. patens}, Arabidopsis, \textit{Medicago truncatula}, and \textit{Populus trichocarpa}. Multiple sequence alignments were performed with MAFFT (Katoh et al., 2005) and subsequent manual curation using JalView (Clamp et al., 2004). Bayesian inference (Huelsenbeck et al., 2001) was conducted for reconstruction of the phylogenetic trees using WAG as the evolutionary model (Whelan and Goldman, 2001) and y-distributed rate categories. Graphics of phylogenetic trees were generated using FigTree version 1.2.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Putative orthologs were considered as reciprocal best BLAST hits (RBBHs) between \textit{P. patens} and Arabidopsis and determined by BLAST search with sequence identity of at least 30% over an alignment length of 100 amino acids and an E-value of e \(=\) 10\(^{-5}\). Annotation of genes belonging to a certain gene family was done by similarity search using Arabidopsis queries for BLAST search with 30% sequence identity over an alignment length of 80 amino acids. Information on tandemly arrayed genes is from Rensing et al. (2008), and genes derived from the whole genome duplication and substitution rate data were determined as described by Rensing et al. (2007).
RNA Isolation and Real-Time PCR

Isolation of total RNA was performed with the RNeasy Plant Mini Kit (Qiagen) from *P. patens* gametophores with on-column DNA digestion using DNAseI according to the manufacturers’ protocol. For real-time PCR, RNA was reverse transcribed using SuperScript III (Invitrogen) using random hexamer primers (Fermentas). Eprimer3 and QuantPrime (Aridvissão et al., 2008) were used for design of specific oligonucleotides. Primer sequences used for amplification of the respective gene models were as in Supplemental Table S4. For each 23-μL reaction, 20 ng of reverse-transcribed RNA was used and the reaction was carried out using SensiMix dT and SYBRGreen (Quantace) on a 7300 Real-Time PCR System (Applied Biosystems). Concentration of cDNA was normalized to 68S ribosomal protein L19 (Physcomitrella 222288), showing expression level and UV-B-independent expression in the microarray analyses. Triplicate measurements were performed for each of three biological replicates. Threshold cycle (Ct) values possessing the 5% highest variance to the median of technical replicates were removed prior to further data processing.

Microarray Experiments

All *P. patens* version 1.2 (www.cosmoss.org) protein-coding gene models were used to design antisense probes (Combimatrix) using the following design parameters: probe length of 35 to 45 bp, melting temperature range of 70.0°C to 75.0°C, as close to the 3′ end as possible, and three to four probes per transcript. Specificity testing was performed using the total set of transcripts as well as the genomic scaffolds. Three spike-in, 19 quality check, and 41 negative control probe sets were included as well. The design resulted in 27,627 probe sets unique to the respective gene and 201 probe sets matching more than one gene (in the case of closely related paralogs). The latter are denoted by more than one gene model identifier enclosed in square brackets and separated by semicolons.

The experimental design for microarray samples is shown in Supplemental Figure S5. *P. patens* gametophores were grown under standardized conditions, and irradiation treatment was performed for 1 h in broad-band and light field with supplemental narrow-band UV-B under WGE03 with WGE345 as a control. UV-B microarray analyses were conducted using 1.5 μg of total RNA for linear amplification; subsequently, 5 μg of amplified RNA were labeled with Cyanin-5 (ampULSe; Kreatech). Microarray hybridization (Combimatrix) was performed using 5 μg of fragmented, labeled amplified RNA following the manufacturer’s protocols. The arrays were imaged using a laser scanner (Genepix 4200A; Molecular Devices) and stripped up to four times using the Combimatrix stripping kit as described previously (Richardt et al., 2010).

Microarray Data Analyses

Microarray expression values were analyzed with the Expressionist Analyst Pro version 5.2 software (Genedata). Probe sets were median condensed, and linear array-to-array normalization was applied using median normalization to a reference value of 10,000. Differentially regulated genes were detected using the Bayesian regularized unpaired CyberT test (Baldi and Long, 2001) with Benjamini-Hochberg false discovery rate correction (*q* ≤ 0.05) and a minimum fold change of 2, as described previously (Richardt et al., 2010). K-means clustering with *k* = 2 identified up-regulated and down-regulated genes. Fisher’s exact test was performed to identify categories enriched or suppressed in the regulated gene set with *P* ≤ 0.01. For comparison of differentially regulated genes between *P. patens* and Arabidopsis, Affymetrix ATH1 expression data with accession numbers E-MEXP-1957 (Favory et al., 2009), E-MEXP-550 (Ulm et al., 2004), and E-MEXP-557 (Oraevicz et al., 2006) were downloaded from Array Express (www.ebi.ac.uk/microarray- as/ae/). Condensing of probes was performed using GeneChip-Robust Multiarray Averaging (Wu et al., 2004) using Expressionist Refiner Array (Genedata). The data were processed further as described for *P. patens*.

Extraction and Analysis of Flavonoids and Chlorophyll

Anthocyanins and flavonols were extracted independently from three biological replicates with 80% methanol and separated as reported previously (Stracke et al., 2009). Chlorophyll content was determined as described by Wiedemann et al. (2007) and referred to gametophore fresh weight. Quantification of total chlorophyll was done using the following equation: Q (chlorophyll) [mg]/fresh weight [g] = (0.00802*A664 + 0.0202*A647)*V aceton solution [mL]/fresh weight [g].

Data Analyses

Measurements of gametophyte colony circumference were carried out using LSM Image Browser 5 (Carl Zeiss). Significant statistical difference between two data sets was determined using Student’s *t* test (*P* ≤ 0.05). Nonnormally distributed populations were considered as significantly statistically different by performing the nonparametric Mann-Whitney rank sum test with *P* ≤ 0.05.

Combinatrix microarray expression data reported in this paper have been deposited in the EMBL-EBI ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae/) under accession number E-MEXP-2508.

Supplemental Data

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

Supplemental Figure S1. Fresh weight and chlorophyll content.
Supplemental Figure S2. TAP families.
Supplemental Figure S3. MYB phylogeny.
Supplemental Figure S4. PAL phylogeny.
Supplemental Figure S5. Experimental design of microarray analysis.
Supplemental Figure S6. CHS and CAB genes.
Supplemental Table S1. Characterization of irradiation fields used.
Supplemental Table S2. Induced gene models under broad-band UV-B.
Supplemental Table S3. Repressed gene models under broad-band UV-B.
Supplemental Table S4. Quantitative real-time PCR gene models.

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LITERATURE CITED


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