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UV-B signalling in rice: Response identification, gene expression profiling and mutant isolation

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Abstract

Responses of rice seedlings to UV-B radiation (UV-B) were investigated, aiming to establish rice as a model plant for UV-B signalling studies. The growth of *japonica* rice coleoptiles, grown under red light, was inhibited by brief irradiation with UV-B, but not with blue light. The effective UV-B fluences (10^{-1}-10^{3} μmol m^{-2}) were much lower than those reported in Arabidopsis. The response was much less in *indica* rice cultivars and its extent varied among *Oryza* species. We next identified UV-B-specific anthocyanin accumulation in the first leaf of purple rice and used this visible phenotype to isolate mutants. Some isolated mutants were further characterized, and one was found to have a defect in the growth response. Using microarrays, we identified a number of genes that are regulated by low-fluence-rate UV-B in *japonica* coleoptiles. Some up-regulated genes were analysed by real-time PCR for UV-B specificity and the difference between *japonica* and *indica*. More than 70% of UV-B-regulated rice genes had no homologs in UV-B-regulated Arabidopsis genes. Many UV-B-regulated rice genes are related to plant hormones and especially to jasmonate biosynthetic and responsive genes in apparent agreement with the growth response. Possible involvement of two rice homologs of UVR8, a UV-B photoreceptor, is discussed.

KEYWORDS
anthocyanin, coleoptile, first leaf, growth inhibition, *Oryza sativa* L., photomorphogenesis, plant hormone, photoreceptor, purple rice, rice phylogeny

1 | INTRODUCTION

Since the 1970s, it has gradually become clear that ultraviolet-B radiation (UV-B; 280–315 nm), known to be harmful to living organisms, induces certain physiological responses at low fluence rates (typically lower than 1 μmol m^{-2} s^{-1}). Their UV-B specificity has been demonstrated by action spectroscopy, interaction with other lights, and by the use of available photoreceptor mutants. The most extensively investigated were suppression of extension growth such as of hypocotyls (Kim, Tennessen, & Last, 1998; Krizek, 1975; Lercari, Sodi, & di Paola, 1990; Tevini, Thoma, & Iwanzik, 1983) and accumulation of anthocyanins in seedling shoots (Beggs & Wellmann, 1985; Brandt, Giannini, & Lercari, 1995; Drumm-Herrel & Mohr, 1981; Hashimoto & Tajima, 1980; Yatsuhashi, Hashimoto, & Shimizu, 1982).

Since the early 2000s, our understanding of UV-B-specific responses has been greatly advanced by the use of Arabidopsis as a molecular genetic model plant. Most significantly, UV RESISTANCE LOCUS 8 (UVR8) was identified, together with other signalling proteins, as the UV-B photoreceptor (Rizzini et al., 2011; Wu et al., 2012; Zeng et al., 2015). Subsequently, it has been shown by a comprehensive phylogenetic analysis that UVR8 is conserved, with a predicted function as a photoreceptor, from green algae to higher plants (Fernandez, Tossi, Lamattina, & Cassia, 2016). With the use of *uvr8* mutants, the involvement of UVR8 has been demonstrated in the

following UV-B-induced responses: hypocotyl growth inhibition (Favory et al., 2009; Gruber et al., 2010), anthocyanin accumulation (Favory et al., 2009; Gruber et al., 2010), suppression of leaf expansion (Wargent, Gegas, Jenkins, Doonan, & Paul, 2009), hypocotyl phototropism (Vanhaelewyn et al., 2016) and accumulation of non-anthocyanin phenolic compounds (Demkura & Ballare, 2012; Kliebenstein, Lim, Landry, & Last, 2002). It has been further shown that expression of a number of genes is regulated by UV-B in a UVR8-dependent manner (Brown et al., 2005; Favory et al., 2009; Oravecz et al., 2006; Tavridou, Pireyre, & Ulm, 2020; Ulm et al., 2004). Although there is no doubt that UVR8 functions as a UV-B photoreceptor, accumulating evidence also indicates that UVR8 may not be the sole UV-B photoreceptor in plants (Biever, Brinkman, & Gardner, 2014; Gardner, Lin, Tobin, Loehrer, & Brinkman, 2009; Kondou et al., 2019; Mazza & Ballare, 2015; O'Hara et al., 2019; Takeda et al., 2014).

Earlier studies of UV-B-induced growth inhibition and anthocyanin accumulation were conducted with seedlings of *Gramineae* species such as sorghum and maize (Beggs & Wellmann, 1985; Drumm-Herrel & Mohr, 1981; Hashimoto & Tajima, 1980; Mohr & Drumm-Herrel, 1983; Tevini, Iwanzik, & Thoma, 1981; Yatsuhashi et al., 1982). However, little progress has been made on signalling mechanisms, except that the maize homolog of UVR8 functions as a UV-B photoreceptor in Arabidopsis (Fernandez, Lamattina, & Cassia, 2020). Rice has proven to be a model plant in many physiological responses and its use in the study of UV-B signalling is anticipated. The growth of rice coleoptiles is strongly inhibited by phytochrome (Biswas, Neumann, Haga, Yatoh, & Iino, 2003; Pjon & Furuya, 1967). It would be of interest to know whether it is also inhibited by UV-B in a UV-B-specific manner. Common rice cultivars do not accumulate anthocyanins in response to light (including UV-B), precluding their use for the study of UV-B-induced anthocyanin accumulation. However, the strains know as purple rice have been shown to accumulate anthocyanins in response to UV-B (Hada, Hidema, Maekawa, & Kumagai, 2003; Maekawa, Sato, Kumagai, & Noda, 2001; Reddy, Goud, Sharma, & Reddy, 1994) and may be useful in the study of UV-B signalling.

In the present study, we investigated UV-B-induced responses of rice seedlings, aiming to establish rice as a model plant for UV-B signalling studies. Seedlings grown under continuous red light (R) were used to exclude possible induction of phytochrome responses by UV-B and the effect of blue light (B) was also examined to evaluate UV-B specificity. Broad-band UV-B lamps, which also emit UV-A, have often been used in the studies of UV-B responses. In order to minimize simultaneous induction of cryptochrome signalling, which interacts with UVR8 signalling (Rai et al., 2020; Tissot & Ulm, 2020), narrow-band UV-B LEDs were used as the UV-B source, except in limited cases. We found that the growth of *japonica* rice coleoptiles is inhibited by low-fluence UV-B and characterized this response in detail. We further explored the use of purple rice, whose visible purple-coloured phenotype, if UV-B-inducible, could be used for mutant screening. Finally, we surveyed and analysed UV-B-regulated genes in rice.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

The main plant materials used were three cultivars of rice (*Oryza sativa* L.): Nipponbare (*japonica*), Habataki (*indica*) and purple rice (*japonica*; provided by Hokuriku National Agricultural Experiment Station, Joetsu, Japan). Other *japonica* cultivars used were Kamenoo, Nihonmasari, Norin-1 and Sasanishiki; other *indica* cultivars used were 93-11, Kasalath, Marich-bati and Surjamkhi. We also used the following plant materials: *O. barthii* A. Chev. (W0562; National Institute of Genetics, Mishima, Japan), *O. glaberrima* Steud. (IRGC104038; International Rice Research Institute, Los Banos, Philippines), *O. glumipatula* Steud. (IRGC105668), *O. longistaminata* A. Chev. & Roehr. (IRGC100930), *O. meridionalis* Ng (W1299), *O. nivara* S.D. Sharma & Shastry (JP242699; National Institute of Agrobiological Science, Tsukuba, Japan), *O. officinalis* Wall. ex Watt (JP226018), *O. rufipogon* Griff. (Asian origin, W106), *O. rufipogon* Griff. (Australian origin, IRGC105303), oat (*Avena sativa* L. cv. Almighty; Snow Brand Seed, Sapporo, Japan), sorghum (*Sorghum bicolor* (L.) Moench ssp. *bicolor*; JP212651), wheat (*Triticum aestivum* L. cv. Shiroganekomugi; provided by Osaka Prefectural Agricultural and Forestry Research Center, Habikino, Japan) and teosinte (*Zea mays ssp. mexicana* (Schrad.) H.H. Iltis., Snow Brand Seed).

Seedlings of *Oryza* species were prepared as described in Iino, Tarui, and Uematsu (1996). In brief, dehusked and surface-sterilized caryopses were surface-sown on 0.7% agar in 4.5-mL acrylic cuvettes (Elkay; Kendall Healthcare, Mansfield, MA, USA). Sown caryopses (one caryopsis per cuvette) were placed inside boxes (340 × 160 mm^2 wide, 105 mm high) made of transparent red acrylic (3 mm thick; No. 102, Mitsubishi Rayon, Tokyo, Japan) and cultured at 25 ± 1°C under R (2.5–-3.0 μmol m^{-2} s^{-1}). The humidity inside the boxes was maintained high with wet paper towel placed on their bottom and sides. The R source was as described in Iino (1995) and its fluence rate was measured with Li-189 photometer (Li-190 sensor; LI-COR, Lincoln, NE, USA).

Sorghum seedlings were prepared as described above for *Oryza*. Seedlings of oats and wheat were prepared as described in Tarui and Iino (1997) and those of teosinte, as described in Liu and Iino (1996) for maize. As for *Oryza* seedlings, all the seedlings were grown under R.

2.2 | Light treatment of coleoptiles and growth measurement

Intact seedlings of *Oryza* and other plants were exposed to UV-B or B when the length of their coleoptiles reached half (within ±10%) of the final mean length achieved under the R growth condition. Seedlings were kept under R during and after light treatment. The high humidity condition was also maintained during light treatment and photographing by placing experimental apparatus inside high-humidity red acrylic boxes. This care was necessary to achieve relatively straight growth of mock-treated *Oryza* coleoptiles.

The entire seedling was irradiated bilaterally for 1 or 10 min with UV-B or B. Bilateral irradiation was conducted in such a way that the coleoptile received the same fluence rate from the two directions; the indicated fluence rates are the sum of bilateral irradiation. Depending on the distance from the light source, two or four seedlings were irradiated each time. In all cases, the direction of light was parallel to the plane of the coleoptile's two vascular bundles. For growth analysis, the coleoptiles were photographed using a digital camera (D-200; Nikon, Tokyo, Japan) equipped with an AF Micro-Nikkor 60 mm f/2.8D lens; the coleoptiles were oriented for photographing so that the camera viewing axis was perpendicular to their long axis and parallel to the plane of their two vascular bundles.

The UV-B source was eight UV-B LEDs (UVTOP290TO39FW; Sensor Electronic Technology, Columbia, SC, USA) placed in a 40 mm-wide and 20 mm-high area and covered with a UV-transmitting filter (UTVAF-34; HOYA, Tokyo, Japan). The B source was 18 blue LEDs (L450-01 U; Epitex, Kyoto) placed in a 30 mm-wide and 15 mm-high area; the basal edge of each LED was slightly trimmed to accommodate all the LEDs within the area. Two sets of each light source were used to allow bilateral irradiation. Figure 1 shows the emission spectra of UV-B and B measured with spectroradiometers USR-40 (Ushio Electric, Tokyo, Japan) and Li-1800 (LI-COR), respectively. The fluence rate of UV-B was controlled by the number of lighted LEDs, the distance from the light source, and the number of UV-B-attenuating polypropylene transparent sheets (0.2-mm thick). The fluence rate of B was controlled by neutral-density glass filters (Vacuum Optics, Tokyo). In daily experiments, the fluence rates of UV-B and B were measured with the photometers PMA2200 (PMA 2101 erythema detector; Solar Light, Glenside, PA, USA) and Li-189, respectively, and the measured values were calibrated to the values determined by the spectroradiometers.

The length of coleoptiles was measured with the image analysis program ImageJ (National Institutes of Health, Bethesda, MD, USA).

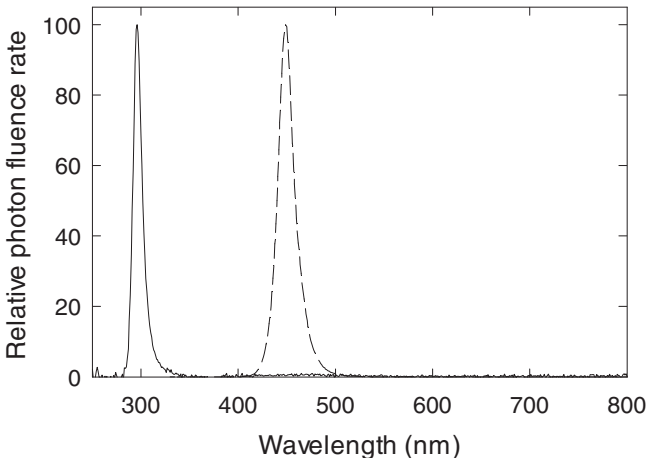

FIGURE 1 Emission spectra of the light sources. Photon fluence rates are relative to the peak value. Solid line: UV-B; dashed line: blue light (B)

The statistical analysis was conducted with Excel (Microsoft, Redmond, WA, USA).

2.3 | Light treatment of purple rice seedlings and anthocyanin measurement

Seedlings of purple rice were raised under continuous R as described above and used for experiments 4 days after sowing. Seedlings were irradiated bilaterally for indicated periods with UV-B or B when the top 5–6 mm portion of the first leaf was above the coleoptile tip. Care was taken so that the entire first-leaf portion above the coleoptile was treated with uniform UV-B or B. Other irradiation conditions were as described above for coleoptile growth analysis. The top 5-mm portion of the first leaf was excised from each seedling after a period of incubation and frozen immediately in liquid N_2. Each first leaf sample, weighing 5–9 mg fresh weight (FW), was collected from eight seedlings and stored at −80°C.

The stored first leaf sample was placed in liquid N_2 and ground for 1 min with a MM300 Mixer Mill (Retsch, Haan, Germany) at 25.0 Hz. Acidified methanol was used to extract anthocyanins (Rabino & Mancinelli, 1986) and chloroform was used to separate chlorophylls (Feinbaum & Ausubel, 1988) as follows. Methanol (750 μL) containing 1% HCl (v/v), ice-cold, was added to the ground tissue and the mixture was incubated for 4 hr at 4°C in the dark, being shaken with a rotary shaker at 80 rpm (Taiyo Rotary Shaker R-II; Taitec, Saitama, Japan). Deionized water (500 μL) and chloroform (750 μL), both ice-cold, were next added to the mixture and, after a vigorous shaking with a Vortex mixer, the mixture was centrifuged for 2 min at 15,000 rpm and 4°C (RT15A2 rotor; Hitachi, Tokyo, Japan). The resulting aqueous methanol phase was taken and the absorbance at 527 nm, the peak of anthocyanin absorption, was measured with a spectrophotometer (U-2000; Hitachi). The total anthocyanin level was assessed as absorbance per g FW of the initial ground tissue. The contaminating level of chlorophylls was negligible judging from the absorption in the red spectral region. This was also supported by the measurement made similarly with R-grown Nipponbare seedlings; the absorbance spectrum of the aqueous methanol phase indicated negligible levels of both anthocyanins and chlorophylls.

2.4 | Isolation of UV-B response mutants from purple rice

About 2,000 caryopses of purple rice were treated with γ rays as described in Biswas et al. (2003). These caryopses were grown in the field and selfed to obtain γ ray-mutagenized M2 caryopses (segregating generation). The M2 caryopses harvested from individual plants were stored separately.

Mutant screening was conducted as follows: M2 caryopses (20 per M2 line) were surface-sterilized and sown in wet vermiculite in grey polypropylene trays (460 x 305 mm wide, 153 mm high). The trays were subsequently covered with UV-B-passing clear acrylic (Delaglas K002, 3 mm thick; Asahi Kasei, Tokyo, Japan) and placed

under continuous R (2.5–3.0 μmol m^{-2} s^{-1}) supplemented with UV-B (0.0015–0.0025 μmol m^{-2} s^{-1}, 280–315 nm). The overhead UV-B source was a fluorescent UV-B lamp (Philips TL40W/12-RS UVB Broadband; Figure S1a) covered with UV-B-attenuating black mesh cloth layers. Four days after sowing, seedlings were observed under white laboratory lighting and those showing non-purple or less purple leaves were isolated.

The isolated M2 seedlings were grown and selfed in the greenhouse to obtain M3 caryopses. Generally, more than three seedlings were selected from each M2 line and M3 caryopses from each plant were stored separately. After confirmation of the mutant phenotype, M3 seedlings were grown to obtain M4 caryopses.

2.5 | Microarray analysis

Nipponbare seedlings were irradiated bilaterally with UV-B (0.065 μmol m^{-2} s^{-1}) for 10 min as described for growth analysis and their coleoptiles were harvested 20 and 60 min after the onset of irradiation (20- and 60-min samples, respectively). Four seedlings were treated simultaneously and their coleoptiles were harvested (without enclosed leaves) within ±1 min of the scheduled time, and this procedure was repeated to obtain eight coleoptiles per sample. Harvested coleoptiles were immediately frozen in liquid N_2 and stored at −80°C. Non-treated controls (eight coleoptiles per sample) were prepared just before and after irradiation treatment (controls 1 and 2, respectively). Three independent sample sets were obtained on different occasions.

Each coleoptile sample (30–40 mg FW) was ground to a fine powder with a mortar and pestle in liquid N_2. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). In this extraction, RLT buffer (1 mL) containing 10 μL β-mercaptoethanol was used for cell lysis, RNase-free DNase (Qiagen) was used to remove contaminating DNA, and the final elution was done with 50 μL RNase-free water. The absorbance at 260 and 280 nm was measured with an Eppendorf Biophotometer (Cambridge Scientific, Watertown, MA, USA). The total RNA concentration was determined from the 260-nm absorbance. The ratio of 260- and 280-nm absorbance was used to assess the purity of RNA. The RNA samples were stored at −80°C.

Two-colour microarray analysis was performed with the Rice Gene Expression 4x44K Microarray (Agilent, Santa Clara, CA, USA) following the manufacturer's protocol. In brief, control and UV-B-treated samples (400 ng RNA each) were labelled with Cy3 and Cy5, respectively, purified with an RNeasy Mini Kit (Qiagen), and measured for cRNA concentration with a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Each UV-B-treated sample (Cy5-labelled, 825 ng cRNA) was mixed with each of the two control samples (Cy3-labelled, 825 ng cRNA) to prepare two mixtures per UV-B-treated sample. The mixtures were applied to microarrays and hybridization was performed at 65°C for 17 hr. The microarrays were washed, dried and scanned for signal intensities with a G2505B scanner (Agilent). One 4x44K microarray slide was used for each sample set.

The scanned images were processed with Agilent Feature Extraction software (version 11.5.1.1.) to extract the signal intensities of each probe. Background subtraction and LOWESS normalization were performed with the default setting. Subsequent analysis was conducted with probes after exclusion of those whose signal intensities in all samples (including the controls) were lower than 3% of the mean signal intensity of all probes. The 3% level (mean signal intensity = 83) corresponded approximately to a signal intensity of 100, which is a commonly used cut-off level (e.g., Chen, Yamaji, Motoyama, Nagamura, & Ma, 2012; Ishiguro, Ogasawara, Fujino, Sato, & Kishima, 2014; Kellermeier et al., 2014). We also conducted subsequent analysis with probes after exclusion at 1 and 10% cut-off levels. Since the results were essentially similar, we will show only those obtained with the 3% cut-off level.

The relative expression levels in UV-B-treated samples (Cy5/Cy3) were calculated for each probe. The values obtained from the two mixtures of each sample (see above) were averaged and the means and standard deviation (SD) were calculated for three samples. When there were more than two probes for the same gene, relative expression data obtained from those probes were averaged. Statistical significance of the differential expression of each gene (treated vs. control) was examined by applying an unpaired *t*-test (with assumption of equal variances) on $\log_2$ values of signal intensities obtained from treated and control samples. The resulting *p*-values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) correction. A gene was considered up- or down-regulated when it showed more than a two-fold expression difference from the control with FDR < 0.05 in either or both of 20- and 60-min samples. Statistical analysis (unpaired t-test and FDR correction) was also conducted between 20- and 60-min samples.

2.6 | Real-time PCR analysis

Nipponbare seedlings were treated with UV-B and B and Habataki coleoptiles were treated with UV-B as described for growth analysis; 20- and 60-min samples and two control samples of coleoptiles (four independent sets) were prepared as described for microarray analysis. In an extended analysis, Nipponbare coleoptiles were treated with 10-min UV-B and B at the same fluence rate (0.1 or 1.0 μmol m^{-2} s^{-1}).

The total RNA was extracted from each coleoptile sample as described above and cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Kusatsu, Japan). A 1-μg aliquot of total RNA was used for cDNA synthesis following the manufacturer's protocol and the synthesized cDNA was stored at −20°C.

Real-time PCR was carried out using the TB Green Premix Ex Taq II Tli RNaseH Plus (Takara Bio) on a Real-time PCR System (Model 7300; Applied Biosystems, Foster City, CA, USA). The reaction mixture (50 μL) contained cDNA (1 ng) and other components supplied by the manufacturer. The PCR conditions were 95°C for 30 s, 35–45 cycles of 95°C (5 s) and 60°C (31 s), followed by a default dissociation stage program. The *18S rRNA* (AK059783) and *OsUBQ10* (AK101547) were used as internal controls. The transcript level of each sample was calculated as a value relative to the mean of the two control samples.

For each investigated gene, primer pair candidates were designed with the Primer-BLAST (Ye et al., 2012) against the Nipponbare full-length cDNA sequence (Ohyanagi et al., 2006) and their specificity was analysed with the MFEprimer-2.0 program (Qu et al., 2012). The candidates were next examined against the full-length cDNA sequences of the *indica* cultivar 93-11 (Zhao et al., 2004) to select those that match with the corresponding gene of 93-11. Finally, a primer pair was selected based on dissociation curve profiles and amplification efficiency values. For each selected primer set, it was also confirmed that a single major band of the expected size is produced after PCR amplification of Nipponbare and Habataki cDNA samples. The size of PCR products ranged from 65 to 140 bp (for primer sequences, see Table S1).

3 | RESULTS

3.1 | UV-B-induced inhibition of coleoptile growth

Effects of brief UV-B irradiation on coleoptile growth were investigated in the *japonica* rice cultivar Nipponbare. Seedlings were grown under continuous R to saturate the phytochrome-mediated inhibition of coleoptile growth (Biswas et al., 2003; Pjon & Furuya, 1967) and to avoid induction of this response by UV-B.

As shown in Figure 2a, 10-min irradiation with bilateral UV-B, which provided a fluence of $10^{2.2}$ μmol m^{-2}, resulted in a clear inhibition of coleoptile growth. The inhibition developed within 1 hr and the slowest growth (about half the control rate) was achieved in the following 2-hr period. Afterwards, the growth tended to recover gradually. The relationship between UV-B fluences and growth inhibition was next investigated by exposing coleoptiles to various fluences provided by 1- or 10-min irradiation and by measuring their growth increments after 8 hr (Figure 2b). Growth inhibition developed in a near log-linear fashion from $10^{-1.2}$ μmol m^{-2} to the highest fluence tested ($10^{2.8}$ μmol m^{-2}). The results obtained with 1- and 10-min irradiation could be well described by a linear regression line and, therefore, the law of reciprocity appeared to hold within this range of irradiation times.

It is possible that the observed UV-B-induced growth inhibition was mediated by B photoreceptors (Baskin & Iino, 1987; Vanhaelewyn, Schumacher, et al., 2016). As shown in Figure 2a,b, however, B fluences as high as $10^{5.1}$ μmol m^{-2} had little effect on coleoptile growth. It was rather surprising that coleoptile growth was unresponsive to such high-fluence B. The results indicated that the UV-B-induced growth inhibition is mediated by UV-B-specific signalling.

We extended the growth analysis to other rice cultivars including *indica* ones. In response to 10-min exposure to UV-B, coleoptiles of all the investigated *japonica* cultivars showed a clear growth inhibition as found in Nipponbare (Figure 3, left). On the other hand, coleoptiles of all the investigated *indica* cultivars showed a much smaller response (Figure 3, right). In fact, a large variation in UV-B responsiveness was observed in other *Oryza* species (mostly wild ones): *O. glumipatula* showed the strongest response, whereas *O. glaberrima* and *O. barthii* showed no response (Figure 4).

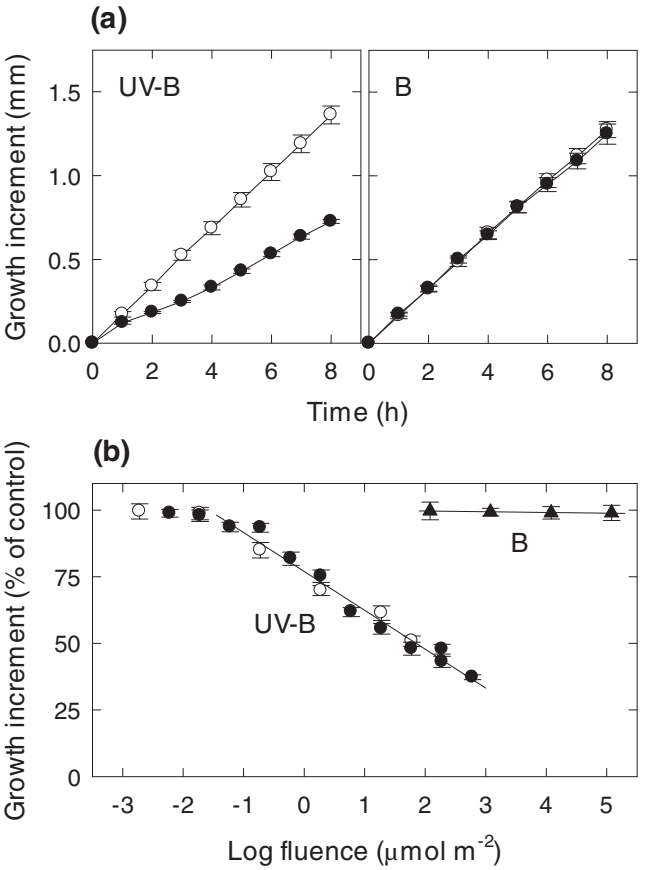

FIGURE 2 Effects of UV-B and blue light (B) on the growth of coleoptiles in red light-grown, 3-day-old seedlings of Nipponbare. (a) Time-courses. Coleoptiles were exposed to bilateral UV-B (0.28 μmol m^{-2} s^{-1}) or B (200 μmol m^{-2} s^{-1}) for 10 min. Closed circles indicate exposed coleoptiles and open circles, mock-treated controls. The abscissa indicates the time after the onset of exposure. The values are means ± SE of 10–12 plants. (b) Fluence-response curves. Coleoptiles were exposed to bilateral UV-B or B at the indicated fluences provided in 1 min (○) or 10 min (●, ▲). The values are means ± SE of 16 plants. Lines were fitted by linear regression (UV-B: $y = -14.6x + 77.0$, $r^2 = 0.974$; B: $y = -0.251x + 100.1$, $r^2 = 0.876$)

We further examined other *Gramineae* species (one species per genera). Sorghum and teosinte coleoptiles showed a clear growth inhibition with a time course similar to that found in rice, although oat and wheat coleoptiles showed little response (Figure 5a–d). In addition, high-fluence B induced little response in sorghum and teosinte (Figure 5e,f). These results indicated that the ability of *Oryza* coleoptiles to show UV-B-induced growth inhibition was inherited from the ancestral genus and that the observed variation in UV-B responsiveness developed within *Oryza*.

3.2 | UV-B-induced accumulation of anthocyanins in purple rice seedlings

Our preliminary investigation indicated that R-grown seedlings of purple rice accumulate clearly visible anthocyanins when background R is

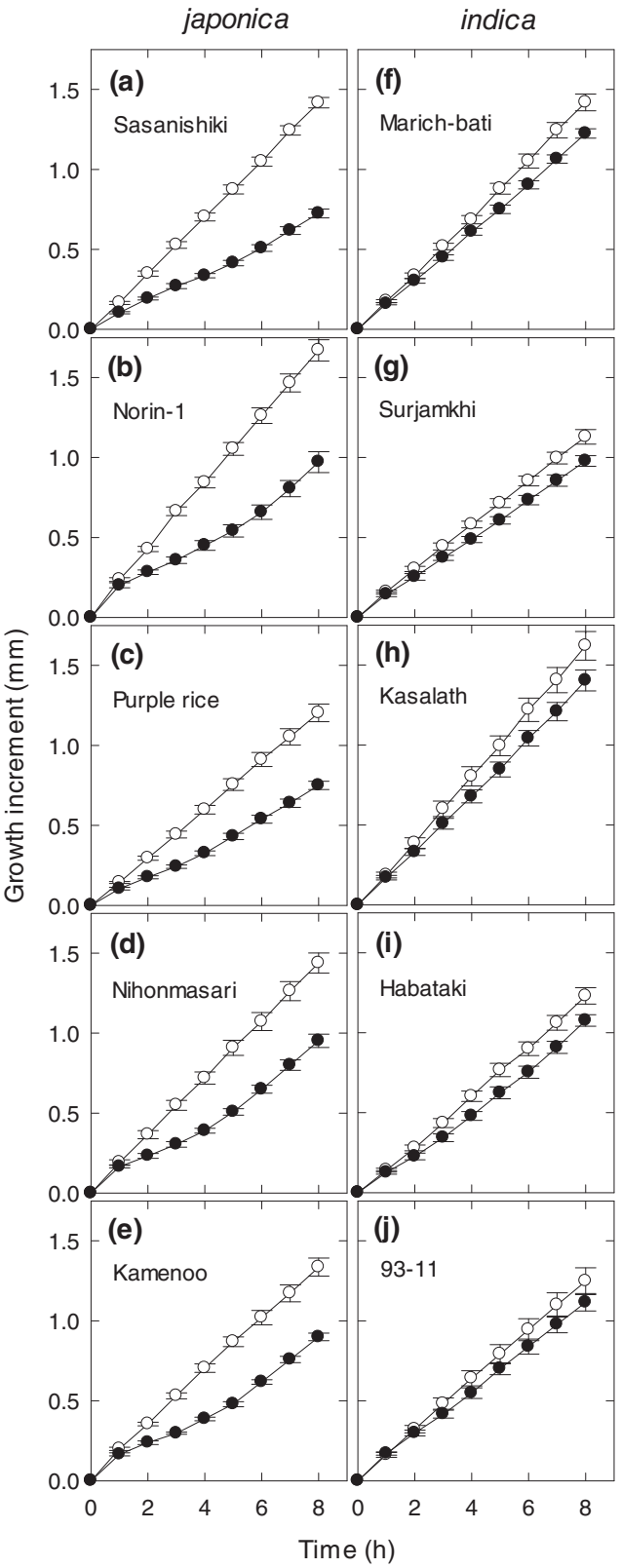

FIGURE 3 Effects of UV-B on the growth of coleoptiles in *O. sativa* ssp. *japonica* and *O. sativa* ssp. *indica* cultivars. The figures are arranged in the order of response magnitude. The values are means ± SE of 10–12 plants. Other details are as for Figure 2a

supplemented with low irradiance from a broad-band UV-B lamp (Figure S1a). In those seedlings, purple colour could be observed in the coleoptile and first leaf with more pronounced colouring in the latter (Figure S1b).

For quantitative analysis, we treated 4-day-old, R-grown seedlings of purple rice with LED UV-B (0.065 μmol m^{-2} s^{-1}) for different periods (up to 60 min). The anthocyanin level in the top 5-mm portion of the first leaf was determined 24 hr after the onset of UV-B irradiation. As shown in Figure 6a, some anthocyanins were detected in non-treated seedlings and this anthocyanin level was enhanced by UV-B treatment; a sharp increase occurred within 10 min of irradiation, followed by a gradual increase. The time course of anthocyanin accumulation was next measured following 10-min UV-B irradiation (Figure 6b). Although an increase of anthocyanins was observed in non-treated seedlings, a greater increase occurred in treated seedlings. The comparison of the time courses obtained with and without UV-B indicated that UV-B-dependent enhancement occurred with a lag of several hours and was near saturated after 24 hr.

The fluence-response relationship was examined with 10-min irradiation. The anthocyanin level was determined 24 hr after irradiation. As shown in Figure 6c, the anthocyanin level increased with fluences up to the highest fluence used (about 10^2 μmol m^{-2}). We further examined the effect of B under comparable conditions. Although the anthocyanin level appeared to increase by about 50% at high fluences (10^2–10^3 μmol m^{-2}), this enhancement was clearly less than that achieved with lower fluences of UV-B (Figure 6c). The results indicated that a large part, if not all, of UV-B-induced anthocyanin accumulation is mediated by UV-B-specific signalling (i.e., not caused by UV-B absorbed by B photoreceptors).

3.3 | Isolation of UV-B response mutants from purple rice

The anthocyanin accumulation phenotype was used to isolate UV-B response mutants from γ ray-mutagenized M2 lines of purple rice (see Materials and Methods section for details). Mutant screening was conducted under continuous R supplemented with broad-band UV-B (Figure S1a). The fluence rate of UV-B was 0.0015 μmol m^{-2} s^{-1}, which was sufficient to cause clearly visible anthocyanin accumulation in the first leaf.

After screening about 2,000 M2 lines, we isolated six independent mutants that do not develop any purple colour and 11 independent mutants that develop clearly less purple colour in the first leaf. In all cases, wild-type (WT) and mutant phenotypes segregated in M2 seedlings (with a lesser proportion of mutant seedlings) indicating that the mutations are recessive. The isolated mutants were selfed to obtain M3 and M4 caryopses.

The mutants showing the non-purple phenotype, named here *n*on-*p*urple under ultraviolet-B 1 through 6 (*npv1* to *npv6*), were characterized further using M4 caryopses. Firstly, the caryopses showed

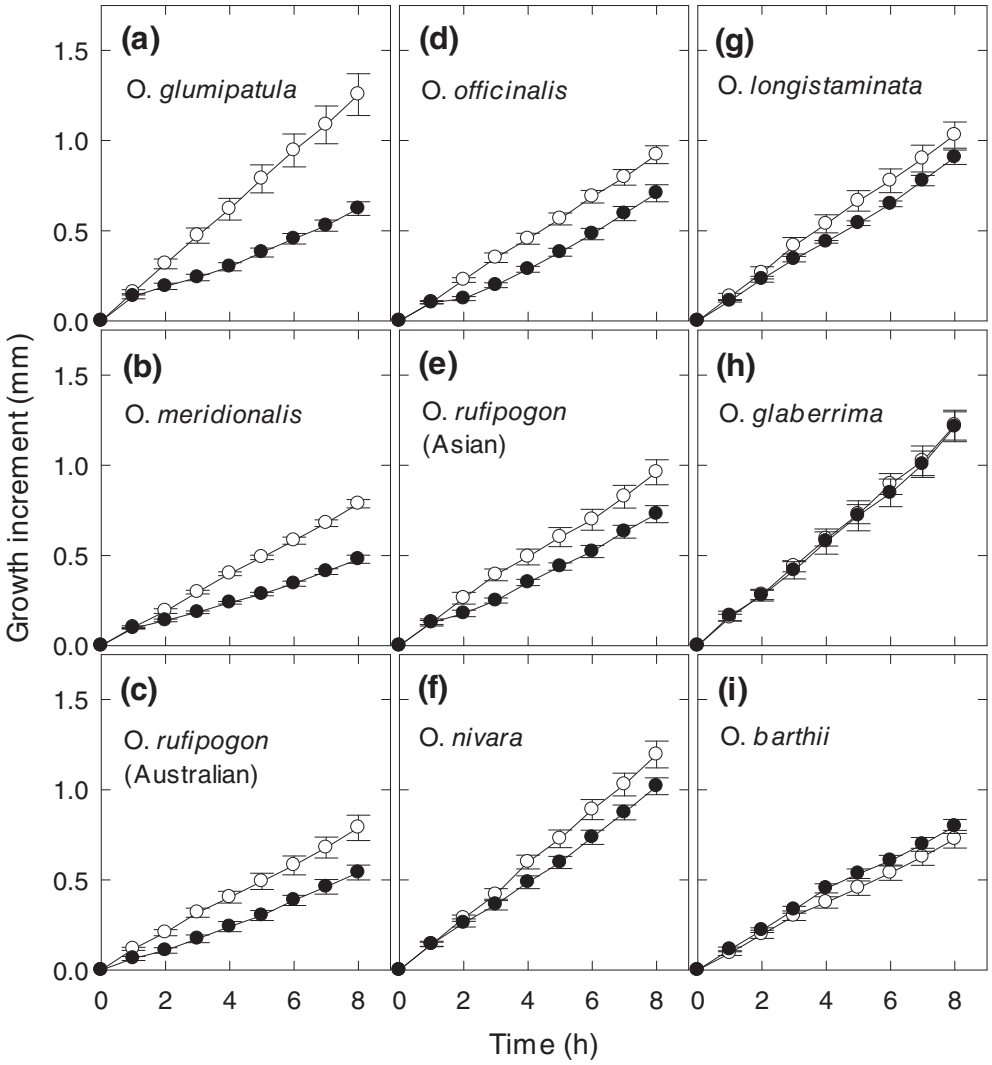

FIGURE 4 Effects of UV-B on the growth of coleoptiles in *Oryza* species other than *O. sativa*. The values are means ± SE of 10–12 plants. Other details are as for Figure 2a

different surface colours: compared to WT, *npv1* to *npv5* had more whitish colour, whereas *npv6* had reddish-brown colour (Figure 7a). The pictures shown in Figure 7b represent the colour appearance of WT and mutant seedlings. Without UV-B, the first leaf was light green in all cases. With UV-B, the first leaf became purple in the WT whereas it remained light green in all the mutants. Table 1 shows the final coleoptile length measured under R without and with UV-B. Without UV-B, *npv6* coleoptiles were clearly longer than WT coleoptiles, whereas in the other cases, the length was similar (*npv1* and *npv2*) or somewhat shorter (*npv3* to *npv5*). The coleoptile length became shorter by UV-B treatment in all cases except *npv3*, in which no significant effect of UV-B could be detected.

We also compared WT and mutant seedlings grown in total darkness. Dark-grown seedlings were observed when most of the seedlings grown simultaneously under R had the second leaf extending 2–3 mm above the first leaf. This observation method did not allow direct comparison of shoot lengths, but it was noted that the relationship between coleoptile and mesocotyl lengths in two mutants differed markedly from that in the WT (Figure S2). As compared to the WT, *npv6* showed shorter coleoptiles but much longer mesocotyls, whereas *npv3* had longer coleoptiles but clearly shorter mesocotyls. These contrasting morphological properties appeared to represent mutant phenotypes. The long mesocotyl phenotype of dark-grown *npv6* seedlings, as well as the long coleoptile phenotype of R-grown *npv6* seedlings (see above), resembles the phenotypes of jasmonate biosynthesis mutants (Riemann et al., 2013).

The effect of UV-B on the growth of mutant coleoptiles was next investigated by exposing the coleoptiles to 10-min UV-B. As shown in Figure 7c–i, the inhibitory effect of UV-B was observed in all the mutants. However, the effect was much less in *npv3* (Figure 7f). This agrees with the result obtained by measuring the final coleoptile length (see above). It appeared that *npv3* is impaired in both UV-B-induced anthocyanin accumulation and growth inhibition.

Greenhouse-grown WT plants developed a purple-coloured feature in all leaves and their leaf sheaths became dark purple-coloured. On the other hand, all the *npv* mutants sustained a green appearance of leaves including their leaf sheaths (Table 1). The awn is another structure that became dark purple in WT plants. In the mutants, the awn colour was either whitish (*npv1*, *npv2*, *npv5* and *npv6*) or light

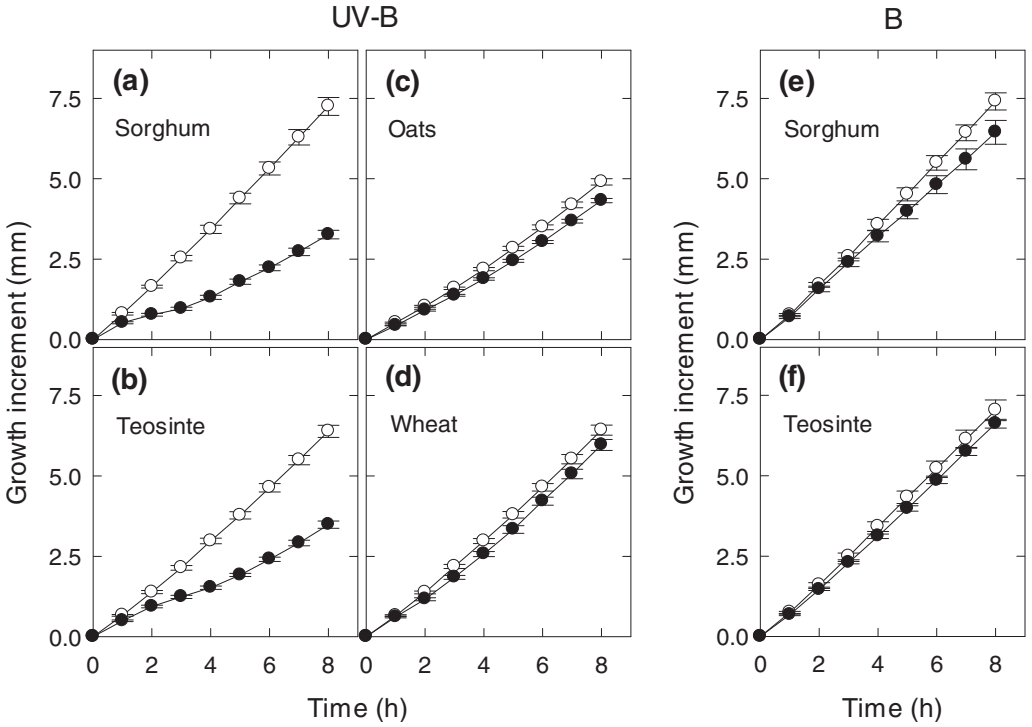

FIGURE 5 Effects of UV-B and blue light (B) on the growth of coleoptiles in *Gramineae* other than *Oryza*. The values are means ± SE of 10–12 plants. Other details are as for Figure 2a

purple (*npv3* and *npv4*), indicating some variation among the mutants (Table 1).

3.4 | UV-B-induced gene expression profiling

A two-colour microarray system was used to resolve genes that are up- or down-regulated in Nipponbare coleoptiles by 10-min UV-B irradiation. This investigation was made with samples harvested 20 and 60 min after the onset of irradiation against non-irradiated controls. Genes that showed more than two-fold changes with statistical significance (FDR < 0.05) were considered differentially expressed (Tables S2-S5). The Venn diagrams in Figure 8 summarize the numbers of genes that are determined to be up- or down-regulated at either or both time points.

The total number of up-regulated genes at either or both time points was 529; among them, 96 and 34 showed more than 5- and 10-fold increase, respectively (Tables S2 and S3). The total number of down-regulated genes was 79, which was much less than up-regulated genes; most of them showed less than five-fold decrease and no genes showed more than 10-fold decrease (Tables S4 and S5).

Comparison of up-regulated genes between 20 and 60 min indicated that a greater number of genes showed a higher expression at 60 min (Figure 8). A small portion of UV-B up-regulated genes were identified at 20 min but not at 60 min (white area in the Venn diagram), suggesting that some genes might be rapidly and transiently up-regulated within 60 min of UV-B irradiation. However, the actual increase at 20 min did not exceed 10-fold in all cases and the relative expression level at 20 min was not significantly different from that at 60 min in most cases (FDR > 0.05). Similar results were obtained with the down-regulated genes. Thus, there was no clear evidence for the occurrence of genes whose expression is rapidly and transiently regulated within 60 min of UV-B irradiation.

UV-B-regulated genes have been extensively studied in Arabidopsis under different growth and UV-B irradiation conditions (Brown et al., 2005; Favory et al., 2009; Oravecz et al., 2006; Qian et al., 2020; Rai et al., 2020; Tavridou et al., 2020; Ulm et al., 2004; Vanhaelewyn, Bernula, Van Der Straeten, Vandenbussche, & Viczian, 2019). We investigated the relationship of UV-B-regulated genes between rice and Arabidopsis (Table 2). Among 529 up-regulated genes of rice, about 25 and 5% were found to have Arabidopsis homologs that were up- and down-regulated, respectively. A large portion of up-regulated rice genes (about 70%) had no homologs reported to be UV-B-regulated in Arabidopsis. Similar results were obtained with the down-regulated genes (Table 2).

We next investigated functions of UV-B-regulated genes with the help of rice genome databases (Kurata & Yamazaki, 2006; Sun et al., 2017). Many of UV-B up-regulated genes (46 in number) were found to be related to the phenylpropanoid pathway as was the case in Arabidopsis (e.g., Oravecz et al., 2006; Stracke et al., 2010). Many of UV-B up- and down-regulated genes were related to plant hormones (Tables 3, S6 and S7). Involvement of plant hormone-related genes was reported in Arabidopsis, but there were some differences between the two plant species as considered in Discussion. We also

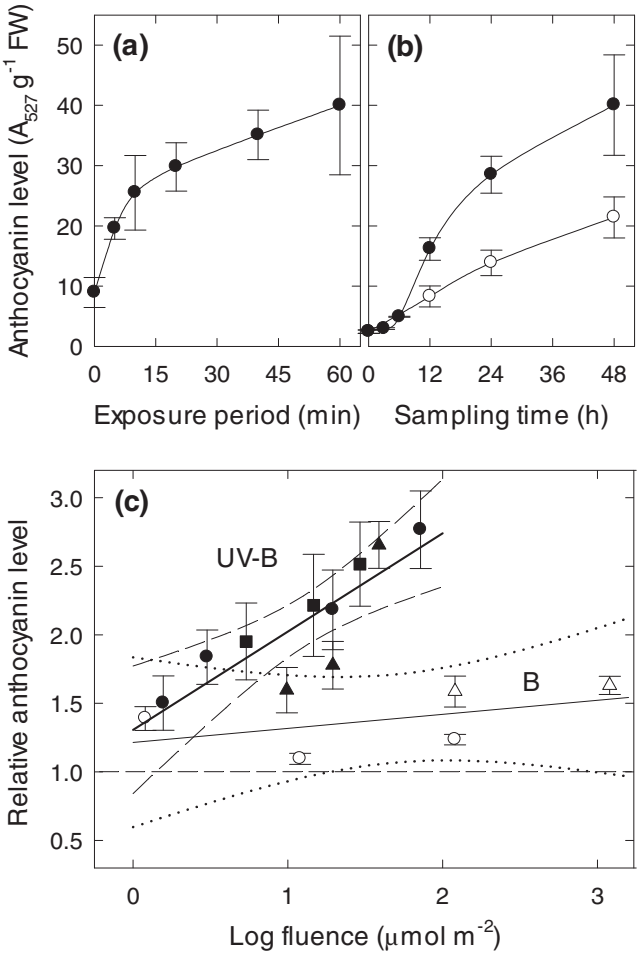

FIGURE 6 Effects of UV-B and blue light (B) on anthocyanin levels in the first leaves of red light-grown, 4-day-old seedlings of purple rice. (a) Anthocyanin accumulation as a function of exposure period. Seedlings were exposed to bilateral UV-B (0.065 μmol m^{-2} s^{-1}) for an indicated time and the level of anthocyanins in first leaves (top 5-mm portion) was determined 24 hr after the onset of exposure. The values are means ± SE of three samples (eight leaves per sample). (b) Time-courses of anthocyanin accumulation after exposure. Seedlings were exposed to bilateral UV-B (0.065 μmol m^{-2} s^{-1}) for 10 min and anthocyanin levels were determined at indicated times after the onset of exposure (○, mock-treated; ●, UV-B-treated). The values are means ± SE of four samples (eight leaves per sample). (c) Fluence-response curves of anthocyanin accumulation induced by UV-B (closed symbols) and B (open symbols). Seedlings were exposed to bilateral UV-B or B for 10 min at indicated fluences and anthocyanin levels were determined 24 hr after exposure. The difference in symbols indicates results obtained on different occasions. The anthocyanin level was expressed relative to the mean of mock-treated controls. The values are means ± SE of three or four independent samples (eight leaves per sample). Solid lines are the linear regression lines (UV-B: $y = 0.717 \times + 1.31$, $r^2 = 0.698$; B: $y = 0.102 \times + 1.22$, $r^2 = 0.262$). Dashed and dotted lines indicate the 95% confidence bands for UV-B- and B-treated samples, respectively

found that 18 of UV-B up-regulated genes are karrikin-responsive genes; four were identified to be karrikin responsive in rice (Zheng et al., 2020) and others are homologs of karrikin-responsive Arabidopsis genes (Nelson et al., 2010). Furthermore, three of UV-B up-regulated genes were biosynthetic genes for the phytoalexins momilactone A and B (Miyamoto et al., 2016).

3.5 | Real-time PCR analysis of UV-B-regulated genes

We extended gene expression analysis concerning UV-B specificity and *japonica-indica* relationship. Tissue samples used were Nipponbare coleoptiles treated with UV-B and B and Habataki coleoptiles treated with UV-B. Transcript levels were analysed by real-time PCR. We selected 12 genes from those found to be up-regulated more than 10-fold with reasonable expression levels in 60-min samples (signal intensity >1,000). The results are summarized in Figure 9. The transcript levels of 18S rRNA and *OsUBQ10*, used as internal controls, remained relatively stable (less than two-fold changes) in all treatments (Figure 9a,b).

The results obtained for UV-B-treated Nipponbare coleoptiles (closed circles, Figure 9) were in general agreement with those found with microarray analysis (Table S3). In all investigated genes, both UV-B and B induced a statistically significant increase in transcript level at least at 60 min (see the legend to Figure 9). In seven genes, the response to UV-B was significantly greater than that to B (Figure 9d–f,h–k). No genes showed a significantly greater response to B than to UV-B. The results indicated that UV-B-specific signalling participates in UV-B-induced transcriptional responses. It remained possible, however, that B photoreceptors contributed, by absorbing UV-B, to the responses.

Compared to Nipponbare coleoptiles, Habataki coleoptiles showed, in response to UV-B, a lower mean expression in all genes (Figure 9). The expression difference was statistically significant in four genes (Figure 9c,e,g,k). Among those showing a significant difference, three genes indicated the mean relative expression less than 25% of that in Nipponbare (Figure 9c,e,g). In particular, two genes showed only about 5% of the expression in Nipponbare (Figure 9c,g). The results demonstrated that some genes respond to a much lesser extent in Habataki as found with the growth response (Figure 2a vs. Figure 3i, see above).

In the above experiments, the fluence rate of B was much higher than that of UV-B (200 vs. 0.28 μmol m^{-2} s^{-1}). To clarify the UV-B specificity further, we compared the effects of UV-B and B at two comparable fluence rates (0.1 and 1.0 μmol m^{-2} s^{-1}) with four genes that showed relatively high responses to B (Figure 9). As shown in Figure 10, all genes responded to UV-B to a much greater extent, especially at the lower fluence rate, as compared to the response to B. The results demonstrated that UV-B-induced transcriptional responses are mediated largely, if not entirely, by UV-B-specific signalling.

4 | DISCUSSION

4.1 | Occurrence of UV-B-specific responses in rice and their UV-B sensitivity

We have demonstrated here using R-grown rice seedlings that UV-B-specific signalling functions in two UV-B-induced responses, that is,

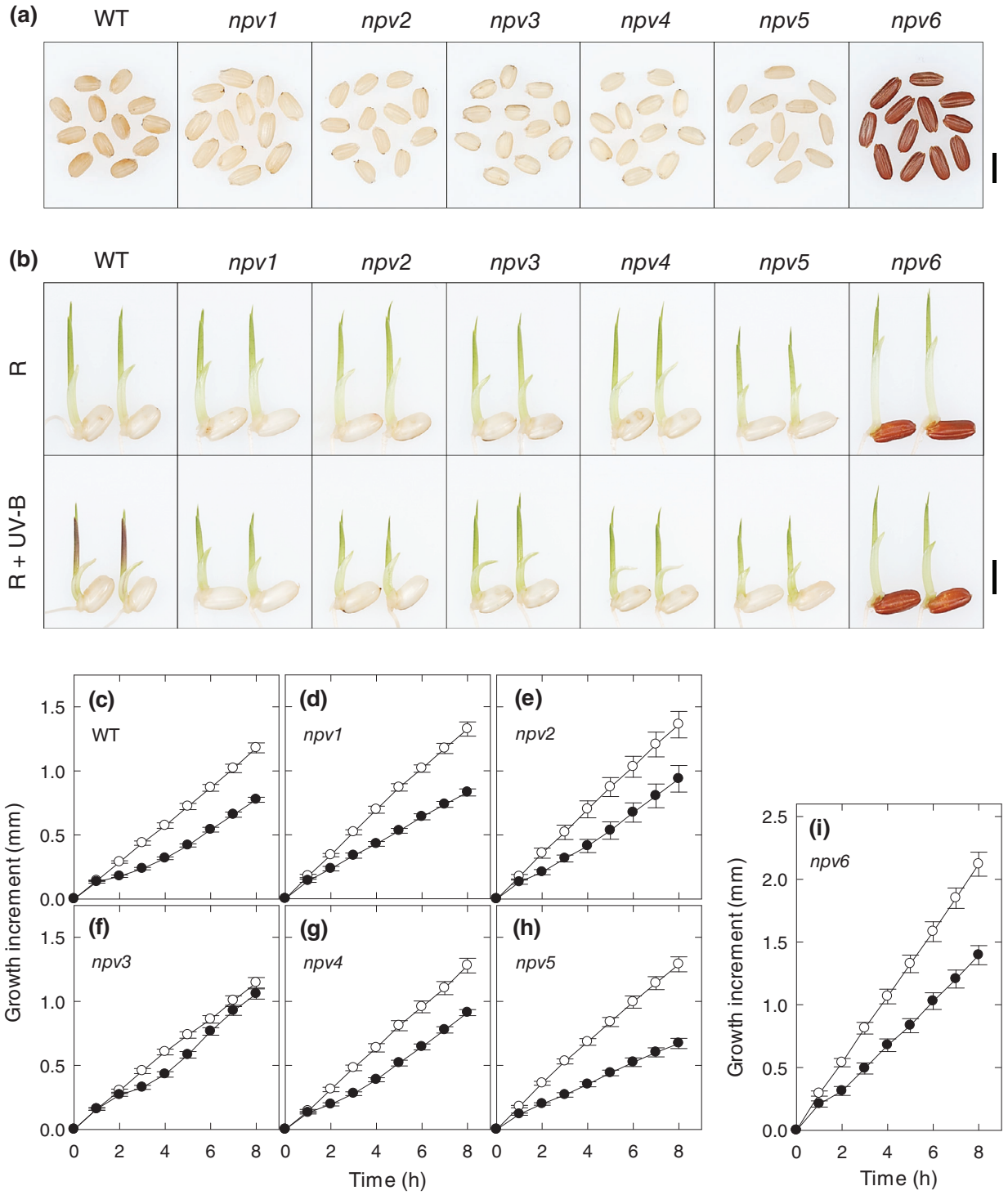

FIGURE 7 Some properties of *npv* mutants showing a non-purple phenotype under UV-B. (a) Caryopses of the wild-type (WT) and mutants. Scale bar = 5 mm. (b) WT and mutant seedlings grown under red light (R, 2.8 μmol m^{-2} s^{-1}) and under R supplemented with UV-B (0.0015 μmol m^{-2} s^{-1}, 280–315 nm). The UV-B source was the UV-B lamp used for mutant screening. Representative 4-day-old seedlings are shown. Scale bar = 5 mm. (c–i) Effects of UV-B on coleoptile growth in R-grown WT and mutant seedlings. The values are means ± SE of 8–16 plants. Other details are as for Figure 2a

inhibition of coleoptile growth and anthocyanin accumulation in first leaves. It is theoretically possible that B photoreceptors also contribute directly, by absorbing UV-B, to UV-B-induced responses. However, B was found to cause almost no coleoptile growth response (Figure 2). Furthermore, B induced some anthocyanin accumulation response but less effectively than UV-B (Figure 6c). It is concluded that the UV-B-induced responses observed in R-grown seedlings are mediated almost solely, if not entirely, by UV-B-specific signalling.

TABLE 1 Characteristics of *non-purple under ultraviolet-B* (*npv*) mutants isolated from purple rice

	Final coleoptile length (mm)[a]			
	R	R + UV-B	Leaf sheath colour[b]	Awn colour[c]
WT	7.56 ± 0.13	5.76 ± 0.13*	Dark purple	Dark purple
npv1	7.59 ± 0.17	5.64 ± 0.17*	Green	Whitish
npv2	7.30 ± 0.20	6.26 ± 0.10*	Green	Whitish
npv3	6.61 ± 0.13	6.11 ± 0.10	Green	Light purple
npv4	6.66 ± 0.20	5.68 ± 0.11*	Green	Light purple
npv5	6.35 ± 0.08	5.33 ± 0.10*	Green	Whitish
npv6	9.63 ± 0.15	7.24 ± 0.16*	Green	Whitish

[a]The means ± SE of 16–20 seedlings. Asterisks indicate a significant difference between "R" and "R + UV-B" samples ($p < 0.001$ by unpaired, two-tailed *t*-test).
[b]Observed during vegetative and flowering stages in greenhouse-grown plants.
[c]Observed in mature spikelets.

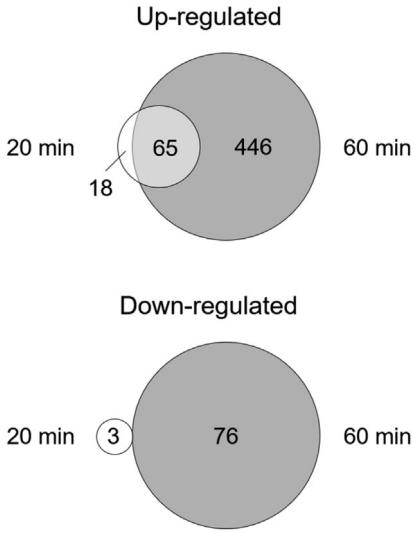

FIGURE 8 Analysis of UV-B-regulated genes in coleoptiles of red light-grown, 3-day-old Nipponbare seedlings. Two-colour microarray analysis was conducted with samples harvested 20 and 60 min after the onset of 10-min exposure to bilateral UV-B (0.065 μmol m^{-2} s^{-1}) against non-exposed samples. Three independent sample sets, each consisting of one 20-min sample, one 60-min sample, and two non-exposed samples (eight coleoptiles per sample), were used for the analysis. Venn diagrams show the numbers of up- and down-regulated genes that showed more than two-fold changes with statistical significance (FDR < 0.05) in 20- and 60-min samples. Numbers of non-overlapping and overlapping genes are indicated in each region of the diagrams. No overlapping was found between genes that were up-regulated at 20 or 60 min and those down-regulated at 60 or 20 min, respectively

TABLE 2 Relationships between UV-B-regulated genes of rice and UV-B responsiveness of their Arabidopsis homologs

	Arabidopsis homologs			
Rice genes	Up	Down	Non	Total
Up	129	28	372	529
Down	5	13	61	79

Note: Rice genes up- and down-regulated by UV-B (in either or both of 20- and 60-min samples, Figure 8) were each divided into three groups in relation to UV-B responsiveness of their Arabidopsis homologs (up-, down-, and non-regulated) and the numbers of those genes are shown. Arabidopsis homologs were searched with the Gramene and MSU rice genome annotation project databases and their UV-B responsiveness was based on the results of Ulm et al. (2004), Brown et al. (2005), Oravecz et al. (2006), Favory et al. (2009), Vanhaelewyn et al. (2019), Tavridou et al. (2020), Qian et al. (2020) and Rai et al. (2020). A given gene homolog was judged up-regulated (or down-regulated) if any of the above reports indicate up-regulation (or down-regulation) and counted non-regulated if none of them indicates up- or down-regulation.

Furthermore, a major contribution of UV-B-specific signalling could be shown in UV-B-induced gene expression responses (Figures 9 and 10).

The coleoptile growth inhibition occurred in the fluence range 10^{-1} to 10^{3} μmol m^{-2} (Figure 2b), which is much lower than the effective fluences ($10^{3.5}$ to 10^{5} μmol m^{-2}) reported for the UV-B-induced inhibition of hypocotyl growth in dark-grown Arabidopsis seedlings (Biever et al., 2014; Gardner et al., 2009). The UV-B-induced anthocyanin accumulation in purple rice occurred in the fluence range 10^{0} to 10^{2} μmol m^{-2} (Figure 6c), which approximately matches the fluence range for coleoptile growth inhibition and agrees with the results reported by Yatsuhashi et al. (1982) for the corresponding response in sorghum mesocotyls. In Arabidopsis, UV-B-induced accumulation of anthocyanins and also of other phenolic compounds has been well studied but only with continuous irradiation; the applied fluence rates ranged from 0.4 to 3 μmol m^{-2} s^{-1} (e.g., Bieza & Lois, 2001; Holl et al., 2019; Jiang et al., 2012; Stracke et al., 2010). We observed clear anthocyanin accumulation in purple rice at fluence rates as low as 0.0015 μmol m^{-2} s^{-1}. As compared to rice and sorghum, Arabidopsis may require much higher fluences for UV-B-dependent anthocyanin accumulation. These results and comparisons suggest that UV-B-specific responses of rice and probably of other *Gramineae* plants are uniquely sensitive to UV-B. Further study is required to resolve the difference in UV-B sensitivity between Arabidopsis (or other dicot species) and rice (or other *Gramineae* species).

TABLE 3 UV-B up- and down-regulated genes of rice that are related to plant hormones

Plant hormone classes and gene categories	Numbers of genes	
	Up-regulated	Down-regulated
Auxin		
Biosynthetic genes	3	0
Responsive genes	26	10
Gibberellins		
Biosynthetic genes	9	0
Responsive genes	10	1
Cytokinins		
Biosynthetic genes	2	0
Responsive genes	3	1
Brassinosteroids		
Biosynthetic genes	5	0
Responsive genes	8	3
Abscisic acid		
Biosynthetic genes	4	0
Responsive genes	41	4
Ethylene		
Biosynthetic genes	3	1
Responsive genes	31	2
Jasmonates		
Biosynthetic genes	14	0
Responsive genes	46	2
Salicylic acid		
Biosynthetic genes	6	0
Responsive genes	35	2

Note: Biosynthetic genes: genes for biosynthetic enzymes. Responsive genes: genes or their products that respond to plant hormones or participate in hormone signalling. Database sources are the integrated rice science database Oryzabase and Rice Pan-genome Browser.

4.2 | Mechanisms underlying UV-B-induced coleoptile growth inhibition

Physicochemical determination of plant hormones in UV-B-treated plants, conducted in more than 10 plant species (dicots and *Gramineae*) under different growth conditions (seedlings to juvenile plants, grown in white light or under natural day/night conditions), have indicated that the levels of growth-promoting hormones (auxin and gibberellins) and growth-inhibiting hormones (abscisic acid, ethylene and jasmonates) are generally reduced and enhanced, respectively (Bandurska & Cieslak, 2013; Chen, Ma, Yang, Gu, & Wang, 2019; Demkura, Abdala, Baldwin, & Ballare, 2010; Dinh, Galis, & Baldwin, 2013; Esringu, Aksakal, Tabay, & Kara, 2016; Fina et al., 2017; He, Zhang, Wang, & Chen, 2011; Katerova et al., 2009; Kovacs et al., 2014; Liu & Zhong, 2009; Mannucci et al., 2020; Pan, Zheng, Tian, Li, & Wang, 2014; Peng & Zhou, 2009; Tossi, Lamattina, & Cassia, 2009; Zhang, Huang, Wang, Zhou, & Huang, 2019). These responses in hormone levels were recorded with UV-B fluence rates ranging from about 0.1 to 10 μmol m^{-2} s^{-1}. The observed hormonal changes can explain growth-inhibiting effects of UV-B (e.g., Fina et al., 2017; Liu & Zhong, 2009; Zhang et al., 2019). It is not clear, however, whether all these plant hormones or particular ones mediate UV-B-induced growth inhibition.

Extensive microarray analysis conducted in Arabidopsis seedlings has indicated that UV-B affects expression of genes that are related to plant hormones, including all those mentioned above (Hectors, Prinsen, De Coen, Jansen, & Guisez, 2007; Morales et al., 2013; Qian et al., 2020; Vanhaelewyn, Prinsen, Van Der Straeten, & Vandenbussche, 2016). In Arabidopsis, auxin has been discussed as a key hormone involved in UV-B-induced inhibition of hypocotyl growth (Mazza & Ballare, 2015; Vandenbussche et al., 2014). Many auxin biosynthetic and responsive genes are down-regulated by UV-B in Arabidopsis seedlings (Fierro et al., 2015; Hectors et al., 2007; Mazza & Ballare, 2015; Qian et al., 2020; Vandenbussche et al., 2014) and evidence has been provided that UV-B promotes degradation of phytochrome interacting factors (PIFs), positive regulators of auxin biosynthesis (Hayes, Velanis, Jenkins, & Franklin, 2014; Qian et al., 2020; Sharma et al., 2019; Tavridou et al., 2020).

Our microarray analysis in rice coleoptiles has also indicated that expression of many plant hormone-related genes is affected by UV-B (Table 3). In contrast to the results reported in Arabidopsis, however, we found that UV-B up-regulates many auxin biosynthetic and responsive genes in rice coleoptiles (Table 3). The results do not suggest a positive involvement of auxin in UV-B-induced coleoptile growth inhibition.

Rice coleoptiles undergo strong phytochrome-mediated growth inhibition and jasmonates have been shown to play a key role in this growth response (Biswas et al., 2003; Riemann et al., 2013; Riemann, Riemann, & Takano, 2008). Our microarray analysis indicated that many jasmonate biosynthetic and responsive genes are up-regulated by UV-B (Table 3). The BASIC HELIX–LOOP–HELIX PROTEIN 006 (bHLH006) is a transcription factor shown to be involved in jasmonate-mediated inhibition of rice shoot growth (Kiribuchi et al., 2004; for the protein name, see Li et al., 2006). In fact, *OsbHLH006* was the gene most highly up-regulated by UV-B (Table S3, Figures 9c and 10a). Furthermore, Habataki coleoptiles showed only a little up-regulation of this gene (Figure 9c) in agreement with the much-reduced level of UV-B-induced growth inhibition in this cultivar (Figure 3i). It is suggested that jasmonates play a central role in UV-B-induced coleoptile growth inhibition.

It is also worth mentioning that members of the *GA2-oxidase* (*GA2ox*) gene family, which encodes GA-inactivating enzymes (Lo et al., 2008), were up-regulated by UV-B in rice coleoptiles; this was found with six members (*OsGA2ox3*, *OsGA2ox4*, *OsGA2ox6*, *OsGA2ox7*, *OsGA2ox8* and *OsGA2ox9*) and, especially, *OsGA2ox6* was one of the most highly up-regulated genes (Table S3). In Arabidopsis, too, *GA2ox* genes were up-regulated by UV-B (Hayes et al., 2014; Oravecz et al., 2006; Tavridou et al., 2020; Ulm et al., 2004) and Hayes et al. (2014) discussed how this up-regulation is correlated with

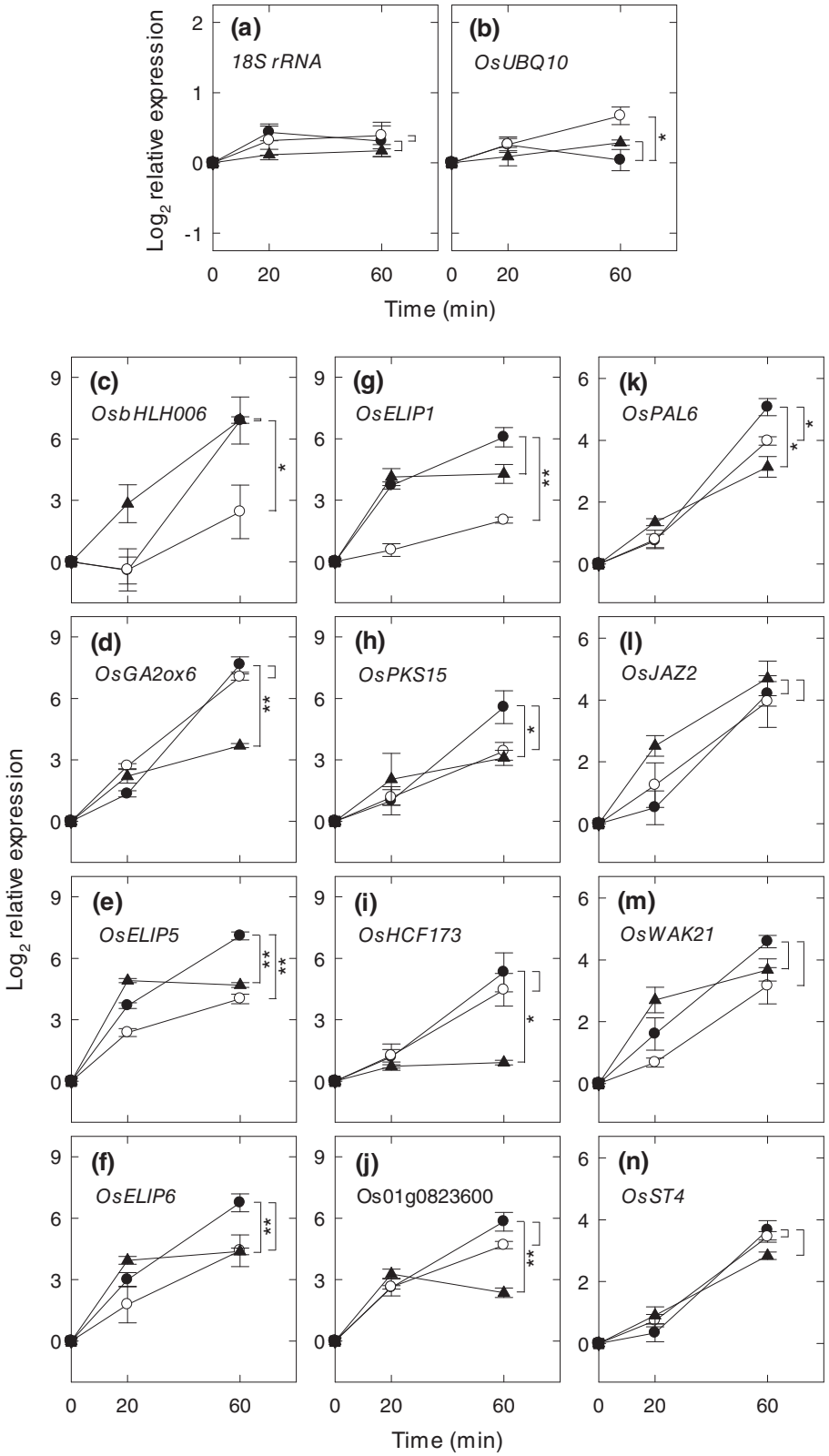

FIGURE 9 Real-time PCR analysis of UV-B up-regulated genes in coleoptiles of red light-grown, 3-day-old Nipponbare seedlings: comparisons with the effect of blue light (B) and the response in Habataki coleoptiles. Nipponbare coleoptiles were exposed to UV-B (●) and B (▲) and Habataki coleoptiles, to UV-B (○) (fluences used were as described for Figure 2a). The coleoptiles were harvested 20 and 60 min after the onset of exposure. The *18S rRNA* (a) and *OsUBQ10* (b) were used as internal controls. Expression levels of each gene at 20 and 60 min were calculated relative to the initial level and transformed to $\log_2$ values. The values are means ± SE of three or four independent samples (eight coleoptiles per sample). The figures (c–n) are arranged in the order of expression levels in UV-B-exposed coleoptiles at 60-min. Asterisks indicate significance between the two indicated means in an unpaired t-test assuming equal variances (* $p < 0.05$ and ** $p < 0.001$; no asterisk: not significant). The significance of each point was also examined in a one-sample t-test against zero (not shown within the figures): In UV-B- and B-treated Nipponbare coleoptiles, all genes (c-n) showed a significant expression enhancement ($p < 0.05$) at least at 60 min. In UV-B-treated Habataki coleoptiles, one gene (c) showed no significant enhancement at both time points and all other genes showed a significant enhancement at least at 60 min

UV-B-regulated auxin biosynthesis. It is possible that *GA2ox* genes are involved in UV-B-induced inhibition of rice coleoptile growth. Unlike the *OsbHLH006* mentioned above, however, *OsGA2ox6* was up-regulated in Nipponbare and Habataki similarly (Figure 9d).

The results described here warrant further investigation of the plant hormones involved in the UV-B-induced inhibition of rice coleoptile growth. At the moment, jasmonates appear to be the key hormone mediating this response.

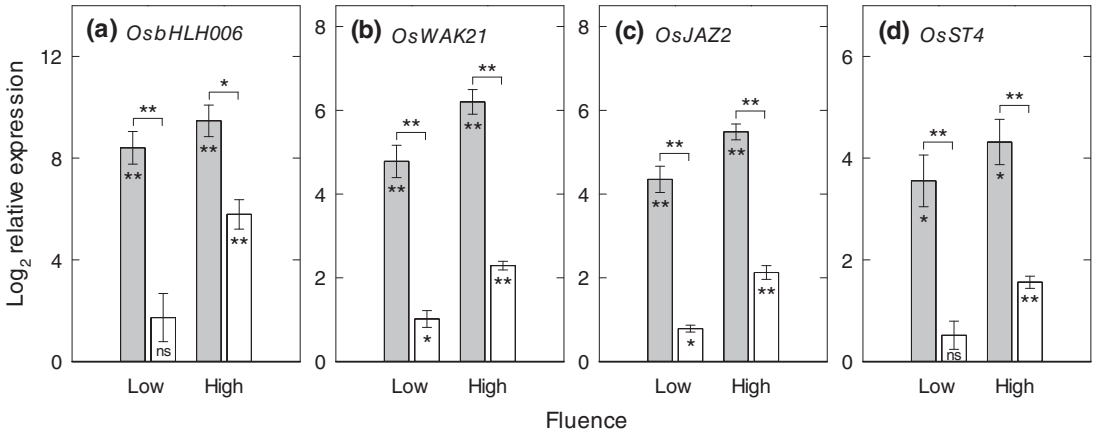

FIGURE 10 Comparisons of the effects of UV-B and blue light (B) on the expressions of four selected genes in coleoptiles of red light-grown, 3-day-old Nipponbare seedlings. Coleoptiles were exposed to bilateral UV-B (grey bar) and B (white bar) at two fluences (Low: 10 min at 0.1 μmol m^{-2} s^{-1}, High: 10 min at 1.0 μmol m^{-2} s^{-1}) and harvested 60 min after the onset of exposure. The values are means ± SE of three independent samples (eight coleoptiles per sample). Asterisks within each bar indicate significance in a one-sample t-test against zero (* $p < 0.05$ and ** $p < 0.01$; ns: not significant). Asterisks above each pair of bars indicate significance between UV-B- and B-treated samples in an unpaired t-test assuming equal variances (* $p < 0.05$ and ** $p < 0.01$)

4.3 | Interspecies variation in UV-B growth response

In recent years, phylogenetic analysis of *Oryza* species (especially of AA-genome species) has progressed by the use of whole chloroplast genome sequences and the major phylogenetic clades of AA-genome species have been correlated with geographical distributions (Moner, Furtado, & Henry, 2018; Song et al., 2017; Wambugu, Brozynska, Furtado, Waters, & Henry, 2015). We compared the phylogenetic tree of *Oryza* with the growth data shown in Figures 2-4 (Figure S3). The extent of UV-B growth response varied even within a clade. The difference observed between *japonica* and *indica* (Figure 3) represents such a variation. In addition, although the South American species *O. glumipatula* and the African species *O. longistaminata* form a clade, the former showed the largest response whereas the latter showed little response. It is noted, however, that *O. barthii* and *O. glaberrima*, which belong to the clade composed of only African species, showed no UV-B growth response. The lack of response might be a property of this clade.

Hidema and co-workers investigated UV-B-induced reduction of shoot dry weight and tiller number in various *Oryza* species and indexed their UV-B stress sensitivity (Hidema & Kumagai, 2006; Iwamatsu et al., 2008; Mmbando, Teranishi, & Hidema, 2020). It is noted that the extent of UV-B-induced growth response (Figures 3 and 4) is partially correlated with the UV-B stress sensitivity. The *japonica* cultivar Sasanishiki, which showed the greatest growth response (Figure 3a), was least sensitive (i.e., most resistant) to UV-B stress (Teranishi, Iwamatsu, Hidema, & Kumagai, 2004). On the other hand, the above-mentioned two African species, which showed no growth response, were recorded to have high stress sensitivity, that is, low resistance, to UV-B stress (Mmbando et al., 2020). The UV-B signalling underlying the growth response might be involved in the establishment of UV-B-stress resistance.

Wild *Oryza* species inhabit in tropical to subtropical regions (Brar & Khush, 2018) where UV-B levels are generally high (Beckmann et al., 2014). It seems that the ability of each *Oryza* species to express the UV-B-induced coleoptile growth inhibition under our laboratory conditions is not positively correlated with the solar UV-B level of its habitat. For example, African rice species that grow under the highest solar UV-B level showed the least UV-B growth response (see above). Therefore, although the UV-B-induced growth response may be functionally correlated with the UV-B-stress resistance as discussed above, we have had no evidence that the measured ability of rice species to show UV-B-induced growth response is correlated with their fitness in natural UV-B conditions.

4.4 | Searching for UV-B signalling mutants of rice

Mutants are useful tools for analysing signal perception and transduction cascades. In Arabidopsis, UV-B response mutants have been isolated and successfully used to elucidate the photoreceptor and signalling components (see the Introduction). Here, we have used a purple rice strain to screen rice mutants that are defective in visible anthocyanin accumulation induced by low-fluence-rate UV-B. With the first trial of mutant screening, we were able to isolate a number of mutants, demonstrating the effectiveness of the screening method.

Our primary purpose of mutant screening is to obtain mutants of UV-B signalling components. However, most isolated mutants may be mutants of anthocyanin biosynthesis, as cultivated "green" strains of rice were generated by mutation in anthocyanin biosynthesis-regulating transcription factors such as OsRb, a member of the bHLH family, and OsC1, a member of R2R3-MYB family (Chin, Wu, Hour, Hong, & Lin, 2016; Zheng et al., 2019). The caryopsis colour phenotypes of *npv* mutants (Figure 7a) are in agreement with this view. Proanthocyanidins accumulate in rice pericarp and are oxidized to

produce tannins that give yellow to reddish-brown colours of rice caryopses (Furukawa et al., 2007). Since anthocyanins and proanthocyanidins share a common biosynthetic pathway (Lepiniec et al., 2006), the mutants impaired in anthocyanin biosynthesis could results in lighter caryopsis colour as observed in *npv1* to *npv5* mutants. If mutation in anthocyanin biosynthetic pathway occurs after the branch point to proanthocyanidin biosynthesis, more accumulation of proanthocyanidins and development of reddish-brown caryopses could occur as noted in the *npv6* mutant. Similarly, anthocyanin biosynthetic mutants of Arabidopsis showed darker as well as lighter seed coat colours (Appelhagen et al., 2014).

Not all phenotypes of *npv* mutants could be explained, however, by mutation in the anthocyanin biosynthetic pathway. Firstly, the *npv3* coleoptile showed a less clear UV-B-induced growth inhibition. It is possible that *npv3* has a mutation in a common UV-B signalling component. Secondly, the morphological mutant phenotypes noted in dark-grown seedlings (i.e., different extents of mesocotyl elongation) cannot easily be linked to the anthocyanin biosynthetic pathway.

A further study should be conducted to isolate more mutants that are defective in both anthocyanin accumulation and coleoptile growth responses. Isolated mutants could then be used to study the UV-B signalling in rice.

4.5 | About UV-B photoreceptors in rice and concluding remarks

The protein UVR8 has been identified as the UV-B photoreceptor in Arabidopsis (see the Introduction). Rice has two genes homologous to *UVR8*: *OsUVR8a* (Os02g0554100) and *OsUVR8b* (Os04g0435700). The predicted products OsUVR8a and OsUVR8b are 82% identical to each other and 74 and 75% identical, respectively, to UVR8 (Figure S4). A *Tos17* insertion line of *OsUVR8a* is available (NF1947, Rice Tos17 Insertion Mutant Database). Our sequence analysis indicated that this line is likely to be an *OsUVR8a* null mutant. Furthermore, RT-PCR analysis showed no expression of *OsUVR8a* but a normal expression of *OsUVR8b* in white light-grown seedling shoots of the insertion line (see Table S1 for the primers used). We found that this insertion line exhibits a clear UV-B-induced inhibition of coleoptile growth (data not shown). A double null mutant of *OsUVR8a* and *OsUVR8b* is needed to clarify whether the UVR8 homologs function as the UV-B photoreceptor in rice.

An increasing body of evidence indicates that UVR8 may not be the sole plant UV-B photoreceptor (for references, see Introduction). Investigation with *uvr8* mutants showed that UVR8 controls almost entirely the UV-B responses including hypocotyl growth inhibition and anthocyanin accumulation (Favory et al., 2009; Gruber et al., 2010). However, Biever et al. (2014) reported that UV-B-induced hypocotyl growth inhibition is not mediated by UVR8. Brown et al. (2005) conducted microarray analysis to demonstrate that a number of genes are up-regulated by UV-B in a UVR8-dependent manner. However, a recent analysis indicated that a greater number of genes are regulated by UV-B in a UVR8-independent manner (O'Hara et al., 2019). Many auxin responsive genes are down-regulated by UV-B in Arabidopsis (see above) and UVR8 dependency of this down-regulation has been demonstrated, at least for some selected genes (Vandenbussche et al., 2014). However, Mazza and Ballare (2015) showed that UV-B regulation of auxin responsive genes is largely conserved in *uvr8* mutants. These conflicting results at least appear to indicate that UVR8 alone cannot explain all the UV-B-induced signalling responses in Arabidopsis seedlings.

Key questions here are whether UVR8 functions as a UV-B photoreceptor in rice and whether rice has a UV-B photoreceptor(s) other than UVR8. The UV-B response phenotypes of rice identified in the present study are basically similar to those of Arabidopsis, but there are certain differences, as discussed above. The most basic difference appears to be the high UV-B sensitivity in rice. It is possible that a UV-B photoreceptor other than UVR8 mainly mediates the investigated UV-B responses at least under our experimental conditions.

In the present study, we have demonstrated that R-grown rice seedlings together with a narrow-band LED UV-B source provide an experimental system with which UV-B-specific signalling responses could be isolated. Furthermore, we were able to establish an effective mutant screening method that could lead to isolation of UV-B signalling mutants. For a better understanding of ecologically relevant functions of UV-B signalling, investigation of UV-B photoreceptors and signalling in species other than Arabidopsis is anticipated (e.g., Robson et al., 2019). Further study of UV-B signalling in rice is expected to provide a wealth of information.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

DATA AVAILABILITY STATEMENT

The raw microarray data are available in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE138450. All other raw data supporting the findings of this study are available from the corresponding author, M. I., upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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