

Original Article

Rapid modulation of ultraviolet shielding in plants is influenced by solar ultraviolet radiation and linked to alterations in flavonoids

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ABSTRACT

The accumulation of ultraviolet (UV)-absorbing compounds (flavonoids and related phenylpropanoids) and the resultant decrease in epidermal UV transmittance (T_{UV}) are primary protective mechanisms employed by plants against potentially damaging solar UV radiation and are critical components of the overall acclimation response of plants to changing solar UV environments. Whether plants can adjust this UV sunscreen protection in response to rapid changes in UV, as occurs on a diurnal basis, is largely unexplored. Here, we use a combination of approaches to demonstrate that plants can modulate their UV-screening properties within minutes to hours, and these changes are driven, in part, by UV radiation. For the cultivated species *Abelmoschus esculentus*, large (30–50%) and reversible changes in T_{UV} occurred on a diurnal basis, and these adjustments were associated with changes in the concentrations of whole-leaf UV-absorbing compounds and several quercetin glycosides. Similar results were found for two other species (*Vicia faba* and *Solanum lycopersicum*), but no such changes were detected in *Zea mays*. These findings reveal a much more dynamic UV-protection mechanism than previously recognized, raise important questions concerning the costs and benefits of UV-protection strategies in plants and have practical implications for employing UV to enhance crop vigor and quality in controlled environments.

Key-words: acclimation; chlorophyll fluorescence; diurnal change; epidermal UV transmittance; quercetin; UV-A; UV-absorbing compounds; UV-B; UV protection.

INTRODUCTION

The epidermis of leaves has long been viewed as a selective filter of sunlight – absorbing much of the potentially deleterious ultraviolet radiation (UV; 280–400 nm) while transmitting visible wavelengths [photosynthetically active radiation (PAR); 400–700 nm] necessary for photosynthesis in the underlying mesophyll tissue (Caldwell *et al.* 1983; Day *et al.* 1992). The

accumulation of UV-absorbing compounds (flavonoids and related phenylpropanoid derivatives; i.e. ‘UV sunscreens’) in epidermal tissue occurs in response to UV exposure (Mazza *et al.* 2000; Bidel *et al.* 2007), and the resultant decrease in epidermal UV transmittance represents a primary mechanism by which plants acclimate to changing UV environments, including altered UV-B (280–315 nm) conditions resulting from stratospheric ozone depletion and climate change (Bornman *et al.* 2015). This UV acclimation response entails a measurable energetic cost (Snell *et al.* 2009; Guidi *et al.* 2011), varies within and among species (e.g. Day *et al.* 1992; Randriamanana *et al.* 2015), is influenced by environmental factors other than UV-B [e.g. UV-A (315–400 nm)], PAR and temperature (Flint *et al.* 2004; Bilger *et al.* 2007; Siipola *et al.* 2015) and is linked with cross-tolerance to other abiotic and biotic stresses (e.g. drought, herbivory and pathogen infection; Mewis *et al.* 2012; Bandurska *et al.* 2013; Zavala *et al.* 2015). Orchestration of UV-B-induced flavonoid biosynthesis appears to involve the UV-B photoreceptor UV RESISTANCE LOCUS 8 (UVR8) (Rizzini *et al.* 2011; Jenkins 2014) with UV-B exposure leading to the expression of UVR8-dependent gene transcripts involved in phenylpropanoid metabolism (Morales *et al.* 2013).

While the ability of plants to accumulate these protective UV-absorbing compounds and change their optical properties in response to days or weeks of exposure to UV is well established (Searles *et al.* 2001), whether they can do so in response to rapid changes in solar UV that occur naturally over the course of a day or as a result of changing cloud cover is largely unexplored. The earliest report suggesting that plants may be capable of rapid adjustment in UV screening came from observations by Lautenschlager-Fleury (1955) that the UV-B transmittance of epidermal peels from fava bean (*Vicia faba*) was low during midday on a sunny day but remained relatively high on a cloudy day. Veit *et al.* (1996) reported measurable midday increases in flavonoid levels in an alpine fern (*Cryptogramma crista*) and a tropical tree (*Anacardium excelsum*), and these changes were not evident when UV-B was filtered out of sunlight. More recently, Barnes *et al.* (2008) used chlorophyll fluorescence to non-invasively measure UV-A shielding and reported small but significant diurnal changes in epidermal UV transmittance in three plant species

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(*V. faba*, *Oenothera stricta* and *Verbascum thapsus*) growing in a high-UV tropical alpine environment. However, these investigators could not detect any associated diurnal changes in UV-absorbing compounds. At present, the prevalence, functional significance and underlying mechanisms responsible for diurnal changes in UV screening remain unclear.

In the studies described here, we used a combination of techniques to demonstrate that large, rapid and reversible changes in epidermal UV screening do indeed occur in some but not all plant species. We further quantify UV-absorbing compounds in species that differ in the degree of diurnal change in epidermal UV transmittance and characterize flavonoid profiles of one of these species to evaluate the relationship between diurnal changes in leaf optical properties and UV-absorbing compounds. Finally, we manipulate the solar UV spectrum to experimentally test whether solar UV radiation drives these rapid changes in leaf UV sunscreen protection.

MATERIALS AND METHODS

Experimental species and growth conditions

Studies were conducted on several cultivated plant species that were identified from a prior survey to exhibit a wide range of variability in diurnal changes in UV shielding and that were readily cultured in the warm subtropical climate of southeastern Louisiana, USA. Plants of okra [*Abelmoschus esculentus* (L.) Moench (syn. *Hibiscus esculentus* L.) cv. Clemson Spineless #80; Malvaceae] and maize (*Zea mays* L. cv. Golden cross bantam T-51; Poaceae) were grown from seed in pots [0.15 L volume containing commercially available organic (compost-based) potting soil]. Tomato [*Solanum lycopersicum* L. (syn. *Lycopersicon esculentum* Mill.) cv. Better Boy and Creole; Solanaceae] plants were transplanted as seedlings into compost-enriched native soil. In one experiment, we used pot-grown fava bean (*V. faba* L. cv. Broad Windsor; Fabaceae), as this species is amenable to manual detachment of epidermal tissue, which was not possible in the other species studied. All plants were grown outdoors in unshaded conditions prior to measurements and were kept well watered and regularly fertilized with commercial fertilizer (10-10-10; N-P-K) throughout the studies. Studies were conducted in the metropolitan area of New Orleans, Louisiana, USA (ca. 1 m elevation; 29.9°N, 90.1°W). Species nomenclature follows the US Department of Agriculture (USDA) Plants Database (<http://plants.usda.gov>).

Measurements of ambient photon flux densities (PFD) of PAR and UV-B irradiances over the dates of studies (May–July) were made using a quantum sensor (LI-185, Li-Cor, Inc., Lincoln, NE, USA) and a broadband UV sensor (Skye UV-B; Skye Instruments, Ltd., Powys, UK), respectively. The UV sensor was calibrated on-site against UV measurements made with a dual-grating scanning UV/Vis spectroradiometer (OL 756; Gooch & Housego, Orlando, FL, USA), and irradiances are reported here as biologically effective UV-B weighted according to the generalized plant action spectrum of Caldwell (1971) and normalized to unity at 300 nm. In one study, on-site UV measurements were not available, so we obtained UV irradiances from a broadband sensor

(UVB-1; Yankee Environmental Systems, Inc., Turners Falls, MA, USA) that was calibrated for human erythral UV response (McKinlay & Diffey 1987) and located at a USDA UV monitoring station (Louisiana State University Central Research Station, Baton Rouge, LA, USA; <http://uvb.nrel.colostate.edu/UVB/index.jsf>) ca. 110 km from the study site. For comparative purposes, we used the conversion factor from McKenzie *et al.* (2004) to convert peak daily erythral UV-B to plant effective UV-B for the 2 days of study.

Measurements of epidermal ultraviolet transmittance

Non-invasive measurements of epidermal UV transmittance (T_{UV}) were made on adaxial (upper) surfaces of mature, healthy leaves with a field-portable pulse amplitude modulation (PAM) chlorophyll fluorometer (UVA-PAM; Gademann Instruments, Würzburg, Germany). This instrument provides estimates of epidermal UV-A transmittance by measuring the fluorescence yield of chlorophyll (Fo; $\lambda > 650$ nm) induced by UV-A (UV; 375 nm) and blue (BL; 470 nm) radiation, as outlined by Kolb *et al.* (2005) and following the precautions and procedures of Barnes *et al.* (2008). Measurements of epidermal UV transmittance using this non-invasive technique have been found to be highly correlated with direct measurements of T_{UV} from epidermal peels (Markstadter *et al.* 2001), and this technique has been widely used to investigate UV sunscreen protection in a diversity of plant species and conditions (Barnes *et al.* 2015; Julkunen-Tiitto *et al.* 2015; and references therein). We also measured T_{UV} directly in epidermal peels of fava bean using a Polytetrafluoroethylene (PTFE)-coated integrating sphere (OL IS-1000, Optronic Laboratories, Orlando, FL, USA) interfaced to a scanning UV/Vis spectroradiometer (OL 756) with UV supplied from a 100-W xenon arc lamp (Oriel Corp., Stratford, CT, USA). Diffuse spectral transmittance was determined using the direct substitution method whereby we placed the sample perpendicular to the beam and beneath a quartz focusing lens. Measurements were then made with and without the sample in place, and transmittance was calculated as the ratio of these two signals. Calibrated PTFE reference standards were placed at the reflectance and reference ports of the integrating sphere for both measurements, and we assumed that the error in spectral efficiency due to the reflectance of the sample to be minimal.

Ultraviolet-exclusion study

For the field study designed to test the influence of solar UV in driving diurnal changes in T_{UV} , okra plants that had been growing under the full solar spectrum were randomly allocated to one of three metal filter frames (ca. $1 \times 1 \times 1$ m; length \times width \times height) that were fully enclosed (tops and sides) with one of three types of clear plastic film. The control frame (+UVB+UVA treatment) was covered with a UV-transparent film (Aclar type 22 A, 0.038 mm thick, Honeywell, Pottsville, PA, USA). Clear polyester (0.051 mm thick, optically equivalent to Mylar, cut-off near 320 nm; DuPont Teijin Films U.S., LLP, Hopewell, VA, USA) was used as the UV-B

blocking film (–UVB+UVA treatment), and clear Llumar (0.13 mm thick, part no. UVCLSRPS, cut-off near 390 nm; CPFilms, Inc., Martinsville, VA, USA) was used as the UV-B and UV-A blocking film (–UVB–UVA treatment). Transmittance spectra for these plastic filters can be found in Ryel *et al.* (2010). Small gaps (width ca. 1 cm) between filter rods in the overlapping end sections and at ground level permitted air movement within these filter ‘tents’. These structures allowed for minimal penetration of ambient UV that did not pass through the appropriate plastic film. Spectral irradiance measurements taken inside these tents indicated that integrated unweighted midday irradiances of UV-B, UV-A and PAR relative to ambient unfiltered conditions for the three types of filter tents were 94.2, 96.1 and 98.5% (Control; +UVB+UVA treatment), 3.9, 69.3 and 89.7% (–UVB+UVA treatment) and 6.5, 6.3 and 90.9% [–UVB–UVA treatment], respectively.

Analysis of ultraviolet-absorbing compounds

Determination of whole-leaf UV-absorbing compounds was made by extracting 1 cm² of fresh foliar tissue in 5 mL of acidified methanol solution (70% methanol, 29% H₂O and 1% HCl) for at least 48 h in a freezer (–20 °C; Beggs & Wellmann 1985). Extract absorbances were measured with a scanning UV/Vis spectrophotometer (Model DU640; Beckman Coulter, Inc., Fullerton, CA, USA), and the concentration of UV-B and UV-A-absorbing compounds is here expressed as the absorbance/cm²/5 mL. Although the relationship varies with species, whole-leaf methanolic extracts have been shown by others to be highly correlated with epidermal UV screening (Barnes *et al.* 2000; Liakoura *et al.* 2003).

Samples for ultra-high pressure liquid chromatography (UHPLC) mass spectrometry (MS) analyses were obtained from okra plants grown from seed outdoors in 2.1 L pots (organic soil medium as aforementioned). Individual, fully expanded leaves of okra were harvested, photographed for digital analysis of leaf area (NIH ImageJ 1.37v) and immediately frozen in liquid nitrogen. Samples were kept at –80 °C, lyophilized and ground + homogenized into a fine powder using a ball mixer/mill (8000M; SPEX SamplePrep, Metuchen, NJ, USA). Ground dried samples were stored at –20 °C until analyses. Immediately prior to analysis, 50.0 ± 1.0 mg of leaf powder was transferred to a 2 mL conical microcentrifuge tube and mixed with 0.5 mL of HPLC-grade MeOH for extraction. The solutions were then sonicated for 5 min and filtered with 0.2 µm PTFE syringe filters, and 2 µL of each solution was injected onto a Waters UHPLC equipped with both a photodiode array detector (PDA) and an electrospray ionization (ESI) single quadrupole MS. Compounds were separated with an Acquity UPLC HSS C18 1.8 µm 2.1 × 100 mm column held at 40 °C with a constant flow rate of 0.5 mL min^{–1} using a binary gradient of a 0.1% v/v aqueous solution of formic acid (mobile A) and acetonitrile with 0.1% v/v formic acid (mobile B). The gradient began with mobile A at 99%, followed by a decrease to 85% over the first 4 min, to 75% over the next 8 min, and then ramped down to a wash at 1% followed a 4 min equilibration period at initial conditions. The PDA was set to collect UV data (210–400 nm) during each run, which was used to obtain

flavonoid UV spectra (Supporting Information Fig. 2). MS conditions consisted of source temperature at 150 °C, desolvation temperature at 250 °C, nebulization gas flow of 500 L h^{–1}, and capillary and cone voltages set at 3 kV (negative ionization mode) and 30 V, respectively. Mass spectra were acquired in centroid mode, *m/z* 200–650. A five-point standard curve of quercetin dihydrate (Sigma-Aldrich Chemical Co. LLC, St. Louis, MO, USA) was used to calculate the amounts of the putative flavonoids as mg of quercetin dihydrate equivalents per g dry weight of leaf tissue g using peak areas integrated from extracted ion chromatograms from both standard and sample runs. Data were converted to concentrations on a leaf area basis using the leaf area/mass ratios determined at the time of leaf harvest.

Statistical analyses

For studies examining changes in UV shielding over time, T_{UV} data were analysed using repeated measures analysis of variance (ANOVA; SAS JMP, Cary, NC, USA) where the experimental unit was the individual plant ($N=10$ –16 plants for most experiments). Mean comparisons of T_{UV} made on the same leaves and plants at pre-dawn and midday were made using paired Student’s *t*-tests. Pigment data and all other T_{UV} data that were not collected on the same plants over time were analysed using individual ANOVAs for completely randomized designs, and mean comparisons were made using Tukey’s multiple range test with statistically significant differences considered at $P < 0.05$. Least square linear regression was used to examine the relationships between T_{UV} and UV-B and PAR, and a logarithmic relationship between T_{UV} and extract absorbance was assumed as per Beer’s law.

RESULTS

Over the course of a mostly sunny summer day in southeastern Louisiana, USA, the adaxial epidermal UV-A transmittance (T_{UV}) of mature leaves of okra, as determined by chlorophyll fluorescence (UVA-PAM), decreased progressively from dawn ($\bar{x} = 43.1\%$) to midday ($\bar{x} = 16.7\%$) and then gradually recovered to near pre-dawn values at sunset (Fig. 1; $P < 0.001$ for repeated measures ANOVA time effect). Diurnal changes in T_{UV} were statistically significant ($P < 0.001$ ANOVA time effect) albeit less pronounced on the following day when intermittent cloud cover reduced UV irradiances, especially during morning and early afternoon periods (Fig. 1a). When data from both days were combined, relative T_{UV} in this species was strongly and negatively related to ambient fluxes of both UV-B and PAR (Fig. 2).

As a test to determine whether UV (UV-B, UV-A or both) drives the diurnal changes in T_{UV} in okra, we placed these plants under three types of plastic film that differed in UV transmittance. Results from this experiment, conducted over two consecutive days under mostly clear skies, indicated that the near-total elimination of ambient solar UV (either UV-B alone or in combination with UV-A) reduced the maximum diurnal change (dawn to midday) in T_{UV} by ca. 45% (Fig. 3; $P < 0.001$; ANOVA). No significant differences in diurnal changes in T_{UV} ($P > 0.05$; Tukey’s multiple range test) were

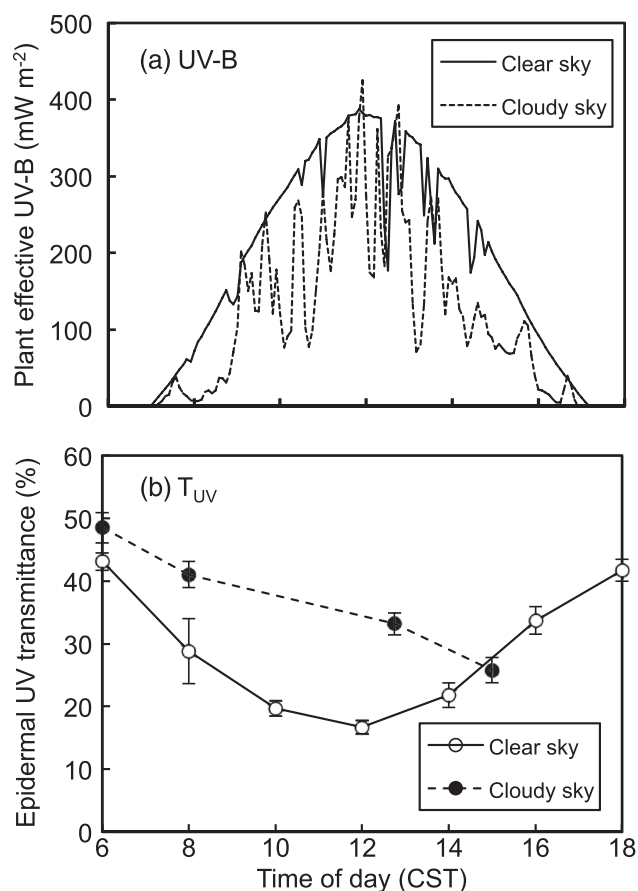


Figure 1. Diurnal changes in (a) solar ultraviolet (UV)-B irradiance and (b) adaxial epidermal UV transmittance (T_{UV}) of okra (*Abelmoschus esculentus*) over two consecutive summer days in New Orleans, Louisiana, USA, that varied in degree of cloud cover. Measurements of T_{UV} were discontinued late on the second day because of precipitation. Solar UV-B irradiances are weighted according to the generalized plant action spectrum of Caldwell (1971). Data are means \pm standard error ($N=15$). CST = Central Standard Time.

detected between plants in the $-UVB+UVA$ and $-UVB-UVA$ treatments.

To confirm that the rapid changes in T_{UV} detected using chlorophyll fluorescence were indeed the result of changes in UV shielding within epidermal tissue, we measured the UV transmittance of epidermal peels of fava bean, a plant whose epidermis can be readily detached and measured using an integrating sphere. Results from this study indicated that the UV transmittance of epidermal peels decreased significantly from dawn to midday [$P < 0.05$, paired Student's t -test for values in the UV-B (305 nm) and UV-A (375 nm); Fig. 4], and the magnitude of the diurnal change was similar to that detected using chlorophyll fluorescence on plants prior to peeling (Fig. 4 inset graph). At 375 nm, the peak excitation wavelength used to measure T_{UV} using chlorophyll fluorescence (UVA-PAM), mean transmittance values were 31.5 and 25.0% for dawn and midday values measured using the UVA-PAM (Fig. 4 inset), respectively, as compared with 38.3 and 29.0% for measurements made with the integrating sphere (Fig. 4). Immediately following midday measurements, plants were

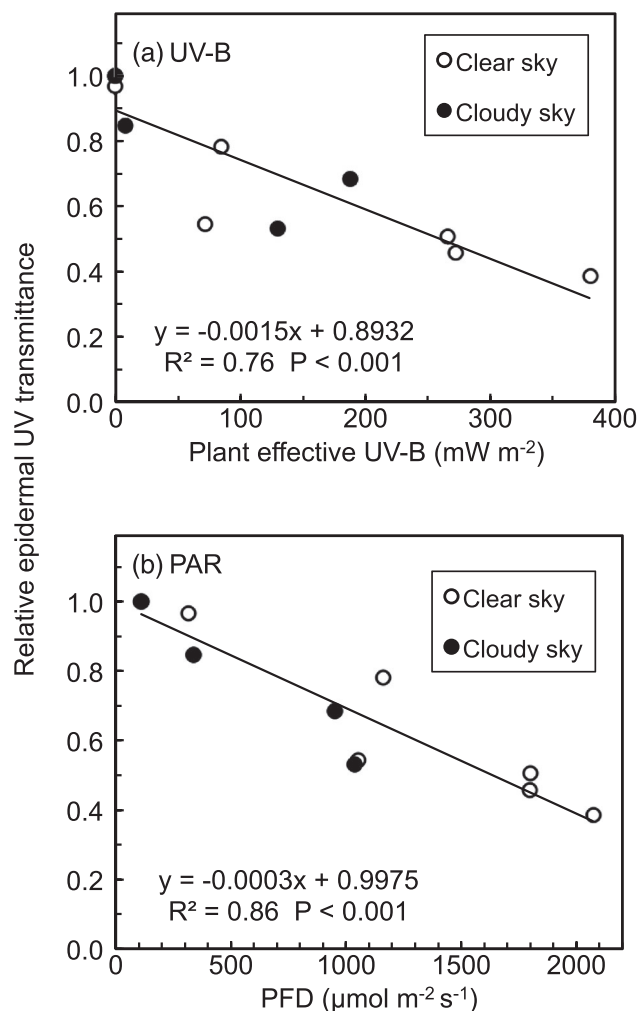


Figure 2. Relative adaxial epidermal ultraviolet (UV) transmittance of okra (*Abelmoschus esculentus*) in relation to (a) incident fluxes of solar UV-B and (b) photosynthetically active radiation (PAR) over the 2 days of measurements shown in Fig. 1. Transmittances were calculated relative to the pre-dawn values for each day. Measurements of UV and PAR were made every 5 min and averaged over the time period of each sampling period (15 min). Equations represent best-fit linear regression models of relative transmittances versus radiation fluxes for combined clear and cloudy sky conditions. PFD, photon flux densities.

placed in a dark room for 1–1.5 h, and then T_{UV} was measured on epidermal peels of adjacent leaves of these same plants. Following this period in the dark, mean T_{UV} values at all UV wavelengths were indistinguishable ($P > 0.87$ for paired Student's t -tests of selected wavelengths in the UV-B and UV-A) from those of leaves measured at dawn (Fig. 4). No UVA-PAM measurements were taken on dark-acclimated plants.

Results from a field study with okra and tomato plants growing in pots and the ground, respectively, over a 2-day period under near clear skies revealed that T_{UV} (Fig. 5a; $P < 0.001$, repeated measures ANOVA time of day effect) and bulk UV-absorbing compounds changed from dawn to midday in both species (Fig. 5b; $P = 0.006$ to 0.060 , repeated measures ANOVA time of day effect). Mean T_{UV} in these species was negatively

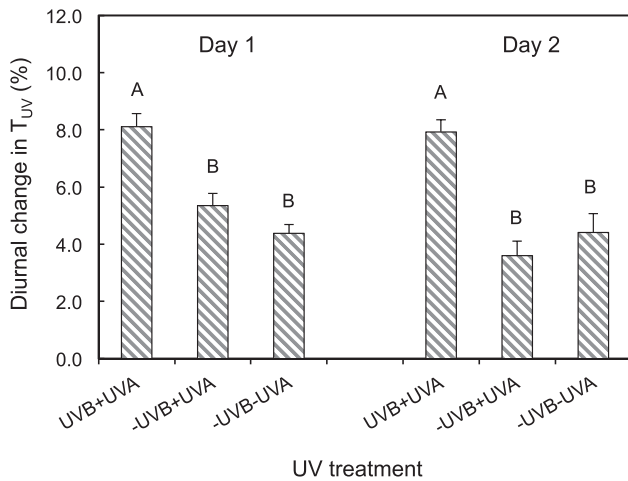


Figure 3. Maximum diurnal change (pre-dawn–midday) of adaxial epidermal ultraviolet (UV) transmittance (T_{UV}) in okra (*Abelmoschus esculentus*) exposed to different solar UV treatments over two consecutive summer days in New Orleans, Louisiana, USA. The three treatments were Controls (+UVB+UVA), attenuated UV-B with near-ambient UV-A (-UVB+UVA) and attenuated UV-B and UV-A (-UVB-UVA). Data are means \pm standard error ($N=16$ plants; 1–2 leaves per plant) with different letters indicating significant differences ($P < 0.05$) among means as determined by Tukey’s multiple range test.

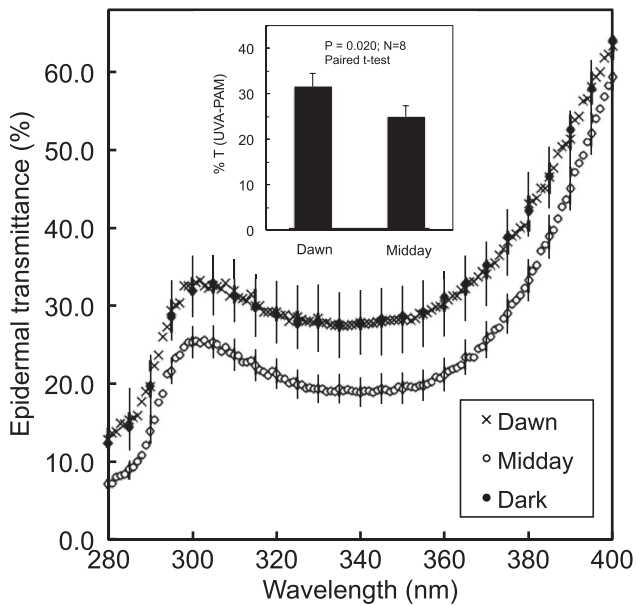


Figure 4. Adaxial epidermal ultraviolet (UV) transmittance of fava bean (*Vicia faba*) measured at different times of day on epidermal peels using an integrating sphere (open, closed and ‘x’ symbols) and using non-invasive chlorophyll fluorescence (UVA-PAM; inset bar graph). Data are means \pm standard error ($N=8$). Integrating sphere measurements were taken every nm from 280–400 nm, but for clarity, only data from every 5 nm are shown for the dark-acclimated plants. No UVA-PAM measurements were made on dark-acclimated plants.

correlated with concentrations of UV-absorbing compounds per area, although this relationship was stronger for okra than tomato [$R^2=0.99$ and 0.92 ($P=0.003$ and 0.04) for linear

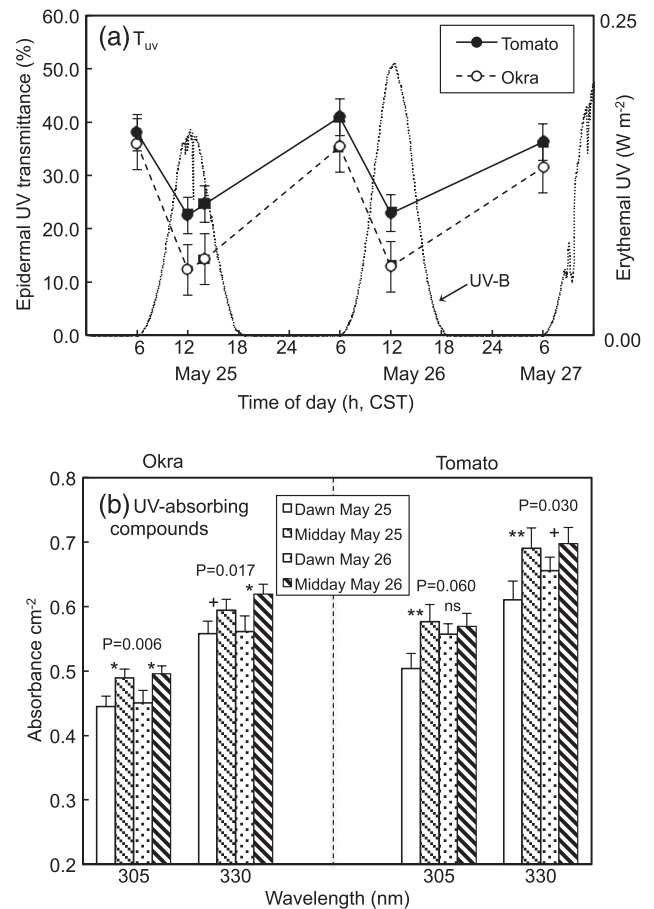


Figure 5. Diurnal changes in (a) adaxial epidermal ultraviolet (UV) transmittance and (b) whole-leaf UV-absorbing compounds for field-grown okra (*Abelmoschus esculentus*) and tomato (*S. lycopersicum*) plants over two mostly clear sky days near New Orleans, Louisiana, USA. Concentrations of UV-absorbing compounds are expressed on a leaf area basis and were measured at wavelengths in the UV-B (305 nm) and UV-A (330 nm). Data are means \pm standard error ($N=14-15$) with P -values above means for each species and wavelength in Panel B indicating levels of statistical significance for the main effect of time of day (repeated measure analysis of variance). Within each species and day, significant increases in UV-absorbing compounds from dawn to midday in Panel B are denoted as +, * and ** for $P < 0.1$, 0.05 and 0.01 , as determined by paired Student’s t -tests (ns=not significant). Two different cultivars were examined in tomato, but no significant differences were detected in the response of the cultivars over time (analysis of variance, $P > 0.32$ for time \times cultivar interactions for T_{UV} and UV-absorbing compounds) so data were pooled. Ambient solar UV-B data are from a near-by US Department of Agriculture UV-monitoring station (see section on Materials and Methods for additional details). Midday peak plant effective UV-B for these 2 days was estimated to be 230 and 304 $mW m^{-2}$.

regressions of $\ln(T_{UV})$ versus extract absorbance at 305 and 330 nm, respectively, in okra; $R^2=0.44$ and 0.70 ($P=0.34$ and 0.17) for linear regressions of $\ln(T_{UV})$ versus extract absorbance at 305 and 330 nm, respectively, in tomato; Supporting Information Fig. 1]. In a separate study with pot-grown maize, a species that exhibited no detectable diurnal changes in T_{UV} ($P=0.51$, paired Student’s t -test), we found no significant diurnal changes in UV-absorbing compounds (ANOVA, $P=0.74$ and

0.70 for extracts measured at 305 and 330 nm, respectively; data not shown).

We identified four methanol soluble quercetin derivatives in okra leaves based on UV and mass spectra data [quercetin-3-*O*-xylosyl (1"→2") glucoside, 2"-*O*-pentosyl-*X*-*C*-hexosyl-luteolin (*X*=6 or 8), quercetin-3-*O*-glucoside and quercetin-3-*O*-rhamnose] that could potentially function as UV sunscreens. The amounts of these compounds ranged from 6.7 g cm⁻² (1.4 mg g⁻¹) for quercetin-3-*O*-xylosyl (1"→2") glucoside to 0.3 g cm⁻² (0.08 mg g⁻¹) for quercetin-3-*O*-rhamnose. All

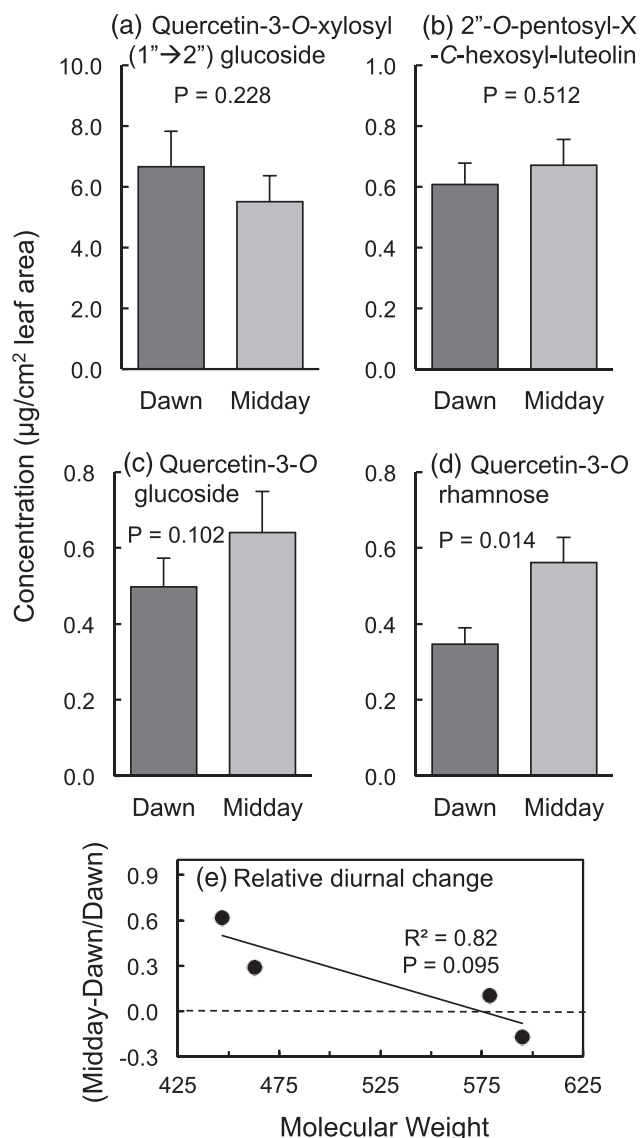


Figure 6. Concentrations of four quercetin derivatives in field-grown okra (*Abelmoschus esculentus*) measured in leaves collected at dawn and midday (a–d) and the relationship between relative diurnal change (midday–pre-dawn/pre-dawn) in concentrations (µg cm⁻²) and the molecular weights of these quercetin derivatives (e). In panel B, *X*=6 or 8. Data are means ± standard error (*N*=15) and are expressed as mass per unit leaf area. *P*-values above means in each panel are from paired Student *t*-tests for each compound. The line and equation in panel E represent the best-fit model as determined by simple linear regression.

four compounds exhibited distinctive flavonoid UV absorbance spectra with dual maxima at approximately 255 and 354 or 265 and 348 nm (Pereira *et al.* 2012; Supporting Information Fig. 2).

Foliar samples collected from field-grown okra plants at midday and the following dawn indicated a significant (*P*=0.014; paired Student's *t*-test) change in the levels of quercetin-3-*O*-rhamnose and a marginally significant (*P*=0.102) change in quercetin-3-*O*-glucoside (Fig. 6c,d). While no (*P*>0.10) diurnal changes were found in the other quercetin derivatives (Fig. 6a,b), there was a negative correlation (*R*²=0.82) between relative changes in the concentrations of all of these compounds (g cm⁻²) and their molecular weights, indicating a tendency for the lower molecular weight compounds to change proportionally more from dawn to midday than the heavier compounds (Fig. 6e). Mean dawn and midday adaxial *T*_{UV} of these leaves at the time of tissue collection were 22.7 and 9.9%, respectively.

DISCUSSION

Our results, based on two different measurement approaches, confirm that some but not all plant species are capable of rapid and reversible changes in epidermal UV transmittance (*T*_{UV}) over time frames of minutes to hours. Our findings further demonstrate, for the first time, that these diurnal changes in *T*_{UV} are coupled with diurnal changes in UV-absorbing compounds, as detected from both crude extracts and the quantification of specific flavonols (i.e. quercetin-3-*O*-rhamnose and quercetin-3-*O*-glucoside). Although we focused on cultivated species in this study, results from a survey of nearly 40 different species of wild and cultivated species representing herbaceous (grasses and dicots) and woody (shrub and tree) growth forms, indicate that this phenomenon is widespread among higher plants (detected in 62% of the species tested so far); however, the magnitude of diurnal changes in *T*_{UV} varies considerably depending on species and growth conditions (P. Barnes ms. in prep.). For example, in comparison with our previous study (Barnes *et al.* 2008) on three herbaceous species (including fava bean) growing in a cool, high elevation, high UV tropical environment, we found that the diurnal changes in *T*_{UV} were ca. 10-fold higher (ca. 30 versus 3% overall and ca. 10 versus 2% in fava bean) for our plants growing in the warmer subtropical climate of southeastern Louisiana. Moreover, we found that the diurnal changes in *T*_{UV} were driven, at least in part, by solar UV radiation. These findings are consistent with those of Veit *et al.* (1996) who reported that removal of solar UV-B eliminated the diurnal changes in flavonoids in two tropical plants, and the studies of Barnes *et al.* (2008) who demonstrated that dense shade could disrupt both the timing and magnitude of diurnal changes in *T*_{UV} in *V. thapsus*. Thus, the diurnal change in UV sunscreen protection we report here most likely represents a specific, rapid and inducible response to sunlight rather than an endogenous circadian rhythm (McClung 2001).

That higher plants can rapidly adjust their UV sunscreen protection in response to short-term changes in UV irradiances may not be surprising as diurnal fluctuations in UV protection have been documented in systems as diverse as human

epidermal stem cells (Janich *et al.* 2013), marine algae (Taira *et al.* 2004) and liverworts (Fabón *et al.* 2012). How plants achieve these rapid changes and what their significance for plant function are not entirely clear. While solar radiation appears to drive this response, it is conceivable that some of the changes in UV-absorbing compounds could be due to diurnal changes in cell turgor (Lee *et al.* 2012), which could then lead to altered concentrations of these compounds in epidermal tissue. Diurnal changes in gene expression, metabolites and the activities of key enzymes involved in phenylpropanoid biosynthesis have been reported by others (Peter *et al.* 1991; Kim *et al.* 2011), and exposure to solar UV is known to increase the levels of phenylpropanoid biosynthetic enzymes and metabolites (Morales *et al.* 2013). Modest exposure to UV-B has also been shown to induce rapid (within minutes) activation of the UV-B photoreceptor (UVR8) (Kaiserli & Jenkins 2007). However, the induction and accumulation of UV-absorbing compounds and resultant increase in epidermal UV screening typically occur over much longer time frames (i.e. days; Hectors *et al.* 2014; Wargent *et al.* 2015). Relocation of flavonoids among different pools in leaf tissue (Schnitzler *et al.* 1996; Burchard *et al.* 2000), the rapid alterations in absorptive properties of individual compounds (e.g. Dean *et al.* 2014) and/or the UV-induced conversion of phenylpropanoid structures would seem more likely mechanisms to account for the rapid changes in these UV sunscreens than *de novo* synthesis and degradation of these compounds. After 1 day of moderate UV-B exposure, Neugart *et al.* (2012) observed that juvenile plants of kale (*Brassica oleracea*) showed a number of structurally dependant changes in flavonol (quercetin and kaempferol) glycosides with some compounds increasing and others declining. Similarly, in the present study, we found significant diurnal changes in the foliar composition of quercetin derivatives in okra with low molecular weight compounds changing to a greater degree than higher weight compounds. Whether these quercetin compounds function as both UV sunscreens and antioxidants (Agati *et al.* 2012) was not assessed in the present study. Furthermore, the concentrations of UV-absorbing compounds we report here are likely an underestimate of total levels of these compounds as our extractions included only methanol-soluble compounds and not wall-bound compounds (Clarke & Robinson 2008).

Irrespective of mechanisms, these diurnal changes in UV shielding likely provide clear benefits to plants in UV protection, at least when compared with hypothetical cases where low pre-dawn levels of UV shielding are maintained throughout the day (Barnes *et al.* 2015). However, the benefits of diurnal adjustment in UV protection relative to plants that maintain consistently high (midday) levels of UV protection over the day are less clear. In comparison with these kinds of plants (e.g. maize), calculations suggest that plants that exhibit diurnal adjustment in UV shielding (e.g. okra) may experience increased penetration of UV to the underlying mesophyll both in the morning and afternoon but not at midday (Barnes *et al.* 2015). It is conceivable that increased penetration of UV to photosynthetic tissue at these times may protect leaves from photoinhibition (Wargent *et al.* 2011) that can occur under high irradiances in the middle of the day. There is evidence that

UV-A can drive photosynthesis (Turnbull *et al.* 2013), and increased penetration of UV-A may therefore increase photosynthesis at times of the day when leaves are light limited. There is also the possibility that maintaining constant high levels of flavonoids could interfere with plant growth during the night. Several of the flavonoids induced by UV (e.g. quercetin and kaempferol) are known to interfere with auxin metabolism and transport (Ringli *et al.* 2008; Kuhn *et al.* 2011), which in turn could influence plant growth and morphology (Hectors *et al.* 2012). Diurnal fluctuations in UV sunscreen protection may also have consequences for the timing of plant responses to other stresses (e.g. herbivory) that can vary in severity over the course of a day (e.g. Goodspeed *et al.* 2012) and which, in some cases, employ similar suites of secondary compounds for both defence and UV protection (Kuhlmann & Müller 2010; König *et al.* 2014). Understanding how UV protection interacts with these, and other physiological and ecological functions, is required to fully evaluate the costs and benefits associated with 'static' versus 'dynamic' UV-protection strategies in plants.

Finally, the existence of UV-driven diurnal adjustments in UV shielding may explain, in part, the oft-reported heightened UV sensitivity of plants grown in controlled environments, such as greenhouses and growth chambers (Caldwell & Flint 1994), where natural diurnal cycles in UV radiation are muted or absent, and hence, UV sunscreen protection would likely be compromised, at least for certain plant species. Increasingly, UV exposure is being considered as a tool to enhance crop quality and vigor (Schreiner *et al.* 2012; Wargent & Jordan 2013) in controlled environments. Our findings suggest that for certain species, this will require developing artificial light and UV exposure systems that promote natural diurnal adjustments in UV screening so as to avoid excessive UV injury under these conditions.

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REFERENCES

- Agati G., Azzarello E., Pollastri S. & Tattini M. (2012) Flavonoids as antioxidants in plants: location and functional significance. *Plant Science* **196**, 67–76.
- Bandurska H., Niedziela J. & Chadzinikolau T. (2013) Separate and combined responses to water deficit and UV-B radiation. *Plant Science* **213**, 98–105.

- Barnes P.W., Flint S.D., Ryel R.J., Tobler M.A., Barkley A.E. & Wargent J.J. (2015) Rediscovering leaf optical properties: new insights into plant acclimation to solar UV radiation. *Plant Physiology and Biochemistry* **93**, 94–100.
- Barnes P.W., Flint S.D., Slusser J.R., Gao W. & Ryel R.J. (2008) Diurnal changes in epidermal UV transmittance of plants in naturally high UV environments. *Physiologia Plantarum* **133**, 363–372.
- Barnes P.W., Searles P.S., Ballaré C.L., Ryel R.J. & Caldwell M.M. (2000) Non-invasive measurements of leaf epidermal transmittance of UV radiation using chlorophyll fluorescence: field and laboratory studies. *Physiologia Plantarum* **109**, 274–283.
- Beggs C.J. & Wellmann E. (1985) Analysis of light-controlled anthocyanin formation in coleoptiles of *Zea mays* L.: the role of UV-B, blue, red and far-red light. *Photochemistry and Photobiology* **41**, 481–486.
- Bidel L.P.R., Meyer S., Goulas Y., Cadot Y. & Cerovic Z.G. (2007) Responses of epidermal phenolic compounds to light acclimation: *in vivo* qualitative and quantitative assessment using chlorophyll fluorescence excitation spectra in leaves of three woody species. *Journal of Photochemistry and Photobiology B: Biology* **88**, 163–179.
- Bilger W., Rolland M. & Nybakken L. (2007) UV screening in higher plants induced by low temperature in the absence of UV-B radiation. *Photochemical & Photobiological Sciences* **6**, 190–195.
- Bornman J.F., Barnes P.W., Robinson S.A., Ballaré C.L., Flint S.D. & Caldwell M.M. (2015) Solar ultraviolet radiation and ozone depletion-driven climate change: effects on terrestrial ecosystems. *Photochemical & Photobiological Sciences* **14**, 88–107.
- Burchard P., Bilger W. & Weissenböck G. (2000) Contribution of hydroxycinnamates and flavonoids to epidermal shielding of UV-A and UV-B radiation in developing rye primary leaves as assessed by ultraviolet-induced chlorophyll fluorescence measurements. *Plant, Cell & Environment* **23**, 1373–1380.
- Caldwell M.M. (1971) Solar UV irradiation and the growth and development of higher plants. *Photophysiology* **6**, 131–177.
- Caldwell M.M. & Flint S.D. (1994) Stratospheric ozone reduction, solar UV-B radiation and terrestrial ecosystems. *Climatic Change* **28**, 375–394.
- Caldwell M.M., Robberecht R. & Flint S.D. (1983) Internal filters: prospects for UV-acclimation in higher plants. *Physiologia Plantarum* **58**, 445–450.
- Clarke L.J. & Robinson S.A. (2008) Cell wall-bound ultraviolet-screening compounds explain the high ultraviolet tolerance of the Antarctic moss, *Ceratodon purpureus*. *New Phytologist* **179**, 776–783.
- Day T.A., Vogelmann T.C. & DeLucia E.H. (1992) Are some plant life forms more effective than others in screening out ultraviolet-B radiation? *Oecologia* **92**, 513–519.
- Dean J.C., Kusaka R., Walsh P.S., Allais F. & Zwier T.S. (2014) Plant sunscreens in the UV-B: ultraviolet spectroscopy of jet-cooled sinapoyl malate, sinapic acid, and sinapate ester derivatives. *Journal of the American Chemical Society* **136**, 14780–14795.
- Fabón G., Monforte L., Tomás-Las-Heras R., Núñez-Olivera E. & Martínez-Abaigar J. (2012) Dynamic response of UV-absorbing compounds, quantum yield and the xanthophyll cycle to diel changes in UV-B and photosynthetic radiations in an aquatic liverwort. *Journal of Plant Physiology* **169**, 20–26.
- Flint S.D., Searles P.S. & Caldwell M.M. (2004) Field testing of biological spectral weighting functions for induction of UV-absorbing compounds in higher plants. *Photochemistry and Photobiology* **79**, 399–403.
- Goodspeed D., Chehab E.W., Min-Venditti A., Braam J. & Covington M.F. (2012) *Arabidopsis* synchronizes jasmonate-mediated defense with insect circadian behavior. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 4674–4677.
- Guidi L., Degl'Innocenti E., Remorini D., Biricolti S., Fini A., Ferrini F., ... Tattini M. (2011) The impact of UV-radiation on the physiology and biochemistry of *Ligustrum vulgare* exposed to different visible-light irradiance. *Environmental and Experimental Botany* **70**, 88–95.
- Hectors K., Van Oevelen S., Geuns J., Guisez Y., Jansen M.A.K. & Prinsen E. (2014) Dynamic changes in plant secondary metabolites during UV acclimation in *Arabidopsis thaliana*. *Physiologia Plantarum* **152**, 219–230.
- Hectors K., van Oevelen S., Guisez Y., Prinsen E. & Jansen M.A.K. (2012) The phytohormone auxin is a component of the regulatory system that controls UV-mediated accumulation of flavonoids and UV-induced morphogenesis. *Physiologia Plantarum* **145**, 594–603.
- Janich P., Toufighi K., Solanas G., Luis N., Minkwitz S., Serrano L., ... Benitah S. (2013) Human epidermal stem cell function is regulated by circadian oscillations. *Cell Stem Cell* **13**, 745–753.
- Jenkins G.I. (2014) The UV-B photoreceptor UVR8: from structure to physiology. *Plant Cell* **26**, 21–37.
- Julkunen-Titto R., Nenadis N., Neugart S., Robson M., Agati G., Vepsäläinen J., ... Jansen M.A.K. (2015) Assessing the response of plant flavonoids to UV radiation: an overview of appropriate techniques. *Phytochemistry Reviews* **14**, 273–297.
- Kaiserli E. & Jenkins G.I. (2007) UV-B promotes rapid nuclear translocation of the *Arabidopsis* UV-B-specific signaling component UVR8 and activates its function in the nucleus. *Plant Cell* **19**, 2662–2673.
- Kim S.-G., Yon F., Gaquerel E., Gulati J. & Baldwin I.T. (2011) Tissue specific diurnal rhythms of metabolites and their regulation during herbivore attack in a native tobacco, *Nicotiana attenuata*. *Plos One* **6**, 1–13.
- Kolb C.A., Schreiber U., Gademann R. & Pfündel E.E. (2005) UV-A screening in plants determined using a new portable fluorimeter. *Photosynthetica* **43**, 371–377.
- König S., Feussner K., Kaever A., Landesfeind M., Thurow C., Karlovsky P., ... Feussner I. (2014) Soluble phenylpropanoids are involved in the defense response of *Arabidopsis* against *Verticillium longisporum*. *New Phytologist* **202**, 823–837.
- Kuhlmann F. & Müller C. (2010) UV-B impact on aphid performance mediated by plant quality and plant changes induced by aphids. *Plant Biology* **12**, 676–684.
- Kuhn B.M., Geisler L., Bigler L. & Ringli C. (2011) Flavonols accumulate asymmetrically and affect auxin transport in *Arabidopsis*. *Plant Physiology* **156**, 585–595.
- Lautenschlager-Fleury D. (1955) Über die Ultravioletturchlässigkeit von Blattepidermen. *Berichte Schweizer Botanischen Gesellschaft* **65**, 343–386.
- Lee K.M., Driever S.M., Heuvelink E., Ruger S., Zimmermann U., de Gelder A. & Marcellis L.F.M. (2012) Evaluation of diel patterns of relative changes in cell turgor of tomato plants using leaf patch clamp pressure probes. *Physiologia Plantarum* **146**, 439–447.
- Liakoura V., Bornman J.E. & Karabourniotis G. (2003) The ability of abaxial and adaxial epidermis of sun and shade leaves to attenuate UV-A and UV-B radiation in relation to the UV absorbing capacity of the whole leaf methanolic extracts. *Physiologia Plantarum* **117**, 33–43.
- Markstadter C., Queck I., Baumeister J., Riederer M., Schreiber U. & Bilger W. (2001) Epidermal transmittance of leaves of *Vicia faba* for UV radiation as determined by two different methods. *Photosynthesis Research* **67**, 17–25.
- Mazza C.A., Boccacchio H.E., Giordano C.V., Battista D., Scopel A.L. & Ballaré C.L. (2000) Functional significance and induction by solar radiation of ultraviolet-absorbing sunscreens in field-grown soybean crops. *Plant Physiology* **122**, 117–125.
- McClung C.R. (2001) Circadian rhythms in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 139–162.
- McKenzie R., Smale D. & Kotkamp M. (2004) Relationship between UVB and erythemally weighted radiation. *Photochemical & Photobiological Sciences* **3**, 252–256.
- McKinlay A. & Diffey B. (1987) A reference action spectrum for ultraviolet induced erythema in human skin. *CIE Journal* **6**, 17–22.
- Mewis I., Schreiner M., Chau N.N., Krumbein A., Ulrichs C., Lohse M. & Zrenner R. (2012) UV-B irradiation changes specifically the secondary metabolite profile in broccoli sprouts: induced signaling overlaps with defense response to biotic stressors. *Plant and Cell Physiology* **53**, 1546–1560.
- Morales L.O., Brosché M., Vainonen J., Jenkins G.I., Wargent J.J., Sipari N., ... Aphalo P.J. (2013) Multiple roles for UV RESISTANCE LOCUS 8 in regulating gene expression and metabolite accumulation in *Arabidopsis* under solar ultraviolet radiation. *Plant Physiology* **161**, 744–759.
- Neugart S., Zietz M., Schreiner M., Rohn S., Kroh L.W. & Krumbein A. (2012) Structurally different flavonol glycosides and hydroxycinnamic acid derivatives respond differently to moderate UV-B radiation exposure. *Physiologia Plantarum* **145**, 582–593.
- Pereira O.R., Silva A.M.S., Domingues M.R.M. & Cardoso S.M. (2012) Identification of phenolic constituents of *Cytisus multiflorus*. *Food Chemistry* **131**, 652–659.
- Peter H.J., Krüger-Alef C., Knogge W., Brinkmann K. & Weissenböck G. (1991) Diurnal periodicity of chalcone-synthase activity during the development of oat primary leaves. *Planta* **183**, 409–415.
- Randriamanana T.R., Nissinen K., Moilanen J., Nybakken L. & Julkunen-Titto R. (2015) Long-term UV-B and temperature enhancements suggest that females of *Salix myrsinifolia* plants are more tolerant to UV-B than males. *Environmental and Experimental Botany* **109**, 296–305.
- Ringli C., Bigler L., Kuhn B.M., Leiber R.M., Diet A., Santelia D., ... Klein M. (2008) The modified flavonol glycosylation profile in the *Arabidopsis rol1* mutants results in alterations in plant growth and cell shape formation. *Plant Cell* **20**, 1470–1481.
- Rizzini L., Favory J.-J., Cloix C., Faggionato D., O'Hara A., Kaiserli E., ... Ulm R. (2011) Perception of UV-B by the *Arabidopsis* UVR8 protein. *Science* **332**, 103–106.

- Ryel R.J., Flint S.D. & Barnes P.W. (2010) Solar UV-B radiation and global dimming: effects on plant growth and UV-shielding. In *UV Radiation in Global Climate Change. Measurements, Modeling and Effects on Ecosystems* (eds W. Gao, D.L. Schmoldt & J. Slusser), pp. 370–394. Springer, New York.
- Schnitzler J.P., Jungblut T.P., Heller W., Kofferlein M., Hutzler P., Heinzmann U., ... Sandermann H. (1996) Tissue localization of UV-B-screening pigments and of chalcone synthase mRNA in needles of Scots pine seedlings. *New Phytologist* **132**, 247–258.
- Schreiner M., Mewis I., Huyskens-Keil S., Jansen M.A.K., Zrenner R., Winkler J.B., ... Krumbein A. (2012) UV-B-induced secondary plant metabolites – potential benefits for plant and human health. *Critical Reviews in Plant Sciences* **31**, 229–240.
- Searles P.S., Flint S.D. & Caldwell M.M. (2001) A meta-analysis of plant field studies simulating stratospheric ozone depletion. *Oecologia* **127**, 1–10.
- Siipola S.M., Kotilainen T., Sipari N., Morales L.O., Lindfors A.V., Robson T.M. & Aphalo P.J. (2015) Epidermal UV-A absorbance and whole-leaf flavonoid composition in pea respond more to solar blue light than to solar UV radiation. *Plant, Cell & Environment* **38**, 941–952.
- Snell K.R.S., Kokubun T., Griffiths H., Convey P., Hodgson D.A. & Newsham K.K. (2009) Quantifying the metabolic cost to an Antarctic liverwort of responding to an abrupt increase in UVB radiation exposure. *Global Change Biology* **15**, 2563–2573.
- Taira H., Aoki S., Yamanoha B. & Taguchi S. (2004) Daily variation in cellular content of UV-absorbing compounds mycosporine-like amino acids in the marine dinoflagellate *Scrippsiella sweeneyae*. *Journal of Photochemistry and Photobiology B: Biology* **75**, 145–155.
- Turnbull T.L., Barlow A.M. & Adams M.A. (2013) Photosynthetic benefits of ultraviolet-A to *Pimelea ligustrina*, a woody shrub of sub-alpine Australia. *Oecologia* **173**, 375–385.
- Veit M., Bilger T., Muhlbauer T., Brummet W. & Winter K. (1996) Diurnal changes in flavonoids. *Journal of Plant Physiology* **148**, 478–482.
- Wargent J.J., Elfadly E.M., Moore J.P. & Paul N.D. (2011) Increased exposure to UV-B radiation during early development leads to enhanced photoprotection and improved long-term performance in *Lactuca sativa*. *Plant, Cell & Environment* **34**, 1401–1413.
- Wargent J.J. & Jordan B.R. (2013) From ozone depletion to agriculture: understanding the role of UV radiation in sustainable crop production. *New Phytologist* **197**, 1058–1076.
- Wargent J.J., Nelson B.C.W., McGhie T.K. & Barnes P.W. (2015) Acclimation to UV-B radiation and visible light in *Lactuca sativa* involves up-regulation of photosynthetic performance and orchestration of metabolome-wide responses. *Plant, Cell & Environment* **38**, 929–940.
- Zavala J.A., Mazza C.A., Dillon F.M., Chludil H.D. & Ballaré C.L. (2015) Soybean resistance to stink bugs (*Nezara viridula* and *Piezodorus guildinii*) increases with exposure to solar UV-B radiation and correlates with isoflavonoid content in pods under field conditions. *Plant, Cell & Environment* **38**, 920–928.

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SUPPORTING INFORMATION

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Figure S1. Relationships between mean adaxial epidermal UV transmittance (T_{UV}) and whole leaf UV-absorbing compounds measured at 305 and 330 nm for the okra (*Abelmoschus esculentus*) and tomato (*Solanum lycopersicum*) leaves shown in Fig. 5. Data are means \pm SE (N=14–15). Equations and lines are shown only for significant ($P < 0.05$) regression models of Absorbance vs. $\ln(T_{UV})$.

Figure S2. Molecular structures, absorption spectra and absorption peaks for the four quercetin derivatives isolated by UPLC-UV-MS from okra (*Abelmoschus esculentus*) leaves. Letters correspond to the panels in Fig. 6; mAU = milli absorption units.