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ORIGINAL ARTICLE

Dynamics of flavonol accumulation in leaf tissues under different UV-B regimes in *Centella asiatica* (Apiaceae)

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Abstract

Main conclusion **A cumulative effect of UV-B doses on epidermal flavonol accumulation was observed during the first week of a time course study in *Centella asiatica* (Apiaceae). However, once flavonol levels had peaked, additional accumulation was possible only if higher daily UV-B irradiances were applied.**

We aimed to understand the dynamics of flavonol accumulation in leaf tissues using non-destructive spectroscopy and HPLC–mass spectrometry. When leaves that had grown without UV-B were given brief daily exposures to low-irradiance UV-B, they accumulated flavonols, predominantly kaempferol-*3-O-β*-D-glucuronopyranoside and quercetin-*3-O-β*-D-glucuronopyranoside, in their exposed epidermis, reaching a plateau after 7 days. More prolonged UV-B exposures or higher doses eventually augmented flavonol concentrations even in non-exposed tissues. If UV-B irradiance was subsequently reduced, leaves appeared to lose their ability to accumulate further flavonols in their epidermis even if the duration of daily exposure was increased. A higher irradiance level was then necessary to further increase flavonol accumulation. When subsequently acclimated to higher UV-B irradiances, mature leaves accumulated less flavonols than did developing ones. Our study suggests that levels of epidermal flavonols in leaves are governed primarily by UV-B irradiance rather than by duration of exposure.

Keywords Biospectroscopy · *Centella asiatica* · Dualex · Light acclimation · Polyphenols · UV-B absorption

Abbreviations

Amu	Atomic mass unit
AU	Absorbance units
ChlF	Chlorophyll *a* fluorescence
ChlF(ex_{375}), (ex_{655}),	Chlorophyll *a* fluorescence induced by 375 nm or by 655 nm excitation wavelength

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COP1	*CONSTITUTIVELY PHOTOMORPHOGENIC1* gene
DPBA	Diphenyl boric acid-2-aminoethyl ester
DA_{375}	Dualex absorbance at 375 nm
LC–MS	Liquid chromatography–mass spectrometry
PAR	Photosynthetically active radiation
ROS	Reactive oxygen species
SIR	Single-ion record
RUP1	REPRESSOR OF UV-B PHOTOMORPHOGENESIS1 protein
RUP2	REPRESSOR OF UV-B PHOTOMORPHOGENESIS2 protein
UVR8	*UV RESISTANCE LOCUS8* gene
UV-A	Ultraviolet-A radiation (315–400 nm)
UV-B	Ultraviolet-B radiation (280–315 nm)
SAA	Systemic acquired acclimation

Introduction

In a wide range of plant species, the accumulation of flavonoids within epidermal cell layers is inducible by light, especially UV-B radiation (Caldwell 1971; Caldwell et al. 1983; Barnes et al. 2000; Solovchenko 2010). A linear relationship has been found between the intensity of UV-B supplied and the extent of flavonoid accumulation, both in cell suspensions (e.g. parsley, Wellmann 1983; Schmelzer et al. 1988), and in intact plants under controlled conditions (e.g. *Arabidopsis*, Lois 1994). UV-B radiation between 290 and 300 nm appears to be the most effective for flavonoid induction; at increasingly longer wavelengths between 300 and 320 nm the effect on flavonoid levels declines exponentially. The action spectra have been well studied for flavonoid and isoflavonoid biosynthesis in *Petroselinum hortense* and *Phaseolus vulgaris* L., respectively (Beggs and Wellman 1994). Moreover, when UV-B induction is followed by a blue light period, flavonoid accumulation increases. UV-B radiation apparently acts synergistically with visible wavelengths (Fuglevand et al. 1996), and in few cases with UV-A radiation (Wang et al. 2012), in this response.

It has been shown for many species that under full sunlight, maximum levels of epidermal flavonoids are attained only after 4–5 days (Kolb et al. 2003; Krause et al. 2003; Cartelat et al. 2005; Bidel et al. 2007; Barnes et al. 2013; Latouche et al. 2013; Louis et al. 2009). The mechanistic reasons for this delay are not entirely understood, but likely relate both to environmental variables such as light, temperature, and abiotic stressors, as well as to the prior conditions under which the plants had acclimated. There is, however, a dearth of information on the dose–response effects of UV-B radiation on flavonoid accumulation in epidermal cells, and many questions remain. For example, are flavonoid concentrations simply a function of the product of UV-B intensity and duration of exposure (i.e. dose)? Does the epidermis continue to accumulate flavonoids if UV-B irradiance is gradually reduced? Does the epidermis retain its sensitivity to UV-B irrespective of the amounts of flavonoids previously accumulated in tissues? And are there kinetic limits to the degree to which flavonoids might accumulate in plants?

This study, therefore, aimed to follow the dynamics of flavonol accumulation in the leaf epidermis. For this we used *Centella asiatica* (Apiaceae), a tropical eudicot widely used in traditional Ayurvedic medicine in Asia (James and Dubery 2009) and eaten by humans as a fresh salad in both Madagascar and Malaysia (Ali 2008). We compare the effects on flavonol levels of both the cumulative duration of exposure to UV-B radiation, and of the UV-B intensity itself. Our data are discussed in relation to recent advances in our understanding of the physiological and genetic mechanisms that control the flavonoid biosynthesis, including the UVR8 receptor that regulates the transcription of a set of target genes mediating UV-B photomorphogenic responses and flavonoid gene induction (Ulm et al. 2004; Brown et al. 2005; Brown and Jenkins 2008; Stracke et al. 2010). When flavonoid accumulates within the epidermis, we might postulate that the UV-B radiation might be too low to activate the UVR8 photoreceptor. Interestingly, any UVR8–COP1 protein complex previously formed would be degraded, the inactive constitutive dimeric UVR8 form becoming predominant. However, since UVR8 is apparently highly sensitive to low UV-B irradiance (Hectors et al. 2007; Singh et al. 2014), even small increases in irradiance might re-activate UVR8 and thereby induce additional flavonol accumulation. It is well-known that flavonoid biosynthesis is mainly controlled at the transcriptional level by genes coding key enzymes such as phenylalanine ammonia-lyase and chalcone synthase (Tevini and Teramura 1989; El Euch et al. 1998; Beritognolo et al. 2001). At low UV-B irradiance, it was recently found that a UVR8 protein regulates the transcription of a set of target genes, such as HY5 and its homolog HYH, two transcription factors mediating UV-B photomorphogenic responses and flavonoid gene induction (Ulm et al. 2004; Brown et al. 2005; Brown and Jenkins 2008; Stracke et al. 2010). Indeed, the UVR8 dimer protein

produces two monomers under UV-B irradiation, which initiate a signalling pathway by a specific interaction with COP1, an E3 ubiquitin ligase (Oravecz et al. 2006). Then, it is regenerated into its inactive dimer form in darkness conditions (Cloix et al. 2012). Therefore, the flavonols accumulating in the epidermis could modulate the function of UVR8 through the quantity of photons that can be absorbed by the dimer protein receptor in cells. In addition, the two WD40-repeat proteins RUP1 and RUP2 act downstream of UVR8–COP1 in a negative feedback loop when they impinge on UVR8 (Gruber et al. 2010). Therefore, a rapid increase in RUP1 and RUP2 abundance seems necessary to prevent overstimulation of secondary metabolism pathway when seedlings are exposed to UV-B radiation. RUP1 and RUP2 could be overexpressed when flavonoids increase in epidermal cells promoting the inactive UVR8 dimer form.

Elevated levels of UV-B-induced flavonoids are maintained for several weeks in the leaves of *C. asiatica* (Müller et al. 2013a). A more comprehensive understanding of the dynamics of flavonoid accumulation in the leaf epidermis may help us to develop new cultural systems conferring to fruits, salads, other vegetables new nutritive values (antioxidant, anti-inflammatory, biostimulant) such as higher concentrations of secondary metabolites (Gould et al. 2008; Poiroux-Gonord et al. 2010; Schreiner et al. 2012).

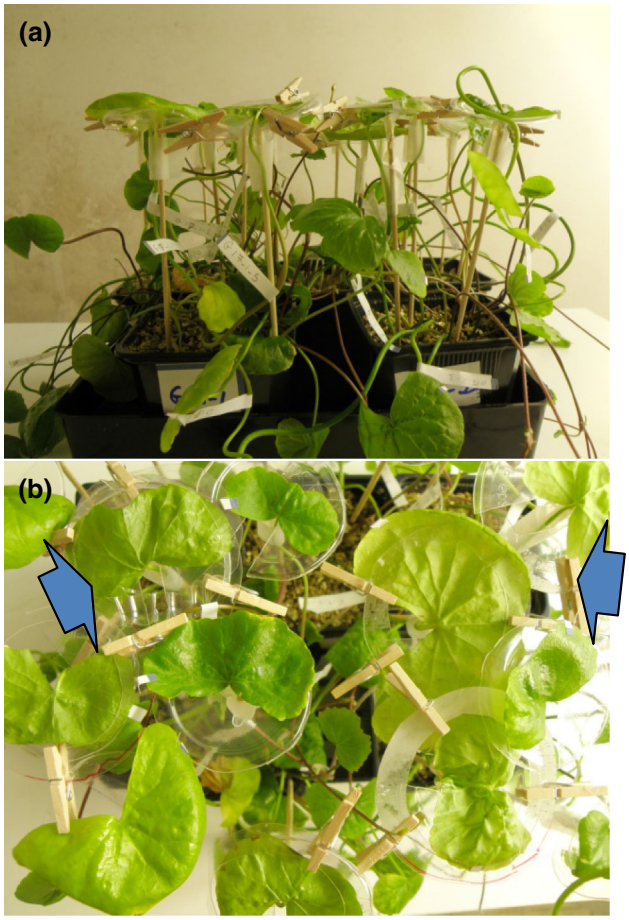

Fig. 1 Clone of *Centella asiatica* grown in pots under various light conditions. **a** Leaves were maintained horizontally using plastic clips. Some of the leaves, indicated by *arrows* in **b**, were inverted, exposing the abaxial surface to the UV-B lamp

Materials and methods

Plant materials

Wild *Centella asiatica* (L.) Urban plants growing in Madagascar were cloned by vegetative propagation in individual pots. A single clone was cultivated in a growth chamber and in the field at Montpellier, France (43°36′39″N, 3°52′38″E). A first set of plants was maintained in a growth chamber under a 12-h photoperiod, 24–25 °C day, 19 °C night and 70–80 % air humidity. A second set was exposed periodically to 312-nm UV-B lamps (30 W Uvitec, Cambridge, UK) in a second growth chamber ("UV-induction growth chamber") and returned to the first growth chamber after each treatment. A third set of 3-month-old plants was transferred outdoors in full sunlight. Only 2-month-old leaves were studied. For each treatment, two fully expanded leaves from six plants were maintained horizontally at a constant distance from the light sources (Fig. 1). To assess the responses of abaxial epidermises to additional UV-B radiation, some leaves were inverted such that their lower (abaxial) epidermis was uppermost. When needed, one half of the leaves was protected from UV-B radiation by two layers of 100-µm-thick cellulose diacetate filter (Rhodoid®) from Rhone-Poulenc (Lyon, France).

Epidermal absorbance measurement

Absorbance at 375 nm (DA_{375}) of the adaxial and the abaxial leaf epidermises was measured non-destructively using a Dualex® FLAV 3.3 leaf-clip (Force-A, Orsay, France). The Dualex measures the ratio of chlorophyll *a* fluorescence (ChlF) emitted at >715 nm when excited at 375 nm (where flavonoids strongly absorb the excitation beam) to that excited at 655 nm, where flavonoid absorbance is negligible (Goulas et al. 2004; Louis et al. 2009):

$$DA_{375} = -\mathrm{Log}_{10}(\mathrm{ChlF}(ex_{375})/\mathrm{ChlF}(ex_{655})) \quad (1)$$

The Dualex also records a chlorophyll transmittance index, which is proportional to chlorophyll content (Cerovic et al. 2012). We recorded the mean of four independent 20 mm^2 acquisitions on each leaf.

LC–MS characterization of leaf tissues

For each set of plants, about 100 mm^2 of abaxial epidermis was removed and immediately extracted in methanol/water (70/30, v/v) acidified with 0.1 % HCl. Interveinal mesophyll located immediately beneath the epidermis was then harvested and similarly extracted. Finally, adaxial epidermis adjacent to this zone was extracted. The surface areas of collected tissues were estimated from images taken with a dissecting microscope. 5-*O*-methoxyflavone, not synthesized by this plant species, was added to each extract as an internal standard. Samples were frozen in liquid nitrogen, sonicated at 0 °C for 10 min and then stored at −20 °C. After thawing and clarification by centrifugation, 20 µL of the supernatant solution was injected into LC–MS.

Chromatographic separation was performed on XTerra MS C_{18} column (3.5 µm particle size, 2.1 × 100 mm) at 40 °C (Gecko 2000, Cluzeau Info Labo, France). A binary mobile phase gradient was delivered at a total flow rate of 210 µL min^{-1} using a binary HPLC pump (Waters 1525µ, Waters, Manchester, UK). It was composed of water (solvent A) and acetonitrile (solvent B), both acidified with 0.1 % (v/v) formic acid to minimize the ionization of phenolics (around pH 3.0). A Micromass ZQ ESCi multimode ionization mass spectrometer (Micromass Ltd, Manchester, UK) equipped with an electrospray ionization ion source (ZSpray MKII) was used. Source and capillary were heated at 90 and 450 °C, respectively. The capillary voltage was set to 2.5 kV. Nitrogen was used as the desolation gas (400 L h^{-1}) and cone gas (50 L h^{-1}). In a first step, spectra were recorded in the full scan mode over the *m/z* 50–1200 range, both in negative and positive modes. In a second step, single-ion records (SIRs) for kaempferol-*3-O-β*-D-glucuronopyranoside (*m/z* 461 $[M–H]^-$), quercetin-*3-O-β*-D-glucuronopyranoside (*m/z* 477 $[M–H]^-$) and caffeoylquinic acid isomers (*m/z* 353 $[M–H]^-$) were also taken. Absorbance spectra over the range of 210–800 nm were acquired using a Waters 996 photodiode array detector. Absorbance and mass spectra were handled using MassLynx 3.5 software (Micromass Ltd).

Flavonoid histological localization

Fresh leaves were sectioned transversely with a vibrating microtome (VT 1000S, Leica, Nanterre, France) according to the procedure of Mondolot et al. (2006). For the same leaf, successive cross sections were immersed in pure water or in aqueous solutions of diphenyl boric acid-2-aminoethyl ester (DPBA) to compare tissue auto-fluorescence and flavonoid fluorescence enhanced by complexation to DPBA (Hutzler et al. 1998). Sections were observed under epi-fluorescence in an Optiphot-200 microscope (Nikon filter set UV-1A: 365 nm excitation filter, 400-nm barrier filter; and filter set B-2A: 450–490-nm excitation filter, 520-nm barrier filter), with 10× and 40× Pan Fluor Nikon objectives. Images were recorded with a digital Nikon Coolpix 4500 camera.

Light treatments and irradiation measurements

UV irradiance was measured using a PMA2106 UV-B sensor and a PMA2110 UV-A sensor from Solar Light Co. (Glenside, PA, USA). Photosynthetically Active Radiation (PAR) was measured using a PAR JYP100 sensor from SDEC (Reignac sur Indre, France). In the growth chamber, leaves were exposed to white light at 250 µmol photons m^{-2} s^{-1} delivered by sodium halide lamps (Table 1). The light lacked UV-B radiation (<3 µW cm^{-2}) and had little UV-A radiation (300 mW cm^{-2}).

Light transitions on growth chamber plants

In experiment 1, six plants were exposed to 100 µW cm^{-2} UV-B for 6 h. Epidermal absorbances were compared to those for plants not exposed to UV-B (control I), and to additional leaves on UV-B exposed plants that had been protected by Rhodoid filters (control II). The Rhodoid filters prevented less than 3 µW cm^{-2} UV-B to be transmitted. Measurements of Dualex absorbance at 375 nm (DA_{375}) were taken every second day for 30 days (Fig. 2).

Plants that had acclimated outdoors to shade were transferred to full sunlight (Fig. 3). In the shade, the adaxial epidermises of horizontal leaves received at solar noon maximum irradiances of 30 and 430 µW cm^{-2} UV-B and UV-A, respectively (Table 1). Under full sunlight they received a maximum of 210 and 4950 µW cm^{-2} UV-B and UV-A, respectively. When leaves had received a cumulative UV-B dose of 17 kJ m^{-2}, plants were returned to the shade. The control plants maintained in the shade received at the same time a UV-B dose of 3.2 kJ m^{-2}. Inverted leaves with the abaxial epidermises exposed to direct sunlight were given the same treatments.

Experiment 2 was designed to compare the effects of UV-B intensity versus UV-B dose. The same daily UV-B dose was applied at three irradiances by reducing the distance between the lamp and leaves, and by varying the duration of exposure. Each light treatment was given to sets of 6 plants, from which 12 mature horizontally oriented leaves were measured (Fig. 1). One set of six plants was held in a growth chamber without UV-B (first control). A second set was transferred to the UV-induction growth chamber, but not exposed to UV-B (second control for potential differences in chamber microclimate). Four sets of UV-B-free grown plants were subjected to 12 h per day of UV-B at 50 µW cm^{-2} for 4 days. Another four sets of six plants received 6 h per day 100 µW cm^{-2}, for 1–4 days

Table 1 Maximum irradiance in UV and PAR as intercepted by leaves outdoors and in the growth chamber

	UV-B [280–315 nm], μW cm^{-2}	UV-A [315–400 nm], μW cm^{-2}	PAR [400–800 nm], μmol m^{-2} s^{-1}
Sunniest August day at solar noon			
Towards solar zenith	220	5345	2150
Flux in north direction (45° elevation)[a]	125	2060	750
Downward flux	210	4950	2030
Downward flux under Rhodoid	>3	4250	2002
Flux reflected upward	5	115	195
Shaded environment under leaves			
Flux in solar zenith direction	40	680	655
Flux in north direction (45° elevation)[a]	50	560	680
Downward flux	30	430	650
Flux reflected upward	3	15	70
In growth chamber, at leaf level under sodium halide lamp			
Downward flux	>3	50	250
Downward flux under Rhodoid	>3	43	247
Flux reflected upward	>3	3	55

[a] Measurements taken due North at 45° elevation angle of incidence

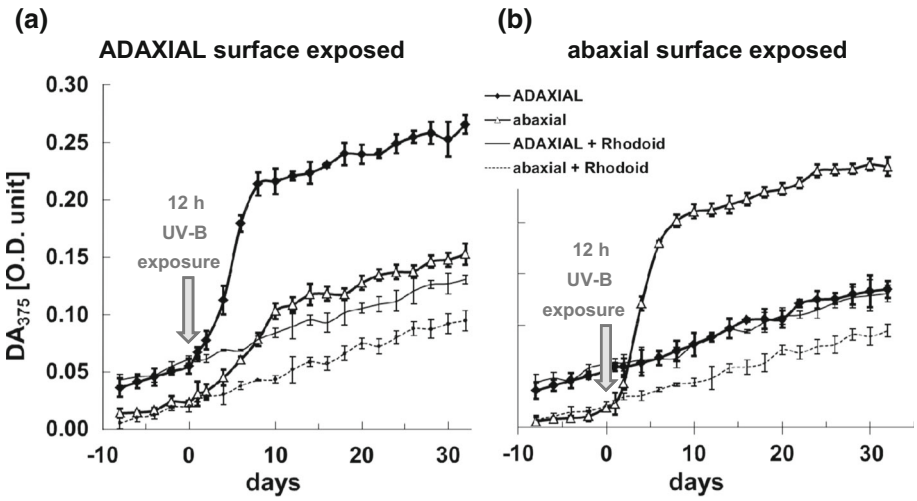

Fig. 2 Kinetics of epidermal absorbance at 375 nm (DA_{375}) for *Centella asiatica* leaves grown without UV-B and then irradiated with 50 μW cm^{-2} of UV-B for 12 h (see *arrows*). This was a daily dose of 2.16 kJ m^{-2}. Half of each leaf was covered by a UV-B-screening Rhodoïd filter. **a** Adaxial surface exposed to UV-B. **b** Abaxial surface of inverted leaf after UV exposure

each. Finally, four sets of six plants received 4 h per day 150 μW cm^{-2} for 4 days. The irradiance of 50 μW cm^{-2} UV-B corresponds to the highest intensity intercepted by shaded leaves outside at solar noon under a clear sky in summer (Table 1); 100 μW cm^{-2} is little lower than the diffuse UV-B intercepted by north-facing leaves under the same conditions. The 150 μW cm^{-2} treatment corresponds to 70 % of the maximal UV-B intercepted by horizontal leaves facing the sun at solar noon. Leaf DA_{375} readings were taken twice before the UV-B treatments to verify epidermal absorbance stability, and also 7 days after the last UV-B dose to account for possible effects of plant disturbance and wounding. Leaves had reached their plateau value then.

Experiment 3 was designed to study leaf acclimation to different UV-B intensities. Three sets of six UV-B-free grown plants were acclimated for 21 days to 6 h per day UV-B at 100 μW cm^{-2}. Thereafter, plants were exposed for 21 days to 6 h per day UV-B at 80, 100 or 120 μW cm^{-2}.

Light treatments to outdoor-grown plants

Leaves of outdoor-grown plants experienced between 10 and 220 μW cm^{-2} UV-B, 500 and 1500 mW cm^{-2} UV-A, and from 400 to 2200 μmol m^{-2} s^{-1} PAR at noon, depending on cloud cover (Table 1). Three sets of these plants with 2-month-old leaves were transferred to growth

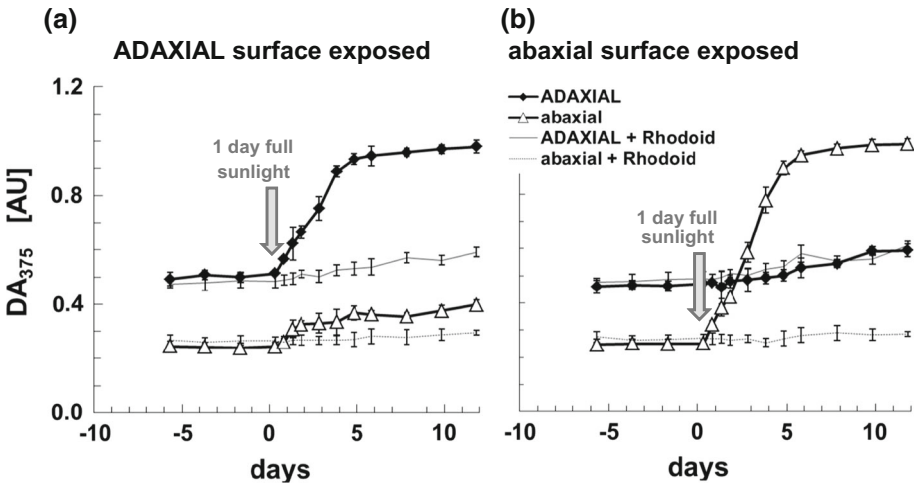

Fig. 3 Kinetics of epidermal absorbance at 375 nm (DA_{375}) of *Centella asiatica* leaves grown outdoors in the shade and then exposed to full sunlight for a single day. Maximum UV-B irradiance was 210 μW cm^{-2} at solar noon, and the daily UV-B dose was 15.3 kJ m^{-2} (see *arrows*). Half of each horizontally held leaf was covered by two UV-screening Rhodoid filters. **a** Adaxial surface exposed to UV-B radiation. **b** Abaxial surface of inverted leaf after UV-B exposure

chambers. One set was maintained under UV-B-free conditions, the second exposed for 6 h per day to 100 μW cm^{-2} UV-B for 15 days, and the third set exposed for 4 h to 150 μW cm^{-2} UV-B for 15 days.

Statistics

Differences between means were assessed by one-way analysis of variance (ANOVA) performed with Statistica 6.1 (StatSoft Inc. Maisons-Alfort, France). Error bars correspond to the mean DA_{375} value ± standard deviation (SD) of 12 leaves in the set of six plants. Bars accompanied by different letters are significantly different at $P < 0.05$, based on a least significant difference of the Fisher's test.

Chemicals and standards

Methanol and acetonitrile from Carlo Erba (Val de Reuil, France) were HPLC-grade quality. Formic acid from Merck (Darmstadt, Germany) was of analytical grade quality. Permuted water was produced using a reverse osmosis Milli-Q system from Millipore (Molsheim, France). Kaempferol-*3-O-β*-D-glucuronopyranoside, kaempferol-*3-O-β*-D-glucopyranoside, quercetin-*3-O-β*-D-glucuronopyranoside, quercetin-*3-O-β*-D-glucopyranoside were purchased from Extrasynthèse (Genay, France). The 5-*O*-metoxyflavone and diphenyl boric acid-2-aminoethyl ester were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France).

Results

Steady state of epidermal absorbance of control leaves

For plants grown in a UV-B-free environment under low PAR and with optimal ferti-irrigation, both the adaxial and abaxial epidermises increased in absorbance at 375 nm (DA_{375}) by about 0.002 AU per day, reaching 0.040 ± 0.03 and 0.015 ± 0.03 AU, respectively, after 60 days (Fig. 2a). These values correspond to 4.1 and 1.5 nmol cm^{-2} of quercetin-*3-O*-glucoside equivalents, respectively. For the inverted leaves, there was no induction of flavonols in the adaxial epidermis, which responded in the same way as adaxial epidermis protected by the Rhodoid filter (control II) and like the adaxial epidermis of leaves from plants grown without UV-B (control I; Fig. 2b).

Kinetics of changes in epidermal absorbance induced by a single UV-B dose

An increase in adaxial DA_{375} in exposed leaves was first recorded 2 days after UV-B exposure (12 h at 50 μW cm^{-2}, 2.16 kJ m^{-2}); levels peaked after 7–8 days. DA_{375} of the directly exposed adaxial epidermis doubled in 4 days whereas the abaxial surface increased much more slowly (Fig. 2a). When one half of the leaf was protected by a Rhodoid UV-B-screening filter, only the exposed leaf-half responded to the UV-B treatment. Epidermal absorbance in the protected leaf-half (control II) increased uniformly at a rate of 0.002 AU per day, comparable to those of non-exposed plants (control I, data not shown). When leaf laminae were inverted, their abaxial epidermis responded to a single UV-B dose in a similar way (Fig. 2b). Rates of DA_{375} increase were similar (0.04 AU per day, corresponding to 4.1 nmol cm^{-2} of quercetin-*3-O*-glucoside equivalents), as were the kinetics and maximum values, notwithstanding the lower initial value of DA_{375}.

Responses of shade leaves to a single-day exposure to full sunlight

Plants acclimated for 3 months outdoors in the shade produced mature leaves with significantly higher adaxial

and abaxial DA_{375} values (0.48 ± 0.08 and 0.26 ± 0.05 AU, respectively; *t* test, $P < 0.01$) compared with leaves grown in a growth chamber without UV-B and with low UV-A and PAR (Fig. 3a). Differences between adaxial and abaxial values were much larger (0.25 AU, corresponding to 25.8 nmol cm^{-2} of quercetin-*3-O*-glucoside equivalents) than those for growth chamber-grown leaves (0.05 AU). When plants were transferred to full sunlight for 1 day only, experiencing 210 μW cm^{-2} maximum UV-B irradiance and a daily dose of 17 kJ m^{-2}, DA_{375} values in both epidermal surfaces increased with kinetics similar to those of leaves grown in growth chambers and exposed to a single UV-B dose. The maximum DA_{375} values were approximately fivefold greater than those of untreated leaves (Fig. 3b). Importantly, the abaxial epidermis when directly exposed to UV-B, although starting from a lower initial value, attained the same maxima after 7 days as reached by the exposed adaxial epidermis (1.02 ± 0.53 AU, corresponding to 105.2 nmol cm^{-2} of quercetin-*3-O*-glucoside equivalents). This maximum value was half of that present in plants grown continuously in full sunlight (cf. Fig. 11), but was fourfold larger than that for growth-room grown plants induced by UV-B (Fig. 2). An increase in DA_{375} could not be induced in the adaxial epidermis when oriented such that the abaxial epidermis was uppermost (Fig. 3b).

Two predominant flavonols contributed to epidermal DA_{375}

Chromatograms of hydro-methanolic extracts revealed that only two major compounds were absorbing at 375 nm (Fig. 4c). The two peaks with retention times of 16.80 and 19.10 min had mass-to-charge ratios (*m/z*) of 477.0 and 461.0 $[M–H]^-$, respectively (Fig. 4d). They shared typical flavonoid absorbance spectra (Fig. 5a). Peak 1 fragmented with *m/z* 301, 179, 151 $[M–H]^-$ (Fig. 5b). Peak 2 fragmented with *m/z* 285 $[M–H]^-$, indicating a 176 amu loss of a glucuronopyranosyl unit (Fig. 5c). Peak 1 co-eluted with quercetin-*3-O-β*-D-glucuronopyranoside (i.e. miquelianin), shared identical absorbance spectra with maxima at 255.1 and 351.1 nm, and had similar pseudo-molecular fragments in the same proportions. Peak 2 co-eluted with kaempferol-*3-O-β*-D-glucuronopyranoside and had similar absorbance spectra (max at 264.1 and 341.1 nm) and fragmentation properties (Fig. 5c). These two flavonols have been

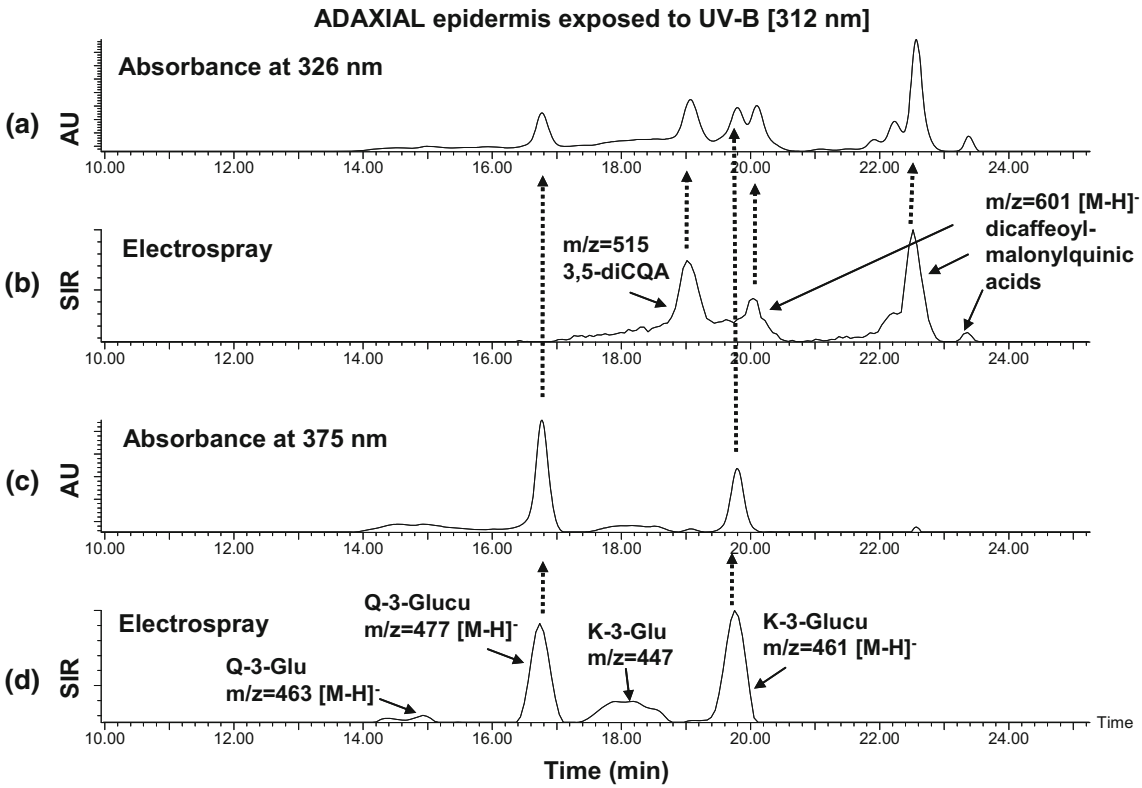

Fig. 4 LC–MS chromatograms of adaxial epidermis of a *Centella asiatica* leaf at 326 nm (**a**) and 375 nm (**c**) expressed in absorbance units (AU). The single-ion records (*SIR*) at $m/z = 353$ and 601 $[M–H]^-$ correspond to caffeoylquinic acid isomers and malonyl-dicaffeoylquinic isomers, respectively (**b**). SIR at $m/z = 477$ and $m/z = 461$ $[M–H]^-$ correspond to quercetin-*3-O-β*-D-glucuronopyranoside and kaempferol-*3-O-β*-D-glucuronopyranoside, respectively (**d**). SIRs are expressed in relative values (%). Caffeoylquinic acid derivatives are major epidermal absorbers at 326 nm, but they do not contribute to the absorbance at 375 nm

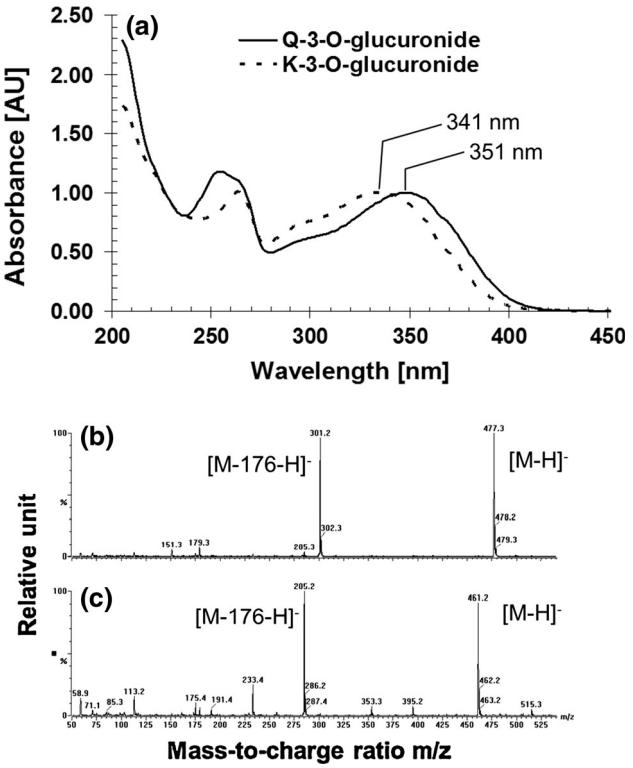

Fig. 5 Normalized absorbance spectra (**a**) and mass spectra for negative electrospray ionization of quercetin-*3-O-β*-D-glucuronopyranoside (**b**) and kaempferol-*3-O-β*-D-glucuronopyranoside (**c**). The loss of 176 amu corresponded to cleavage of the glucuronopyranoside fragment (**b**, **c**)

identified previously by Ali (2008) and Nhiem et al. (2011) for whole leaf extracts of *C. asiatica*. Under UV-B-free conditions (control I), these two flavonols did not accumulate at measurable amounts in the leaf mesophyll (Fig. 6), which provided a cross-validation of our sampling method, confirming that no epidermal cell was present in the mesophyll samples. Under UV-B-free conditions only kaempferol-*3-O-β*-D-glucuronopyranoside accumulated in the epidermises. After a single UV-B dose, the exposed epidermis accumulated larger amounts of kaempferol-*3-O-β*-D-glucuronopyranoside as well as quercetin-*3-O-β*-D-glucuronopyranoside (Fig. 6).

For the leaves grown under full sun, two additional flavonols contributed to epidermal absorbance at 375 nm, albeit at very low proportions (Fig. 4c). Quercetin-*3-O-β*-D-glucopyranoside (i.e. isoquercetin, *m/z* 463 $[M–H]^-$) and kaempferol-*3-O-β*-D-glucopyranoside (i.e. astragalin, *m/z* 447 $[M–H]^-$) were identified by comparison with authentic standards. They were previously characterized in *C. asiatica* leaves by Nhiem et al. (2011). These two latter compounds represented less than 4 % of DA_{375} (Fig. 4c). Their contribution to DA_{375} values was always less than 10 %. Castiliferol (i.e. kaempferol-*3-O-p*-coumarate, *m/z* 447 $[M–H]^-$) and castillicetin (quercetin-*3-O*-caffeate, *m/z* 463 $[M–H]^-$) described by Subban et al. (2008) in *C. asiatica* leaves were also found in epidermises, but only in trace amounts (less than 1 % of DA_{375}) irrespective of the light treatment given. For each UV-B treatment, the flavonol content measured by mass spectrometry was fivefold higher than DA_{375} values. They are consistent with those assessed by Dualex 3.3, which measured an area fivefold smaller (i.e. 20 mm^2).

Large amounts of caffeoylquinic acid derivatives, which also accumulated in epidermises, were observed in the 326-nm chromatograms (Fig. 4a, b). Since these did not contribute to the 375-nm chromatograms (Fig. 4c), they were not recorded in DA_{375} values. Thus, under our experimental conditions, the DA_{375} measurements on *Centella* leaves corresponded almost exclusively to the absorbance of the sum of two major flavonols, quercetin-*3-O-β*-D-glucuronopyranoside and kaempferol-*3-O-β*-D-glucuronopyranoside.

Cumulative effect of UV-B dose modulated by daily exposure duration or UV-B irradiance during time-lapse response

Ten days after a single-day exposure to UV-B in the growth chamber, DA_{375} readings surpassed their initial values and remained stable, even if a subsequent UV-B exposure was applied at the same intensity (data not shown). By contrast, if UV-B was supplied at the same intensity on consecutive days immediately after the first exposure, DA_{375} values increased (Figs. 7, 8). Final values of adaxial DA_{375} and, therefore, flavonol content were greater as the daily duration of exposure increased. They were also greater as the number of daily exposures to UV-B increased (Figs. 7, 8).

We observed that the maximum DA_{375} values were ultimately lower in leaves for which UV-B irradiance had been increased than in leaves that had developed under this higher UV-B irradiance (Fig. 9). Consistent with the DA_{375} measurements, transverse sections showed lower yellow fluorescence of DPBA–flavonol complexes within cell walls in both epidermal and mesophyll cells of these acclimated leaves compared to leaves grown under higher UV-B light from the beginning (data not shown). It is noteworthy that the plants which developed in the absence of UV-B were the more susceptible to necrosis. Brown necrotic patches appeared on the surface of exposed epidermises at 150 μW cm^{-2}. After four doses at 150 μW cm^{-2}, some leaves senesced or partially dried within a week after the end of the treatment. UV-B irradiance caused damage to *C. asiatica* leaves with DA_{375} lower than 0.50 AU. When applied to leaves grown

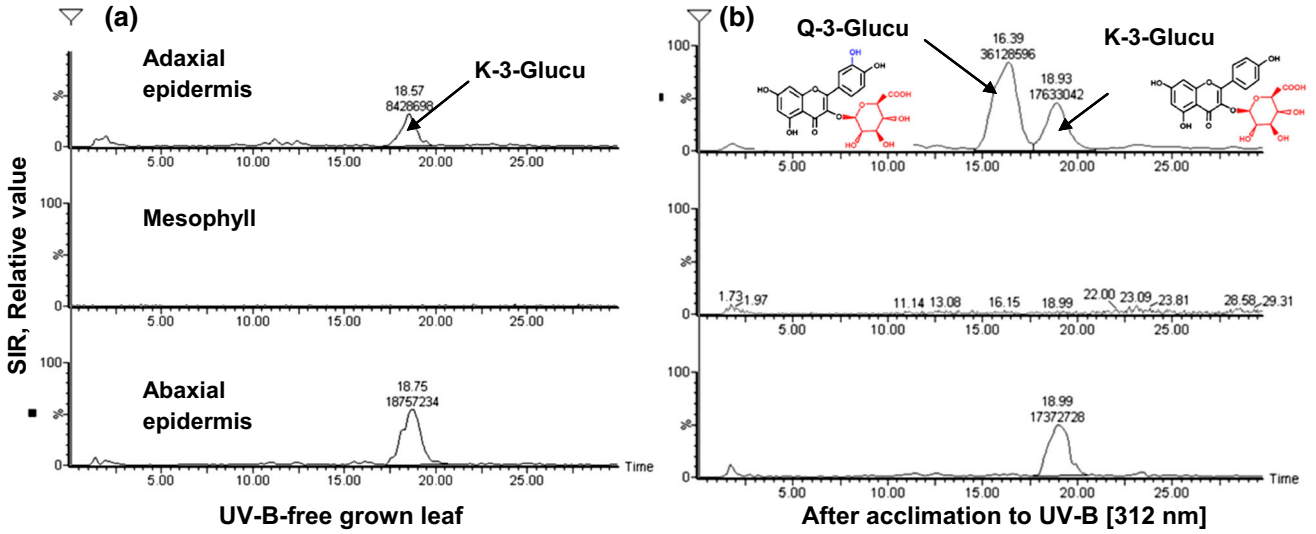

Fig. 6 Single-ion records (SIR) at $m/z = 477$ and 461 $[M–H]^-$ corresponding to quercetin-*3-O-β*-D-glucuronopyranoside and kaempferol-*3-O-β*-D-glucuronopyranoside, respectively, for a *Centella asiatica* leaf grown without UV-B radiation (**a**) and exposed to UV-B radiation for 12 h at 100 μW cm^{-2} (**b**)

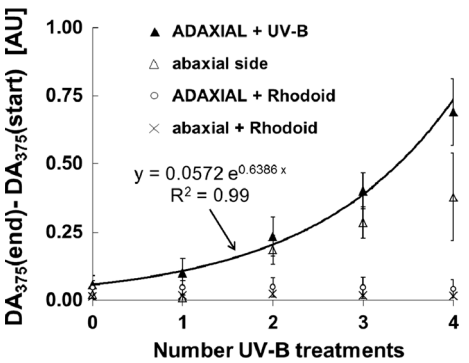

Fig. 7 Differences in leaf epidermal absorbance at 375 nm (DA_{375}) before and after leaves were given up to 4 daily 12 h doses of 2.16 kJ m^{-2} of UV-B radiation at 50 μW cm^{-2}. Measurements indicate the quantitative changes of major flavonols (Fig. 4). Points are mean DA_{375} values ± SD of 12 leaves recorded 7 days after the last UV-B treatment. Points accompanied by *different letters* are significantly different at $P < 0.05$ (Fisher's LSD)

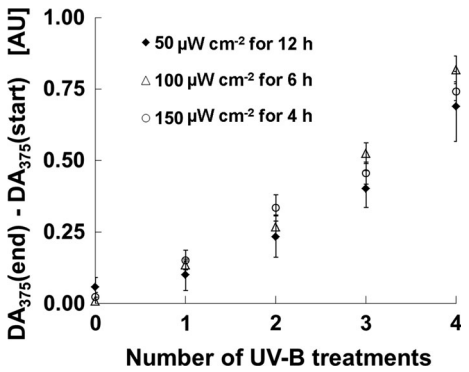

Fig. 8 Increase in adaxial epidermal absorbance at 375 nm (DA_{375}) for leaves given up to 4 daily doses of UV-B radiation, corresponding to 2.16 kJ m^{-2} for three different time-irradiances combinations. Measurements indicate the quantitative changes of major flavonols in epidermises (Fig. 4). Points are mean DA_{375} values ± SD for 12 leaves recorded 7 days after the last UV-B treatment

outdoors with adaxial DA_{375} higher than 1.00 AU, no necrosis was observed after even 15 days.

Non-cumulative effect of diminishing UV-B irradiance

After its initial acclimation in a growth chamber to 100 μW cm^{-2} UV-B, the adaxial epidermis reached a stable DA_{375} value. It retained the capacity to respond to higher irradiances, reaching a significantly higher mean value (0.67 ± 0.08 AU, *t* test, $P < 0.05$) when exposed to 120 μW cm^{-2} (Fig. 10). In contrast, if irradiance was lowered to 80 μW cm^{-2} UV-B, the adaxial DA_{375} (0.49 ± 0.07 AU) no longer increased irrespective of the duration of exposure. Similarly, when plants grown under full sunlight (maximum UV-B irradiance of 220 μW cm^{-2}) were transferred to the UV-B-induction growth chamber with only 150 μW cm^{-2}, adaxial DA_{375} slowly declined by 0.04 AU per week (4.1 nmol cm^{-2} per week of quercetin-3-*O*-glucoside equivalents). We conclude that when UV-B irradiance decreases, adaxial flavonol levels in exposed leaves cease to increase, irrespective of the number of daily UV-B doses given and independent of the duration of the irradiance.

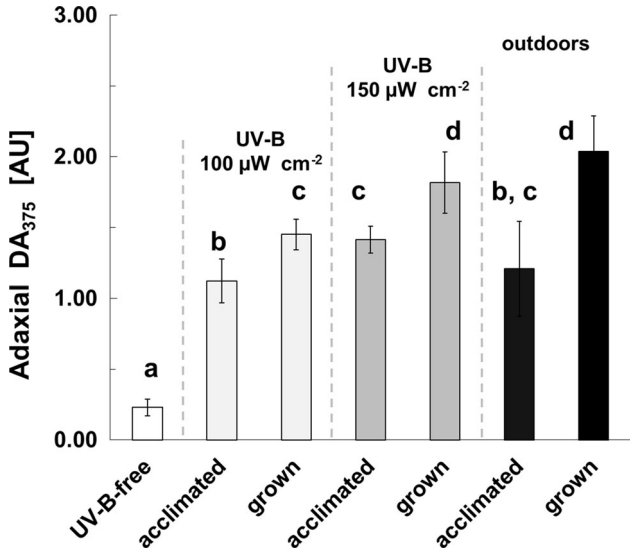

Fig. 9 Effect of UV-B irradiance intercepted during leaf growth phase (21 days) on adaxial epidermal absorbance at 375 nm (DA_{375}) of fully expended leaves measured at 42 days. Plants cultivated without UV-B were used for this experiment. Leaves grown in UV-B-free conditions (control I) were compared to leaves grown under 100 µW cm^{-2} UV-B for 21 days and then transferred when leaves reached their final size to 100, 150 and up to 210 µW cm^{-2} UV-B radiation for 21 days. Mature leaves "Acclimated" to the higher irradiance accumulated less flavonoids than leaves "grown" at this irradiance. *Bars* show mean DA_{375} values ± SD for 12 leaves. *Different letters* indicate significant differences at $P < 0.05$ (Fisher's LSD)

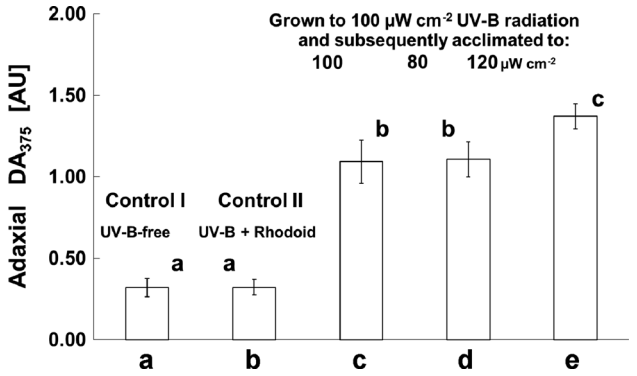

Fig. 10 Adaxial epidermal absorbance at 375 nm (DA_{375}) for leaves acclimated to different UV-B irradiances. Leaves were grown in a UV-B-free environment (**a**), under UV-B-screening Rhodoid filters (**b**), under daily doses of 2.16 kJ m^{-2} UV-B (6 h at 100 µW cm^{-2}) and then submitted to various UV-B irradiances (80, 100 and 120 µW cm^{-2}) for 21 days (**c**–**e**). *Bars* show mean DA_{375} values ± SD for 12 leaves recorded 7 days after the last UV-B treatment. *Bars* with *different letters* are significantly different at $P < 0.05$ (Fisher's LSD)

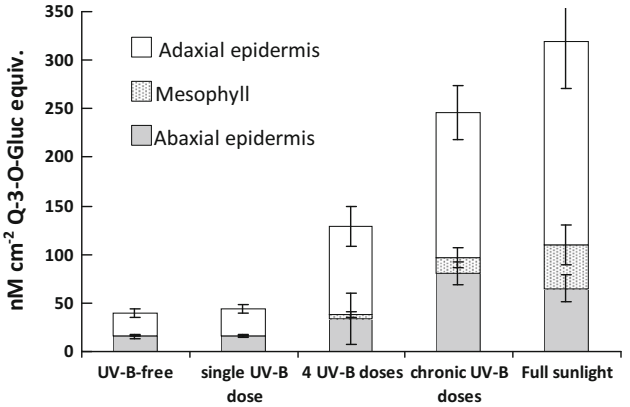

Fig. 11 Distribution of flavonol accumulation within adaxial epidermis, mesophyll and abaxial epidermis of leaves of *Centella asiatica*, (expressed in nmol quercetin-*3-O-β*-D-glucopyranoside equivalents for 100-mm^{-2} leaf areas). Leaves were grown in UV-B-free condition (**a**), exposed to a single UV-B dose (**b**), 4 doses (**c**), acclimated to chronic daily UV-B doses of 100 µW cm^{-2} UV-B for 12 h (**d**), and compared to leaves grown in full sunlight (**e**). Mean ± SD of three samples per leaf from three plants

Flavonol distribution within the leaf in reaction to UV-B irradiance level

A single low UV-B dose (2.16 kJ m^{-2}) at low UV-B irradiance induced the accumulation of flavonols in the exposed epidermis without any parallel increase in the mesophyll or opposite lamina surface (Figs. 2, 6). Under higher UV-B irradiances, the epidermis that was not directly exposed to UV-B also began to accumulate flavonols, albeit in lower amounts than the exposed epidermis (Fig. 7). When leaves were given 150 µW cm^{-2} UV-B irradiance every day, or when they were exposed to full sunlight, areas protected under the Rhodoid filter also started to accumulate flavonols, although in lower amounts than adjacent exposed regions. Mass spectrometry analyses revealed that the accumulation of flavonols in the mesophyll began in the growth chamber with chronic daily UV-B irradiance of 100 µW cm^{-2} (Fig. 11).

Microscopy and necrotic changes

Under our experimental conditions, when leaves were exposed to 100 µW cm^{-2} of UV-B for 6 h, infrequent necrotic spots appeared on the epidermises within 2 days. The chlorophyll index measured by Dualex FLAV 3.3 decreased significantly by 18 % relative to healthy tissue. Under a light microscope, brown tissue zones appeared within both epidermises and underlying cells, probably corresponding to the presence of oxidized compounds. Tissue structure was highly modified (Fig. 12c). Their leaf cells appeared less turgid and palisade parenchyma was less prominent. The palisade mesophyll of plants grown without UV-B showed blue fluorescence in vacuoles characteristic of hydroxycinnamic acids (UV-1A filter set) (Fig. 12a). These caffeoylquinic acid derivatives were also

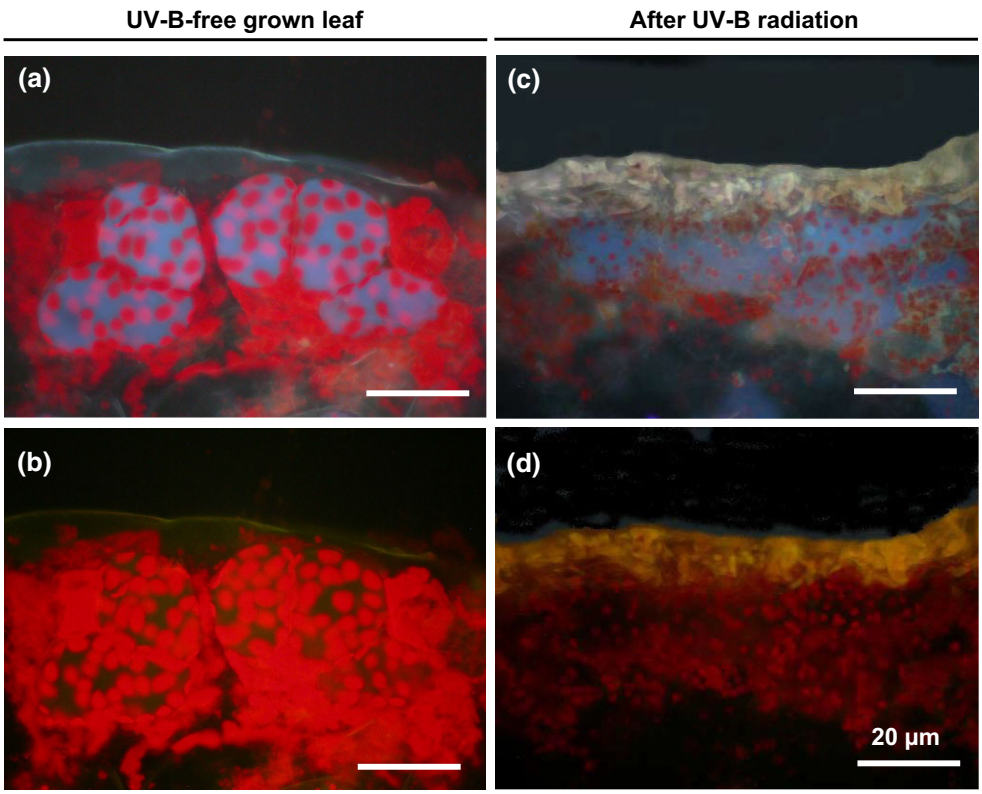

Fig. 12 Histological localization of major phenolics in transverse section of *Centella asiatica* leaf before (**a**, **b**) and 2 days after (**c**, **d**) exposure to UV-B radiation (6 h at 100 µW cm^{-2}). Caffeoylquinic acid esters fluoresced blue with the Nikon UV-1A filter set (**a**, **c**). Flavonols complexed to DPBA fluoresced yellow with the Nikon UV-1A filter set (**c**) and orange under the B-2A filter set (**b**, **d**). Red chlorophyll *a* fluorescence decreased in mesophyll after UV-B exposure

present after radiation with UV-B (Fig. 12c). Flavonoids were not visible microscopically in a UV-B-free grown leaf (Fig. 12a, b). Two days after UV-B treatment, we observed a thicker epidermis, grey-yellow fluorescence with UV-1A filter set (Fig. 12c), and yellow fluorescence with DPBA and the B-2A filter set (Fig. 12d), confirming the presence of high amount of flavonoids becoming bright yellow after DPBA complexation. Red auto-fluorescence of chlorophyll was greatly reduced after UV-B treatment. UV-B exposure induced the formation of a translucent and glossy adaxial layer as previously observed in cucumber cotyledons (Fukuda et al. 2008).

Discussion

There are three key findings from our study that have not been reported previously. (1) Epidermal flavonol contents correlate with the highest UV-B irradiance intercepted during the previous 7–10 days. Irradiance appears more important than duration of exposure. (2) Leaves that have matured under a given UV-B regime have only a limited capacity to upregulate flavonol production when exposed to even higher UV-B levels. The capacity for accumulation appears to be largely dependent on the original acclimation. (3) With a single, low UV-B dose, flavonol accumulation is highly localized but with multiple and successive daily exposures or at higher intensities, flavonols accumulate progressively throughout all leaf tissues, even those that have not been exposed. *C asiatica* is a useful plant model, accumulating only two major flavonols in their leaves when UV-B-free grown plants are exposed to UV-B light.

Epidermal flavonol content depends upon the UV-B irradiance during acclimation

Figure 13 was obtained by compiling results of our experiments in which plants grown under various conditions received long-term daily UV-B doses. After 7–10 days the highest flavonol content in epidermises correlated with the highest UV-B irradiance applied on leaves. This relationship applies for healthy leaves before the start of senescence and when plants are healthy and grown in optimal ferti-irrigation conditions. For the same species, Müller et al. (2013b) also found that epidermal accumulation of flavonoids increased with nitrate, phosphate or potassium starvation during the early growth of young cuttings. In our experiment, *C. asiatica* attained full UV protection by flavonols when exposed to the highest irradiance, consistent with previous work in other species (Liakoura et al. 2003; Barthold et al. 2007; Morales et al. 2011). We

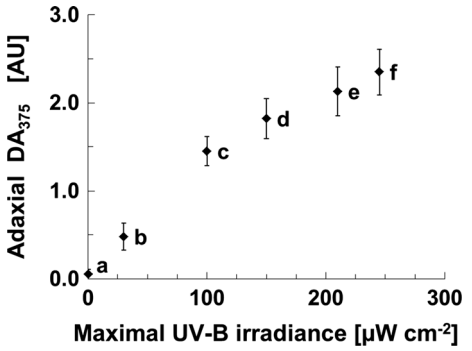

Fig. 13 Adaxial epidermal absorbance at 375 nm (DA_{375}) as a function of the maximum UV-B irradiance experienced by leaves of *Centella asiatica*. **a** UV-B-free grown leaves (control I). **b** Leaves grown outdoors in the shade (up to 50 µW cm^{-2} UV-B). **c** Leaves grown in growth chamber with 100 µW cm^{-2} UV-B. **d** Leaves grown in growth chamber at 150 µW cm^{-2} UV-B radiation. **e** Leaves oriented horizontally under full sunlight. **f** Leaves grown oriented towards the sun at noon. Data show mean DA_{375} values ± SD for 12 leaves. *Different letters* indicate significant differences at $P < 0.05$ (Fisher's LSD)

observed that rates of daily flavonol accumulation decreased during the UV-B-acclimation period (Figs. 2, 3) in agreement with previous works (Kolb et al. 2003, 2005; Agati et al. 2011; Latouche et al. 2013). The 7-day delay to maximum flavonol levels is comparable to those in leaves of *Vitis vinifera* L. (Kolb et al. 2003; Latouche et al. 2013), *Hordeum vulgare* L. (Kolb et al. 2005), *Morus nigra* L. (Bidel, unpublished), although a delay of 10 days was found for leaves of *Ligustrum vulgare* L. (Agati et al. 2011), and of 16 days for *Nothofagus solandri* and *Leptospermum scoparium* (Hunt and McSeveny 2002). In *C. asiatica*, Müller et al. (2013b) found significant increases in absorbance due to flavonoid accumulation under UV-B exposure up to 3 weeks. Although it seems much longer than shown here, it is not contradictory because the sampling area of Multiplex (30 cm^2) used in their study probably contained young expanding leaves in addition to mature acclimating leaves.

Although flavonol glucuronides of *C. asiatica* afford a high protection against UV-A radiation, they also contribute to epidermis UV-B absorbance (see Fig. 5a). Daily flavonol accumulation progressively decreased UV-B transmittance through epidermis cells. Under a constant irradiance, UV-B radiation passing through the epidermis decreased exponentially day by day. This mechanism could be considered as negative feedback regulation of flavonol biosynthesis, controlling flavonol content in leaves that receive variable UV-B fluxes. Once the level of flavonols has peaked, they could not be augmented further unless the intensity of UV-B was increased (Fig. 11). This explains the non-cumulative effect of UV-B radiation under conditions of diminishing UV-B irradiance (Fig. 10). The cumulative effects of UV-B dose, UV-B irradiance and exposure duration during the time course likely indicate that full flavonol protection had not yet been achieved and that UV-B radiation had penetrated the epidermis (Figs. 7, 8). Indeed, flavonoids might play a role in the putative "UV-B memory" process described by Müller-Xing et al. (2014), by decreasing UV-B transmittance through epidermis cells. Thus, when submitted to high UV-B irradiance, leaves previously exposed to low UV-B irradiance accumulated flavonols more slowly than did UV-B-protected leaves; the leaves apparently "remember" their previous light microclimate. Flavonoid screening effect may be a major part of this apparent "UV-B memory".

Original acclimation and subsequent acclimation to UV-B light

Epidermal flavonol content varied greatly between plants grown in a growth chamber and those grown outside under similar UV-B irradiance. Indeed, DA_{375} values determined for indoor-grown plants remained significantly lower. However, we observed that leaves grown in full sunlight had thicker cuticles, more lignified epidermal cell walls and more abundant trichomes, all of which contribute to UV protection. Our study was restricted to fully developed leaves and, therefore, evaluates only the acclimation potential of plants previously grown under low UV-B or UV-B-free conditions. The capacity of original acclimation of young growing leaves is higher (Fig. 9).

Flavonols accumulate progressively throughout all leaf tissues in di-hydroxylated forms

With a single low UV-B dose, flavonol accumulation is highly localized (Fig. 6), but with multiple and successive daily exposures or at higher intensities, flavonols accumulate progressively throughout all leaf tissues (Figs. 11, 12). Constitutive kaempferol-*3-O-β*-D-glucuronopyranoside is complemented by its hydroxylated form, quercetin-*3-O-β*-D-glucuronopyranoside, which accumulates in response to UV-B (Fig. 7b). An increasing proportion of di- and tri-hydroxylated flavonoids under stress conditions have been observed in various eudicotyledon species, conferring elevated antioxidant capacities of plants compared to the mono-hydroxylated forms (Agati et al. 2011, 2013; Brunetti et al. 2013). Therefore, the UV-B-induced di-hydroxylated flavonol (quercetin-3-*O-β*-D-glucuronopyranoside) in *C. asiatica*, suggests that flavonoid-mediated UV-B protection may be conferred both by UV-B screening and the quenching of reactive oxygen species (ROS) (Bidel et al. 2010). Our previous work on deciduous

species, such as *Morus nigra* L., *Prunus mahaleb* L., *Lagerstroemia indica* L., has shown that proportions of secondary metabolites depend on local leaf microclimate, with higher flavonoid amounts accumulating under higher UV-B irradiances (Bidel et al. 2007).

At the highest UV-B irradiance, mesophyll tissue preferentially accumulated quercetin-*3-O-β*-D-glucuronopyranoside and, in a smaller amounts kaempferol-*3-O-β*-D-glucuronopyranoside. The presence of quercetin-*3-O-β*-D-glucuronopyranoside in leaf mesophyll potentially explains the weak correlation usually observed between DA_{375} readings and the flavonoid content of whole leaf extracts (Barnes et al. 2000). In *C. asiatica* di-hydroxylated flavonols (quercetin derivatives) preferentially accumulate in response to UV-B (Fig. 5), which is consistent with the antioxidant role of the 3′,4′-catechol structure (Agati and Tattini 2010; Agati et al. 2012).

Our results obtained with inverted leaves (Figs. 2b, 3b) also indicate that the palisade mesophyll tissues may function as a conduit to facilitate UV-B transmission to subjacent cells, as it has been previously shown for visible light (Day et al. 1993; Vogelmann and Evans 2002). Indeed, UV-B radiation applied to the adaxial leaf surface can, if sufficiently intense, induce flavonol accumulation in the abaxial epidermis. However, this is not the case for the adaxial epidermis when the abaxial side of the leaf is illuminated.

Are epidermal DA_{375} values reliable indicators of UV-B acclimation?

By analysing different cell layers of leaves by LC–MS, we confirmed that flavonols are the main contributors to epidermal absorbance at 375 nm. DA_{375} can, therefore, be used for the non-destructive quantification of flavonols in *C. asiatica* leaf epidermises, as has been proposed for other species (Cerovic et al. 2002). By contrast, the contribution of hydroxycinnamates to DA_{375} measurements was negligible for *C. asiatica*. Thus, it was possible to express the flavonol content as molar quercetin-*3-O-β*-D-glucopyranoside equivalent units that correlated directly with DA_{375} values (Louis et al. 2009). The adaxial and abaxial epidermises similarly accumulated flavonols in response to UV-B. DA_{375} as recorded by the Dualex leaf-clip provided a reliable measure of epidermal flavonols accumulated in response to maximal UV-B irradiance experienced by each individual leaf during growth. However, the relationship between DA_{375} and flavonol content may not necessarily hold true for other species because histolocalization of flavonols is species specific. We, therefore, recommend that LC–MS analyses of epidermal tissues and lower tissues be done to validate the use of DA_{375} in other species.

Can epidermal UV absorbance by flavonols affect the UV-B sensing mechanism?

When DA_{375} increased from 0.5 to 1.0 AU, corresponding to an increase from 51.5 to 103.1 nmol cm^{-2} of quercetin-*3-O*-glucoside equivalents, epidermis UV-B-transmittance declined such that mesophyll cells received less than 0.010 μW cm^{-2} UV-B radiation. Thus, we can postulate that the UV-B radiation might be too low to activate the UVR8 photoreceptor. The distribution of UVR8 (monomers/dimers) and its associated regulators (COP1, HY5, HYH, RUP1, RUP2) in the leaf tissues under various light conditions should be investigated to provide a better understanding of these regulation processes.

Under relatively high solar UV-B irradiance, Long and Jenkins (1998) have shown for *Arabidopsis* that UV-B photoperception can modify the redox status of the plasma membrane, which could play a role in the induction of phenylpropanoid and flavonoid biosynthetic genes (Jenkins et al. 2001; Brown and Jenkins 2008; Jenkins 2009; Hideg et al. 2013). This could occur through (1) the jasmonate pathway resulting in degradation of phospholids (Demkura et al. 2010) or (2) in the generation of H_2O_2 under combinations of high light and low temperatures which induce an excess of electrons in chloroplasts accepted by O_2 (Moullineaux et al. 2002; Gordon et al. 2013; Karpinski et al. 2013). Hence, the irradiance level and the speed of synthesis/accumulation of flavonols in the different leaf tissues could be key factors that determine the final amounts of flavonols in leaves.

As epidermal absorbance can now easily be probed with portable sensors such as Dualex (Cerovic et al. 2008; Meyer et al. 2006, 2009) or Multiplex (Ben Ghozlen et al. 2010; Müller et al. 2013a), differences between the DA_{375} response curves to UV-B irradiance obtained under different agronomic conditions might be studied to identify new stress indicators for crop monitoring.

Author contribution All authors participated in the design and critical reviewing of the manuscript. LPRB, GC, FB, LM, JS, MC, PLF conducted experiments. LPRB, GC, MC, ZGC and CJA wrote manuscript. KSG improved the manuscript.

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