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REGULAR PAPER

Investigating UV screening in leaves by two different types of portable UV fluorimeters reveals in vivo screening by anthocyanins and carotenoids

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Abstract Two portable instruments, designed to evaluate epidermal UV screening in leaves, were compared: the Dualex and the UV-A-PAM fluorimeter. Both instruments excite chlorophyll fluorescence at the same UV wavelengths but reference excitation is in the red and the blue spectral range in the former and the latter fluorimeter, respectively. When analyzing green leaves, general agreement of the data is obtained with the two instruments. In the presence of anthocyanins, the UV-A-PAM fluorimeter provided higher estimates for epidermal UV transmittance than the Dualex fluorimeter, which was attributed to absorption of blue excitation light by anthocyanins. By comparing data from the instruments, anthocyanin-dependent transmittance of 50% was determined in adaxial sides of some autumn leaves, and also in abaxial sides of tropical shade plants. Further, with leaves of chlorophyll *b*-less mutants of *H. vulgare*, unusually high epidermal UV transmittance was detected but this was attributed to the lack of chlorophyll *b* absorption and, in addition, to absorption of blue radiation by xanthophylls which are not functionally connected to photosystems.

Keywords Anthocyanins · Chlorophyll fluorescence · Chlorophyll *b* · Flavonoid · UV screening

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Abbreviations

AD and AB	adaxial and abaxial
Chl	chlorophyll
FER	fluorescence excitation ratio
Fλ	fluorescence intensity at wavelength λ
Φ_F	quantum yield for chlorophyll fluorescence
PS II	photosystem II
UV	ultraviolet radiation

Introduction

Measurements of spectral and kinetic behavior of chlorophyll fluorescence provide a plethora of information on the state and performance of the photosynthetic apparatus (Govindjee 1995; Govindjee and Seufferheld 2002; Papageorgiou and Govindjee 2004). Certainly, one important advantage of recording chlorophyll fluorescence is the capability to non-intrusively retrieve information on photosynthesis in the intact leaf.

Fluorescence analysis of leaves works well with a constant excitation wavelength. However, when the wavelength of excitation radiation varies, the optical properties of the epidermis become involved. In particular, epidermal absorption in the ultraviolet (UV) by phenolics interferes critically with fluorescence excitation (Cerovic et al. 1993; Stober and Lichtenthaler 1993; Sheahan 1996; Mazza et al. 2000). Epidermal effects on fluorescence excitation, on the other hand, were exploited by Bilger et al. (1997) to determine epidermal UV screening from the UV/blue-green-excited

fluorescence excitation ratio (FER) with the rationale that blue radiation can freely penetrate into the leaf and, hence, blue-excited fluorescence can serve as a reference signal to which UV-excited fluorescence can be related.

In the field, epidermal phenolics serve as an indispensable screen that protects the leaf from damage by natural UV radiation (Caldwell et al. 1983; Tevini et al. 1991; Bornman and Teramura 1993; Jordan 1996). Hence, information on the epidermal transmittance for UV radiation is prerequisite to understand plant functioning in nature. In response to demands by field researchers, portable fluorimeters that measure UV screening have been developed: the Dualex (Goulas et al. 2004) and the UV-A-PAM fluorimeter (Bilger et al. 2001; Kolb et al. 2005). These two instruments are differently designed: both use the same wavelengths of UV excitation but the reference wavelength in the visible is in the blue and red spectral range in the UV-A-PAM and the Dualex fluorimeter, respectively. Further, the UV-A-PAM fluorimeter excites and detects fluorescence on the same side but in the Dualex fluorimeter detection of fluorescence occurs on the opposite side with respect to excitation.

Both UV fluorimeters represent new and promising techniques with a wide potential of applications in basic and applied research; however, we do not know if the two instruments produce comparable data despite their spectral and constructional differences. Indeed, it has been proposed that using blue, but not red, radiation to excite reference fluorescence yields false estimates for UV screening in the presence of anthocyanins (Barnes et al. 2000; Goulas et al. 2004; Hagen et al. 2006). Therefore, in this work, we compare both fluorimeters by analyzing a wide range of different samples. Our results reveal that the data from the two fluorimeters are largely comparable when normal green leaves are investigated. In addition, the difference in the reference wavelength allowed us to retrieve information on leaf anthocyanins and carotenoids.

Material and methods

Plants

Table 1 compiles all plant species investigated along with information on their systematic classification. The table arranges species according to leaf coloration (green or red), and indicates whether plants are grown outdoors or in a greenhouse of the Botanical garden of the University of Würzburg, because under our greenhouse conditions natural UV radiation is greatly diminished (Kolb et al. 2001). Leaves were harvested during September and October 2005 and immediately transferred to the laboratory for subsequent analyses.

Table 1 Species investigated

Species (Family)	Order	Growth condition
Green-leaf species		
Aeonium haworthii Webb & Berth. (Crassulaceae)	Saxifragales	Greenhouse
Crassula ovata (Mill.) Druce (Crassulaceae)	Saxifragales	Greenhouse
Hordeum agriocrithon Åberg (Poaceae)	Poales	Outdoors
Hordeum vulgare L. var. Donaria (Poaceae)	Poales	Greenhouse
Hordeum vulgare L. var. Donaria, Chl *b*-less mutants chlorina-f2 2800 and 3613 (Poaceae)	Poales	Greenhouse
Hordeum vulgare var. Hege 550/75 (Poaceae)	Poales	Greenhouse
Hordeum vulgare L. var. Hege 550/75, flavonoid-deficient mutant Ant 287 (Poaceae)	Poales	Greenhouse
Regnellidium diphyllum Lindm. (Marsileaceae)	Marsileales	Greenhouse
Sedum telephium L. (Crassulaceae)	Saxifragales	Outdoors
Vitis vinifera L. var. Silvaner (Vitaceae)	Vitales	Outdoors and greenhouse
Red-leaf species		
Red autumn leaves		
Kolkwitzia amabilis Graebner (Caprifoliaceae)	Dipsacales	Outdoors
Parthenocissus tricuspidata (Sieb. & Zucc.) Planch. (Vitaceae)	Vitales	Outdoors
Viburnum lentago L. (Caprifoliaceae)	Dipsacales	Outdoors
Viburnum phlebotrichum Sieb. & Zucc. (Caprifoliaceae)	Dipsacales	Outdoors
Red-leaf vegetables		
Lactuca sativa L. var. crispa L. Lollo Rosso (Asteraceae)	Asterales	Outdoors
Brassica oleracea L. var. capitata f. rubra DC (Brassicaceae)	Brassicales	Outdoors
Tropical understorey plants (abaxially red)		
Calathea spec. (Marantaceae)	Zingiberales	Greenhouse
Ctenanthe oppenheimiana (E. Morr.) K. Schum cv. Tricolor (Marantaceae)	Zingiberales	Greenhouse
Dipteracanthus devosianus (Makoy) Boom (Acanthaceae)	Lamiales	Greenhouse

Green-leaf plants included: *Regnellidium diphyllum*, which is a semi-aquatic fern; *Vitis vinifera* var. Silvaner; three species of the Crassulaceae family, and two species of the genus *Hordeum* (Table 1). *H. vulgare* was represented by two varieties: Donaria and Hege 550/75. Also, three mutants of *H. vulgare* were investigated: the Chl *b*-less mutants chlorina-f2 2800 and chlorina-f2 3613, which are rooted in the Donaria variety (Simpson et al. 1985), and the flavonoid-deficient mutant Ant 287, which is derived from the Hege 550/75 variety (Reuber et al. 1996). Seeds of the Donaria variety and their mutants were obtained from the seed collection of the Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany, and seeds of the Hege 550/75 variety and the Ant 287 mutant were provided by Prof. G. Weissenböck, Universität zu Köln, Köln, Germany. In our experiments, the two chlorina-f2 mutants behaved identically and, consequently, they will not be differentiated. All plants of *H. vulgare* were grown in the greenhouse in pots of 20 cm diameter containing Torfsubstrat Cultural F (Euflor, Munich, Germany), watered daily and fertilized weekly using Flory 3 (Euflor). Leaves of *Hordeum agriocrithon* were collected from the outdoor crop plant collection of the Botanical Garden.

Plants of *Vitis vinifera* cv. Silvaner were grown either in the greenhouse or were permanently cultivated outdoors in the Botanical Garden's crop plant collection. In the greenhouse, the vines were grown in pots of 50 cm diameter containing Torfsubstrat Cultural F. They were watered daily and fertilized twice a week using Hakaphos blau 15 + 10 + 15(+2) (Compo, Münster, Germany). *Regnellidium diphyllum* was permanently cultivated in a ''tropical greenhouse'', *Aeonium haworthii* and *Crassula ovata* were grown in a ''Mediterranean'' greenhouse, and *Sedum telephium* was planted outdoors in an ornamental plot.

Four species grown outside exhibited red autumnal leaf coloration: one vine (*Parthenocissus tricuspidata*) and three shrubs or trees (*Kolkwitzia amabilis*, *Viburnum lentago* and *Viburnum phlebotrichum*). Additional red-leaved species were the vegetables *Lactuca sativa* and *Brassica oleracea*, both grown outdoors, and three understory plants, which are continuously cultured in a tropical greenhouse (*Calathea spec.*, *Ctenanthe oppenheimiana*, and *Dipteracanthus devosianus*).

Portable UV fluorimeters

Both, artificial and natural UV screening of leaves was determined by a Dualex (FORCE-A, Orsay, France) and a UV-A-PAM fluorimeter (Gademann Messgeräte, Würzburg, Germany). To compare instruments, identical leaf positions were analyzed sequentially by the Dualex and the UV-A-PAM.

A detailed description of the Dualex fluorimeter was published earlier (Goulas et al. 2004; for further information contact: info@force-a.fr). Detailed information on the UV-A -PAM fluorimeter is available on request (mail@gademann.de). Some significant characteristics of both fluorimeters are outlined in Fig. 1. The Dualex and the UV-A-PAM fluorimeter stimulate chlorophyll fluorescence by UV-A and visible radiation from light-emitting diodes (LED). The same waveband is used for UV excitation (375 nm, bandwidth 10 nm), but excitation in the visible occurs at 655 nm (bandwidth 12 nm) and 470 nm (bandwidth 50 nm) in case of the Dualex and the UV-A-PAM fluorimeter, respectively. Further, the Dualex fluorimeter measures fluorescence at wavelengths >700 nm, but the UV-A-PAM fluorimeter records fluorescence >645 nm. In the Dualex fluorimeter, fluorescence excitation and detection units are situated in opposite parts of the ''Dualex leaf clip'' in which the sample is inserted for measurements; in contrast, the UV-A-PAM fluorimeter uses a flexible fluid light guide to carry excitation radiation to the sample and fluorescence back to the instrument.

Both instruments derive information on UV screening by relating the UV to the visible radiation-excited fluorescence intensity (Bilger et al. 2001; Kolb et al. 2003; Goulas et al. 2004; Kolb et al. 2005). This concept requires that radiation absorbed by chlorophyll-protein complexes is converted into chlorophyll fluorescence with the same quantum yield (Φ_F) for both excitation conditions, and that the reference visible beam is not affected by the screening. Since the former prerequisite is met differently by the two fluorimeters, their primary data output differs as now explained.

With the rationale to measure reasonable signal levels of UV-induced fluorescence, even in extremely UV-shielded samples, the Dualex fluorimeter applies relatively high excitation intensities that can affect photosynthesis and, thus, can change Φ_F (Goulas et al. 2004). Variable Φ_F, however, would interfere with fluorimetric determination of UV screening if significant changes in Φ_F occur between measurements of F375 and F655. Therefore, the Dualex fluorimeter switches rapidly (i.e., at a frequency of 1 kHz) between UV-A and visible excitation of fluorescence. Moreover, the instrument achieves equal fluorescence levels for both excitation conditions (F375 = F655) by keeping the UV-A intensity constant but continuously adjusting the intensity of visible excitation. During an

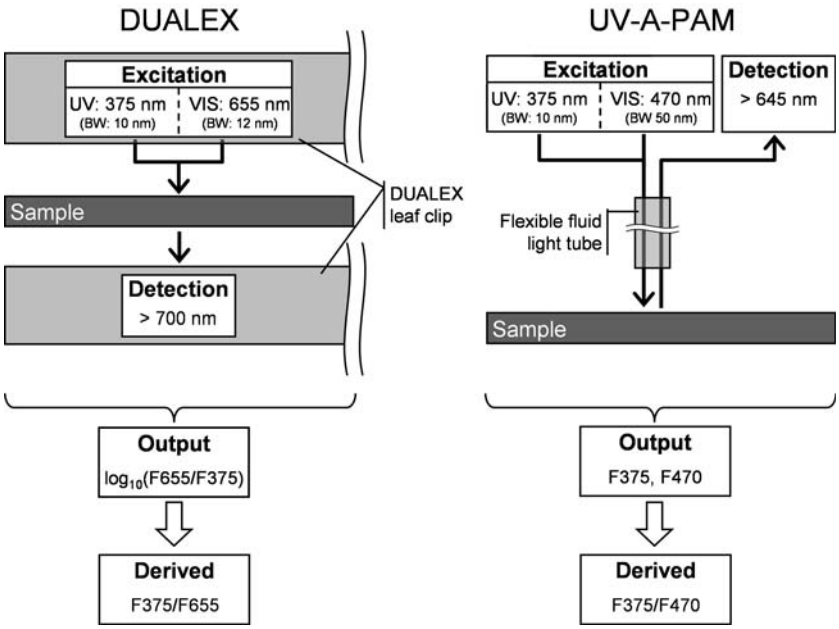

Fig. 1 Key features of the Dualex and the UV-A-PAM fluorimeters. In the Dualex fluorimeter, samples are held by a leaf clip, and excitation and detection of fluorescence occur at opposite sides of the sample. The UV-A-PAM fluorimeter employs a flexible light guide as the optical connection between instrument and sample so that excitation and fluorescence detection occurs on the same side of the sample. Although spectral distributions of ultraviolet (UV) excitation radiation are comparable between the two instruments, the excitation wavelengths in the visible (VIS) and of fluorescence band detected differ. BW: bandwidth of excitation radiation; Output: primary output of the instrument; Derived: data calculated from primary output (see Figs. 4–6 and 11); F375, F470, and F655: fluorescence excited at 375, 470, and 655 nm, respectively

initial period of adaptation to excitation radiation, the ratio between Φ_F for F375 and Φ_F for F655 may vary; throughout the actual measurement phase, which is characterized by a smoothly changing fluorescence signal in the presence of a virtually constant output by the 655 nm reference LED, it is safe to assume similar values for Φ_F under both excitation conditions (Goulas et al. 2004).

To achieve equal fluorescence conditions (F375 = F655), low visible excitation intensities are required in the case of the low F375 emissions from efficiently UV-screened samples and *vice versa*. Hence, the intensity of the visible reference LED is associated with the degree of UV-screening of a sample. In fact, the Dualex fluorimeter relates the reference excitation intensity measured with an unknown sample to the behavior of a standard sample devoid of UV screening to derive data of $\log_{10}$(F375/F655). The actual factory-calibration of the instrument employs a blue fluorescent film (Walz, Effeltrich, Germany) as the standard. The Walz fluorescence film was chosen for standardization as it corresponds to a tolerable compromise for the actual variable fluorescence properties of different preparations of non-UV-screened leaf mesophyll (Barnes et al. 2000; Kolb et al. 2005).

In contrast to the Dualex fluorimeter, the UV-A-PAM employs weak measuring light, which does not induce any significant closure of PS II reaction centers, so that chlorophyll fluorescence is measured with the constant fluorescence yield of dark-adapted samples. The ''pulse amplitude modulation'' technique of UV-A-PAM fluorimeter permits determination of fluorescence yields even in the presence of background radiation, and the instrument switches between UV and visible excitation to correct for variations in fluorescence yields caused by background radiation. Prior to each experiment, excitation intensities of the UV-A-PAM fluorimeter were adjusted to yield equal fluorescence intensities with a blue fluorescence standard film glued on a reflective metal foil (Walz). After adjustment of excitation intensities, the measured intensities of chlorophyll fluorescence excited by 375 and 470 nm (i.e., F375 and F470, respectively) represent the primary output of the UV-A-PAM fluorimeter.

Artificial screening

Standard square glass filters (50 × 50 mm) were placed on adaxial (upper) sides of detached leaves. Leaves of

H. vulgare var. Hege 550/75 mutant Ant 287 were used in these experiments because they exhibit high epidermal UV transmittance due to deficient flavonoid synthesis (Reuber et al. 1996). In addition, leaves of *S. telephium* were chosen because of their easily removable epidermis, which permitted preparations of leaf samples with high adaxial UV transmittance. The epidermis-free leaves were wrapped in UV-transparent Teflon foil of 30-μm thickness (type ET 6235, Novofol, Siegsdorf, Germany) to prevent dehydration.

Seventeen different filters obtained from Schott (Mainz, Germany) were selected as artificial screens and the same leaf sample was subjected to 17 consecutive measurements employing these filters. Figure 2 shows all filter names together with filter transmittance

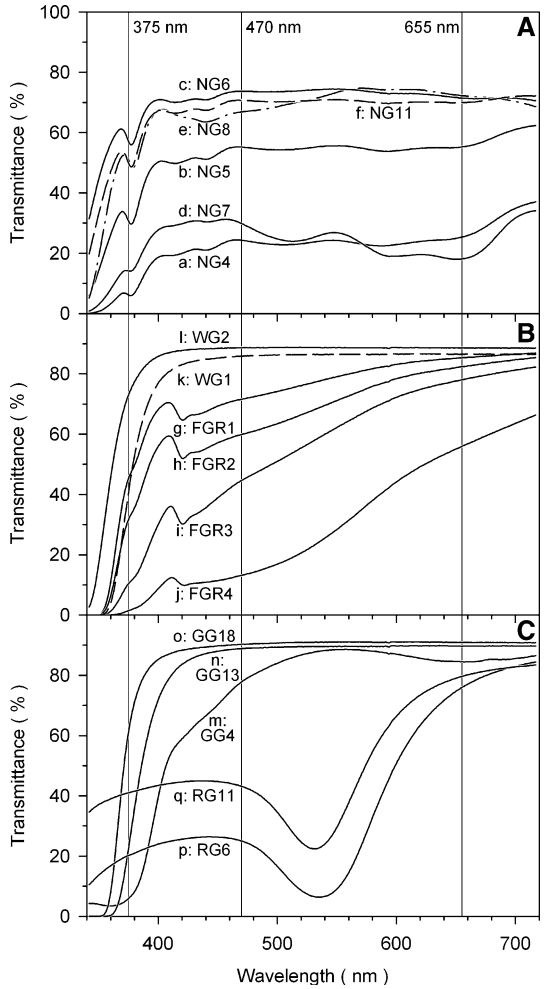

Fig. 2 Transmittance spectra of glass filters. Transmittance spectra of glass filters that were employed to screen leaves. Spectra are labeled by the original filter designations together with small letters (a to q), which, in Figs. 3 and 4, indicate for each data point the filter used. Vertical lines specify the central wavelengths of fluorescence excitation (compare Fig. 1)

spectra measured against air in a UV4 spectrometer (ATI Unicam, Cambridge, UK).

Fluorescence excitation and absorptance spectra

Fluorescence excitation spectra in the range from 280 to 650 nm of chlorophyll fluorescence at 685 nm were recorded using a spectrofluorimeter (Cary Eclipse, Varian, Les Ulis, France) and corrected using rhodamine B as a quantum counter as described by Cerovic et al. (1999).

Reflectance and transmittance spectra of leaves were recorded with leaves of *V. lentago* and *H. vulgare* var. Donaria using an external integrating sphere (type 1800-12, Li-Cor, Lincoln, Nebraska) in combination with an LI-1800 portable spectrometer (Li-Cor) using a barium sulfate block as a reference (see Daughtry et al. (1989) for details). Absorptance spectra were calculated from reflectance and transmittance spectra according to:

$$\text{Absorptance (\%)} = 100\ (\%) - \text{Reflectance (\%)} - \text{Transmittance (\%)}.$$

The sample port (diameter = 14.5 mm) was fully covered by a leaf of *V. lentago* but, in the case of *H. vulgare*, several leaves were carefully mounted in parallel to avoid light leaks but also taking care to minimize leaf overlap.

Leaf extracts

Phenolics were extracted using 50% methanol (v/v) containing 0.01% (w/v) phosphoric acid. Chlorophylls and carotenoids were extracted with buffered 80% acetone (Porra et al. 1989). For both extractions, a disk of 9 mm diameter was punched out a leaf using a cork borer. Subsequently, the disk was transferred into a 2 ml round-bottom tube containing a 5 mm diameter metal ball. The closed tube was frozen in liquid nitrogen and immediately ground using a Retsch MM 301 mixer mill (Düsseldorf, Germany) operating at a shaking frequency of 20 s^{-1} for 20 s. Subsequently, the powder was suspended in 0.5 ml of extraction solvent and, after centrifugation (4 min at 12000*g* and 4°C), the supernatant was collected and the sediment homogenized twice more with a further 0.5 ml of extraction solvent, and the supernatants pooled and clarified by centrifugation. The present work presents original absorbance spectra of the combined supernatants recorded by a UV4 spectrometer (ATI Unicam).

Microscopy

Cross sections of freshly-harvested leaves of 100–200 μm thickness were prepared using a hand microtome (Leica Instruments GmbH, Nußloch, Germany) and immediately examined in water using a Leica DMR microscope equipped with a DC500 digital camera system controlled by the IM1000 Image Manager version 1.20 (both Leica).

Leaf thickness

Thickness of leaves between veins was determined using an electronic sliding caliper or it was derived from microscopic thin-sections in the case of *V. vinifera*.

Statistics

Linear regression analysis was performed using SigmaStat for Windows (version 2.03, SPSS, München, Germany). To compare coefficients (slope or *y* axis-intercept) of two different regression lines, 95% confidence intervals (95%CI) were calculated according to: $95\%CI = t_{n-2} \cdot SE$, where SE is the standard error of the coefficient, and t_{n-2} represents the t value for $n - 2$ degrees of freedom and 5% error level. The difference between coefficients was considered as statistically significant if the intervals of coefficients ± 95%CI did not overlap. If the Y axis-intercept ± 95%CI did not include the $Y = 0$, it was concluded that the linear regressions did not extrapolate to the origin.

To test, if sample means of apparent transmittance (T_{APP}; Fig. 9) differ meaningfully from 100 % transmittance, t values were calculated according to $t = |T_{APP}\text{-}100| \cdot SD^{-1} \cdot n^{1/2}$, where SD and n corresponds to standard deviation and sample size, respectively. If the calculated t value was greater than the tabulated t_{n-1} for 5% error level and $n - 1$ degrees of freedom, the mean of T_{APP} was considered as significantly different from 100%. Statistical comparison of T_{APP} of adaxial with that of abaxial leaf sides was carried out using Student's *t*-test (SigmaStat for Windows).

Results and discussion

Screening of leaves by glass filters

The transmittance spectra of 17 different glass filters used as artificial screens on adaxial leaf sides are shown in Fig. 2. Transmittance spectra are labeled with the filter designation and a small letter (a to q) which, in Figs. 3 and 4, indicates the filter used for each specific data point. All filters exhibit lower transmittance at 375 nm, the central wavelength of UV excitation of both fluorimeters, than at 470 and 655 nm, the central wavelengths of visible excitation radiation of the UV-A-PAM and the Dualex fluorimeters, respectively. Further, the transmittance at 655 nm was similar, or higher, than that at 470 nm except for filter ''d'', which showed higher transmittance at 470 than at 655 nm.

Screening of chlorophyll excitation by filters was quantified with a leaf from the flavonoid-free mutant Ant 287 of *H. vulgare* (Fig. 3). Using the UV-A-PAM fluorimeter, fluorescence intensity in the presence of a filter ($F\lambda_{FLT}$) and without filter ($F\lambda_{REF}$) was measured, and the ratio of $F\lambda_{FLT}/F\lambda_{REF}$ was used to assess filter transmittance: here, the λ represents the central wavelength of fluorescence excitation by the UV-A-PAM fluorimeter, either 375 or 470 nm. The $F\lambda_{FLT}/F\lambda_{REF}$ ratios were plotted against the filter transmittance at excitation wavelength λ, $T\lambda$ (Fig. 3A, C), or against the product of $T\lambda$ and the filter transmittance at wavelengths of chlorophyll fluorescence emission, $T\lambda \cdot T710$ (Fig. 3B, D). In Figs. 3 and 4, the filter transmittances ($T\lambda$) correspond to the mean values of transmittance, derived from the spectra shown in Fig. 2, within intervals defined by λ, the central wavelength of excitation radiation, ± the corresponding half-bandwidth (compare Fig. 1). In the case of T710, transmittance was averaged in the interval 710 ± 25 nm that includes most of the chlorophyll emission.

Almost ideal proportionalities were observed between $F\lambda_{FLT}/F\lambda_{REF}$ and the product, $T\lambda \cdot T710$, (see Fig. 3B, D) but not when the $F\lambda_{FLT}/F\lambda_{REF}$ was plotted against $T\lambda$ alone (Fig. 3A, C). This was expected because, in the UV-A-PAM apparatus (Fig. 1), both the excitation and fluorescence radiation have to pass the screening filter so that the $T\lambda$ value alone is insufficient to describe the behavior of $F\lambda_{FLT}/F\lambda_{REF}$. Slopes of regression lines in plots of $F\lambda_{FLT}/F\lambda_{REF}$ versus the product, $T\lambda \cdot T710$, were, however, smaller than 1 (Fig. 3B, D). We suspect that the lower transmittances reported by fluorescence measurements, when compared to spectrophotometric data, arises from the escape of some radiation (particularly diffuse fluorescence radiation) at the level of our screening filters. In general, the data in Fig. 3 clearly confirm the capacity of fluorescence techniques to evaluate absorption properties of optical layers.

In practice, both, the Dualex and the UV-A-PAM fluorimeters, do not evaluate screening properties of leaves by excitation at a single waveband but derive

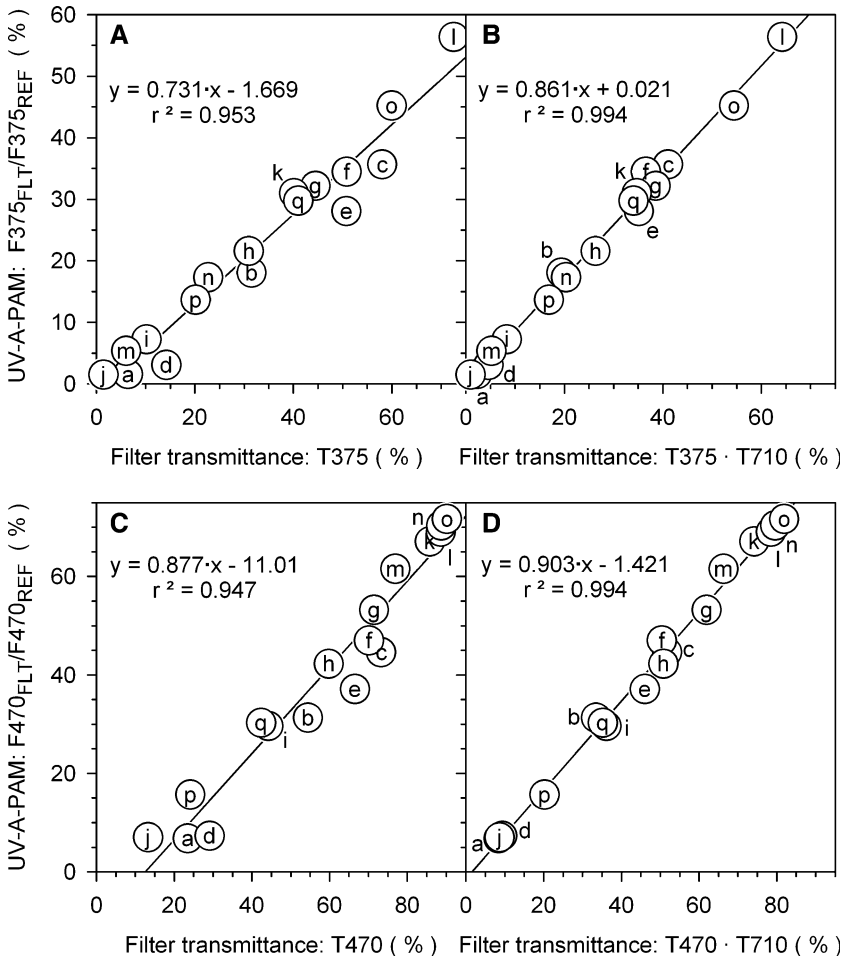

Fig. 3 Fluorimetrically and spectrophotometrically determined filter transmittances. Ordinate data correspond to fluorescence ratios obtained from the adaxial side of a leaf of *H. vulgare* Ant mutant 287 by a UV-A-PAM fluorimeter. Fluorescence was recorded with the leaf covered by a glass filter ($F\lambda_{FLT}$) or uncovered ($F\lambda_{REF}$). The λ (central wavelength of fluorescence excitation) was 375 (**A** and **B**) or 470 nm (**C** and **D**; compare Fig. 1). In panels (**A**) and (**C**), the ratios of $F\lambda_{FLT}/F\lambda_{REF}$ are plotted against filter transmittances, Tλ. The Tλ were derived from Fig. 2 as mean transmittance in the intervals 375 ± 5 nm (T375, panel **A**) or 470 ± 25 nm (T470, panel **C**). Abscissa data in (**B**) and (**D**) are the product of T375 · T710 and T470 · T710, respectively, where the T710 represents filter transmittance at wavelengths of chlorophyll emission and was derived from Fig. 2 as the mean in the interval 710 ± 25 nm. Small letters indicate the filter used to measure $F\lambda_{FLT}$ (see Fig. 2). For data of each panel, the results of linear regression analysis are represented by a straight line, the corresponding equation and the coefficient of determination, r^2

information on UV screening at wavelength λ1 from the ratio Fλ1/Fλ2, which corresponds to fluorescence excited at λ1 divided by fluorescence excited in the visible region at wavelength λ2. Similarity, we measured ratios of Fλ1/Fλ2 of leaves covered by glass filters to compare the latter data with ratios of filter transmittances (Tλ1/Tλ2): mean values used for filter transmittances (Tλ1 and Tλ2) were principally calculated as described above. Since the Fλ1 and Fλ2 are measured with similar optical paths for fluorescence, absorption of chlorophyll fluorescence by filters cancels out and does not need to be considered here.

Figure 4 demonstrates the close linear associations between fluorescence and transmittance ratios that extrapolate near the origin for both fluorimeters, and for both a leaf from *H. vulgare* mutant Ant 287 and from *S. telephium*, even though the latter leaf was much thicker (1 mm) than the former (0.2 mm). Moreover, for the same leaf, both the slope and intercept of regression lines obtained with the two instruments did not differ in any statistically meaningful way. The slope of regression lines, and also the ratios of Fλ1/Fλ2 of leaves not covered by a filter (grey symbols in Fig. 4), was much higher for *H. vulgare* mutant Ant 287 than for *S. telephium*. The ratio of Fλ1/Fλ2 recorded in the presence of a screening filter depends on the filter's transmittance properties and the specific ratio of Fλ1/Fλ2 of the leaf beneath. Within a

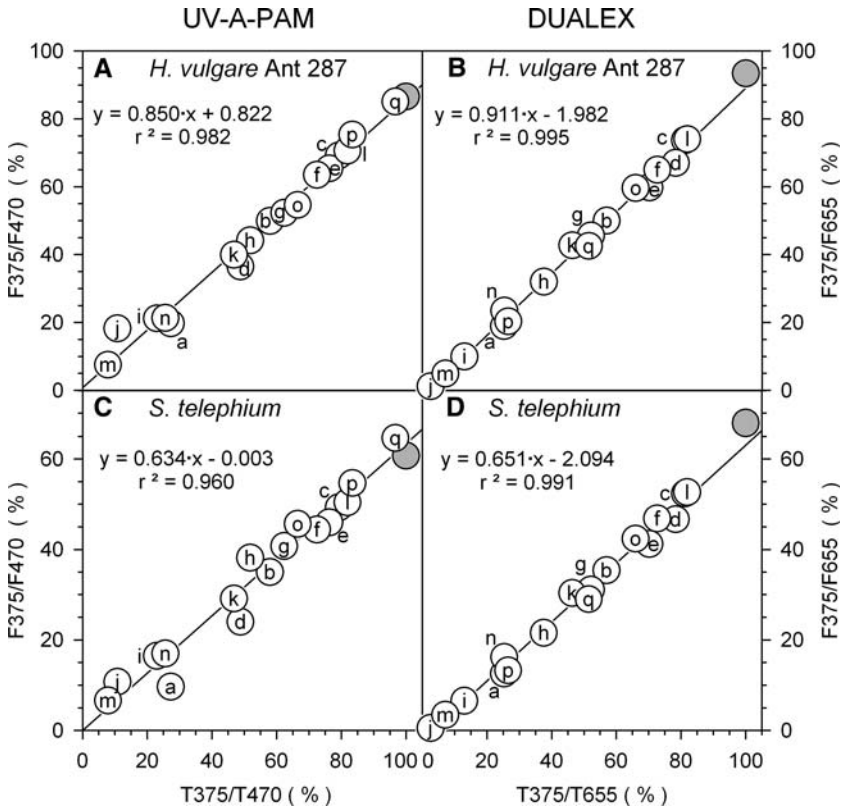

Fig. 4 Fluorescence excitation ratios (FER) and transmittance ratios of filters. UV to visible FER ratios measured by a UV-A-PAM fluorimeter are the ordinate data of (**A**) and (**C**) (F375/F470) but, in (**B**) and (**D**), the ordinates are the corresponding ratios recorded by the Dualex fluorimeter (F375/F655). The fluorescence excitation ratios are plotted against transmittance ratios at matching wavelengths of filters. Data drawn by open symbols are recorded with a filter placed on the adaxial side of a leaf from *H. vulgare* mutant Ant 287 (panels **A** and **B**) or an epidermis-free leaf from *S. telephium* (panels **C** and **D**). Grey-filled symbols represent data from these leaves obtained without a filter. The T655 represents the mean transmittance in the interval 655 ± 6 nm and was derived from Fig. 2. See Fig. 3 for information on the T375 and T470, labeling of data points and on linear regression analysis

series of measurements, the Fλ1/Fλ2 ratio of a leaf affects each measurement in a proportional fashion and, hence, influences the slope of the relationship between Fλ1/Fλ2 and transmittance ratios. Therefore, the different slopes of regression lines obtained with *H. vulgare* mutant Ant 287 and *S. telephium* simply reflect different fluorescence properties (Fλ1/Fλ2) of leaves used.

In short, since the ratio of Fλ1/Fλ2 recorded in the presence of a screening filter integrates both the filter's transmittance properties and the specific ratio of Fλ1/Fλ2 of the leaf beneath, different slopes of regression lines arose with the particular fluorescence properties of the leaf used.

To summarize, the relationships observed between ratios of fluorescence and ratios of filter transmittances suggest compatible evaluation of artificial screening by Dualex and UV-A-PAM fluorimeters despite the different excitation wavelengths in the visible, and the different optical and electronic designs (see Fig. 1 and Material and methods).

Comparison of fluorimeters using green leaves

In the preceding experiment, the direct comparison of ratios Fλ1/Fλ2 from the two fluorimeters was meaningless, since the visible excitation wavelength (λ2) differed between fluorimeters (470 and 655 nm) implying that the Fλ1/Fλ2 of the same filter differed for those filters exhibiting different transmittances at 470 and 655 nm (Fig. 2). When green leaves are investigated, however, similar high epidermal transmittances can be assumed at 470 and 655 nm (cf. Pfündel et al. 2006). Therefore, we can directly compare data from the two fluorimeters by plotting the Fλ1/Fλ2 recorded by the Dualex against that of the UV-A-PAM fluorimeter.

Figure 5 depicts data obtained with three groups of leaves: Fig. 5A shows data from plants of the genus *Hordeum*. Here, considerable variation of data arises particularly from extremely low UV screening in *H. vulgare* mutant Ant 287 and efficient screening of field-grown *H. agriocrithon*. Figure 5B summarizes the

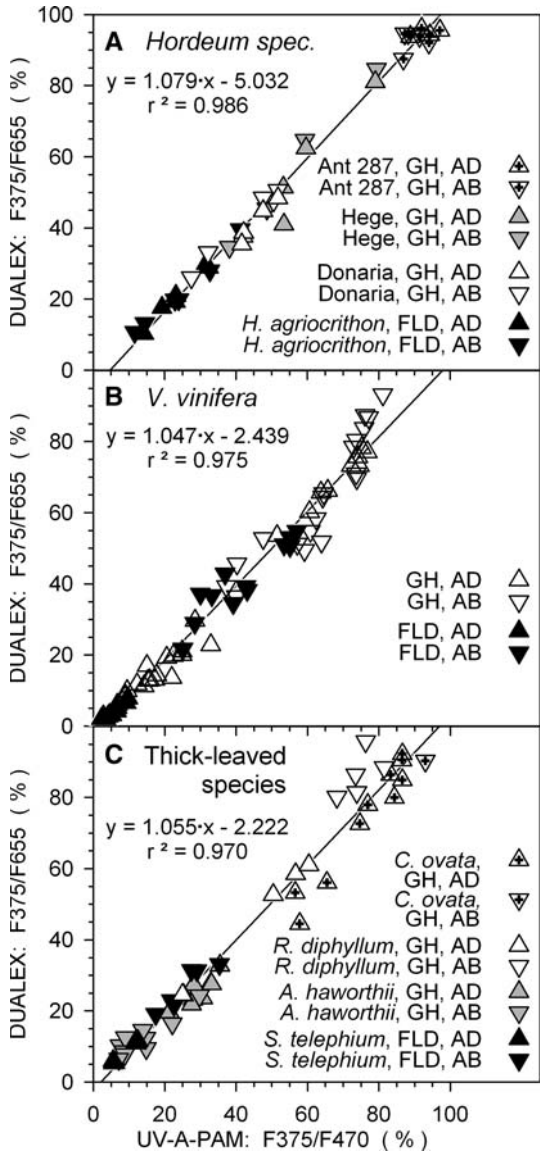

Fig. 5 Fluorescence excitation ratios (FER) measured with green leaves. In all panels, FER values measured with a Dualex fluorimeter (F375/F655) are plotted against that recorded by a UV-A-PAM fluorimeter (F375/F470). Leaves of different plants were probed: **A**, species of the genus *Hordeum*; **B**, the species *V. vinifera*; and **C**, thick-leaved species including species of the Crassulaceae family with leaf thickness up to 3.8 mm. Different symbols indicate the plant investigated, its growth condition (GH, greenhouse; FLD, field) and the leaf side investigated (AD, adaxial leaf side; AB, abaxial leaf side). Ant 287, flavonoid-deficient mutant of *H. vulgare*; Donaria and Hege, varieties of *H. vulgare* (compare Table 1). See Fig. 3 for information on linear regression analysis

results achieved with leaves from *V. vinifera* where the variability in the Fλ1/Fλ2 arises from both stimulation of UV screening by natural UV radiation in the field and by better UV screening on the adaxial than on the abaxial side (Kolb and Pfündel 2005). Figure 5C depicts data from various thick-leaved species. Thickness of the leaves from *R. diphyllum* ranged from 0.3 ± 0.1 mm (mean value ± standard deviation) and in *A. haworthii* up to 3.8 ± 0.2 mm. In comparison, leaf thickness was 0.2 ± 0.01 and 0.2 ± 0.04 in *Hordeum* and *V. vinifera* leaves, respectively. In Fig. 5C, variations in the Fλ1/Fλ2 arise from mixed effects including exposure conditions, leaf side, and species-dependent traits.

For the three groups of leaves, clear linear associations were observed between data obtained by Dualex and the UV-A-PAM fluorimeter. Further, neither slopes nor intercepts of the three regression lines differed in a statistically meaningful way. Thus, the consistency of regression analyses suggests that the relationship between data from the two fluorimeters is robust and not affected by the different leaf anatomies existing in monocot (*Hordeum* species) and dicot leaves (*V. vinifera*), or by leaf thickness.

All three regression lines in Fig. 5 exhibited slopes > 1 and ordinate intercepts < 0, and these deviations were all statistically significant except for the ordinate intercept in Fig. 5C. The behavior of the data is consistent with a higher contribution of strayed or reflected excitation radiation to the measured signal in the UV-A-PAM than in the Dualex fluorimeter. For example, different filter sets or optical arrangements might have caused a different degree of false signal detection. Indeed, by assuming the same chlorophyll fluorescence yield of 0.02 in both fluorimeters, and that 0.0 and 0.1% of excitation intensity is falsely detected by the Dualex and the UV-A-PAM fluorimeter, respectively, simple model calculations (not shown) reproduced the behavior of the data observed in Fig. 5. On the other hand, chlorophyll fluorescence yield is probably higher during measurements in the Dualex than in the UV-A-PAM fluorimeter because of partial reaction center closure under relatively high excitation intensities in the Dualex fluorimeter (see Material and methods). Hence, we also calculated the effect of higher fluorescence yield in the Dualex than in the UV-A-PAM fluorimeter (0.06 and 0.02, respectively), but presupposed that both fluorimeters detect the same percentage of excitation radiation as false signal (0.1%). These calculations also yielded a relationship that was very similar to those shown in Fig. 5.

In summary, both calculations agree in suggesting a higher influence of stray excitation radiation on the total signal in the UV-A-PAM than in the Dualex fluorimeter; but, this difference could originate in the optical peculiarities of the instruments or in variations in chlorophyll fluorescence yield, or a combination of both. Certainly, our data does not

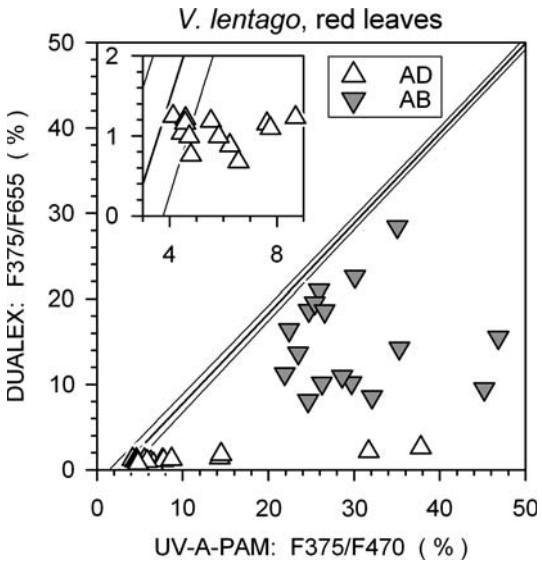

Fig. 6 Fluorescence excitation ratios (FER) measured with red leaves of *V. lentago*. FER values obtained by a Dualex fluorimeter and a UV-A-PAM fluorimeter are plotted against each other as explained for Fig. 5. The insert shows data with F375/F470 <10% at amplified scales. Data from adaxial and abaxial leaf sides are drawn as open and grey-filled symbols, respectively. For comparison, the result of regression analysis of all data points in Fig. 5 is shown as straight line with 95% confidence intervals (see text for equation and statistical information on regression results)

rule out the possibility that strayed excitation radiation is negligible in the Dualex fluorimeter. Therefore, it is always advisable to assess the amount of falsely detected radiation using suitable non-fluorescing samples.

Comparison of fluorimeters using red leaves

When red leaves were investigated, the relationships between the Fλ1/Fλ2 measured by the two fluorimeters often diverged from those established with green leaves as illustrated in Fig. 6, which compares the data from *V. lentago* with a consensus regression line for green leaves obtained by considering all data points in Fig. 5.

To explore the latter phenomenon, quantum-corrected fluorescence excitation spectra were recorded with a green and a red leaf from shaded and exposed regions, respectively, of the same *V. lentago* plant (Fig. 7A). With the green leaf, the ratio of efficiency for fluorescence excitation at 470 nm to that at 647 nm (F470/F647) was 1.3:647 nm was the upper wavelength limit of our excitation spectra (Fig. 7A). In the red leaf, the F470/F647 ratio was only 0.85 because excitation efficiency at 470 nm was decreased by 40% in the red compared to the green leaf but the value at 647 nm dropped only slightly. Therefore, particularly inefficient fluorescence excitation at 470 nm in red leaves of *V. lentago* explains why the FER measured with the UV-A-PAM fluorimeter (F375/F470) are conspicuously higher than those recorded by Dualex fluorimeter (F375/F655; Fig. 6).

In variance to excitation spectra, absorptance spectra exhibited comparable values at 470 nm for both leaf types (Fig. 7B). Further, the red leaf compared to the green leaf showed clearly higher absorptance in the range from 500 to 600 nm but lower values at longer wavelengths at which chlorophyll absorption predominates. This means that red leaves contain less chlorophylls than green ones but have synthesized pigments that absorb at wavelengths <600 nm. Consistent with absorptance spectra, acetonic leaf extracts revealed smaller absorbance of chlorophylls in the red than in the green leaf but acidic methanolic extracts of red, but not of green leaves, yielded a distinct absorbance spectrum peaking at 525 nm (Fig. 7C). Taken together, Fig. 7 suggests that absorption by red pigments in leaves of *V. lentago* screens chlorophylls from excitation radiation of 470 nm wavelength so that fluorimetric determinations of epidermal UV-screening by the UV-A-PAM become erroneous.

Absorbance spectra of methanolic extracts from six red-leaf species including *V. lentago* (Fig. 8) agree with spectral characteristics of anthocyanins (Harborne 1988; Strack and Wray 1989; Stintzing and Carle 2004) but could also result from absorption of betalain pigments (cf. Pedreño and Escribano 2000). However, for all the red-leaf species investigated here, we excluded the presence of betalains because they are confined to the order of Caryophyllales (Clement et al. 1994; Stafford 1994), and our 9 red-leaf species are all associated with families of different orders (Table 1). Further, the presence of anthocyanins has been demonstrated for *K. amabilis* and *V. lentago* (Lawrence et al. 1938), for the genus *Parthenocissus* (Bate-Smith 1962), for red varieties of *B. oleraceae* and *L. sativa* (Hrazdina et al. 1977; Kleinhenz et al. 2003) and for the Acanthaceae and Marantaceae families of our tropical under-story plants (Forsyth and Simmonds 1954; Bloom 1976; Nakayama et al. 2000; Lee and Collins 2001).

In summary, screening of excitation radiation at 470 nm by anthocyanins has the potential to distort evaluation of epidermal UV screening by the UV-A-PAM fluorimeter as has previously been reported (Barnes et al. 2000; Goulas et al. 2004) and exploited (Hagen et al. 2006). This is not the case for Dualex measurements that uses a red excitation as reference.

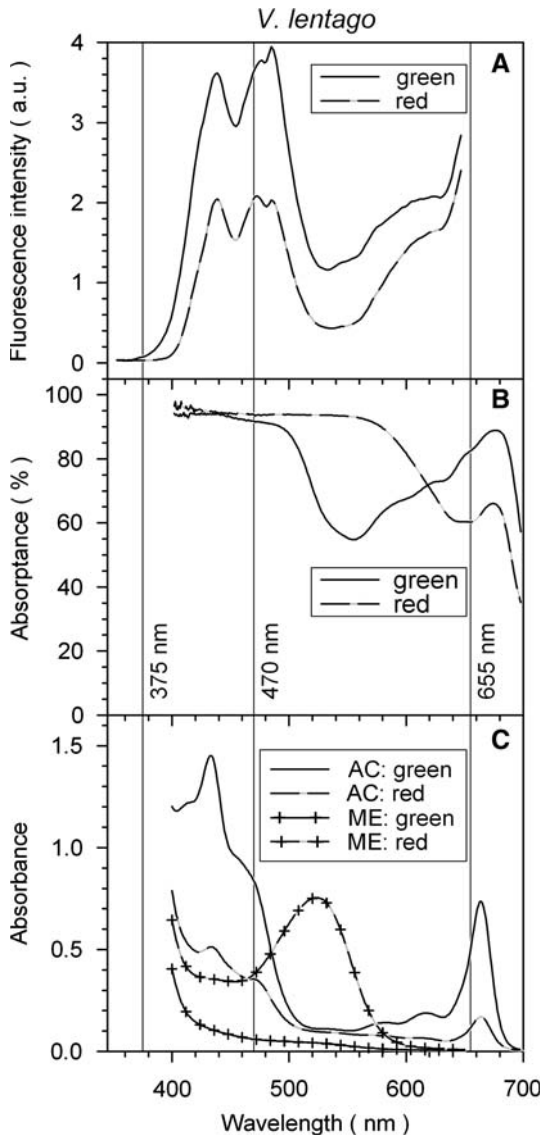

Fig. 7 Spectroscopic characteristics of green and red leaves of *V. lentago*. Panels (**A**) and (**B**) depict quantum-corrected fluorescence excitation spectra and absorptance spectra, respectively, of adaxial sides of green (solid lines) and red (dashed lines) leaves. Panel **C** shows absorbance spectra of acetonic (AC) and acidic methanolic (ME, marked by crosses) extracts from green (solid lines) and red leaves (hatched lines). Central wavelengths of excitation by the Dualex and the UV-A-PAM fluorimeters are indicated by vertical lines

Apparent screening at 375 and 470 nm in red leaves

By introducing the apparent transmittance at 470 nm ($T_{APP}470$), to quantify anthocyanin-dependent screening at 470 nm, we can relate the F375/F470 measured with red leaves to the theoretical $(F375/F470)_{TH}$, which would have been recorded by the UV-A-PAM fluorimeter in the absence of anthocyanins, by using Eq. 1:

$$(F375/F470) = (F375/F470)_{TH} \cdot T_{APP}470^{-1} \quad (1)$$

or

$$T_{APP}470 = (F375/F470)_{TH} \cdot (F375/F470)^{-1} \quad (2)$$

The $(F375/F470)_{TH}$ can be derived from the F375/F655 ratio by considering the relationship between the two fluorescence ratios in green leaves, which was quantified by regression analysis that included all available data points in Fig. 5: the resulting equation is given below but the regression line, together with 95% confidence intervals, is drawn in Figs. 6 and 11:

$$(F375/F655) = 1.053 \cdot (F375/F470)_{TH} - 2.765 \quad (3)$$

The standard deviation of slope and ordinate intercept is 0.013 and 0.646, respectively, and a coefficient of determination of 0.977 was calculated. After rearrangement, we arrive at:

$$(F375/F470)_{TH} = ((F375/F655) + 2.765) \cdot 1.053^{-1} \quad (4)$$

Therefore, by expressing in Eq. 2 the $(F375/F470)_{TH}$ using Eq. 4, the $T_{APP}470$ can be computed. As anthocyanins do not absorb significantly at 655 nm (Fig. 8), the ratio F375/F655 reported by the Dualex fluorimeter is suited to evaluate UV screening in red leaves. To be consistent with the nomenclature introduced for screening at 470 nm, the latter FER will be designated $T_{APP}375$. For all red leaves investigated here, the $T_{APP}375$ and the $T_{APP}470$ expressed as percentages are depicted in Fig. 9. These data are paralleled by the microscope images shown in Fig. 10.

All of our four species showing autumnal leaf coloration exhibited low values for $T_{APP}375$ on adaxial leaf sides that ranged between 1 and 6% but abaxial sides were clearly more UV transparent (Fig. 9A–D). This observation agrees with previous reports of more efficient UV screening in sun-exposed, upper leaf sides than on lower leaf sides (Bilger et al. 1997; Grammatikopoulos et al. 1999; Barnes et al. 2000; Markstädter et al. 2001; Ounis et al. 2001; Krause et al. 2003; Kolb and Pfündel, 2005).

Three red-leaf species (*K. amabilis*, *V. lentago*, and *V. phlebotrichon*) showed adaxially measured $T_{APP}470$ of about 50% indicating significant screening at 470 nm by anthocyanins (Fig. 9A, C and D) but the adaxially measured $T_{APP}470$ of *P. tricuspidata* was 80% (Fig. 9B). Red coloration indicates anthocyanins in the palisade parenchyma in *P. tricuspidata*, *V. lentago* and *V. phlebotrichon* but this was less evident in *K. amabilis* (Fig. 10 A–D). Reddish tints

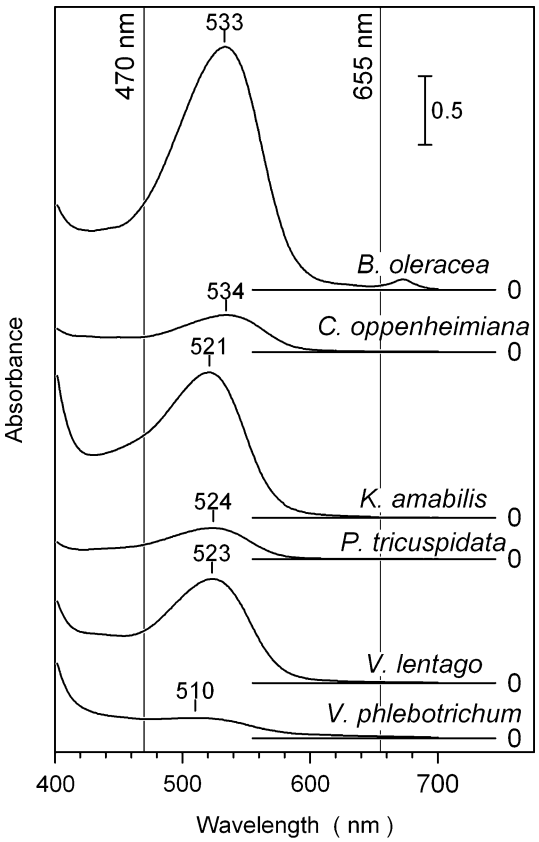

Fig. 8 Absorbance spectra of acidic methanolic extracts of six red leaves. Original absorbance spectra and central wavelengths of visible excitation (vertical lines) by the Dualex and the UV-A-PAM fluorimeter are shown

again suggest the presence of anthocyanins in the adaxial epidermis of *K. amabilis* and *V. phlebotrichon* (Fig. 10A, D). Unequivocally, epidermal layers of leaves of *P. tricuspidata* appear transparent suggesting that epidermal anthocyanins are absent (Fig. 10B). Whether epidermally located anthocyanins also occur in *V. lentago* is undecided because the relatively small epidermal cells were difficult to analyze by microscopy (Fig. 10C). However, similar values of $T_{APP}470$ for both leaf sides of *V. lentago* (Fig. 9C) are difficult to explain by adaxially located palisade-parenchyma anthocyanin screening (Fig. 10c) and would be more readily explained by comparable screening of upper and lower epidermis.

In autumn leaves, therefore, we propose that efficient adaxial screening at 470 nm occurs when anthocyanins are present in both the epidermis and the palisade parenchyma (*K. amabilis* and *V. phlebotrichon*), but when anthocyanins are confined the palisade parenchyma (*P. tricuspidata*) screening is less effective. Accordingly, the importance of epidermal location of UV-absorbing phenolics for efficient UV screening in leaves has been reported (Caldwell et al. 1983; Hutzler et al. 1998; Kolb et al. 2001).

In contrast to autumn leaves, leaves of tropical shade plants and of *L. sativa* restrict anthocyanins exclusively to the epidermis (Fig. 10F–I). Therefore, in these leaves, significant reduction in $T_{APP}470$ solely arises from epidermal screening. The ''epidermal'' $T_{APP}470$ was approximately 50% in the abaxial sides of tropical shade leaves but was 70–80% in *L. sativa* (Fig. 9F–I). This means that epidermally located anthocyanins have a considerable potential for screening out radiation at 470 nm but the degree of screening is variable. The precise reasons for these variations are unidentified. Moreover, the $T_{APP}470$ was lowered to 35% in *B. oleracea* leaves, which possess narrow layers of anthocyanins just below the adaxial and abaxial epidermis: this suggests that efficient screening at 470 nm can also be brought about by non-epidermal anthocyanins (Figs. 9E and 10E).

To summarize, our data show that autumnal anthocyanins can screen out about half of radiation intensities at 470 nm. Since radiation of 470 nm is well absorbed by the major antenna of PS II (the light-harvesting complex II), anthocyanin screening of radiation can effectively reduce excitation energy in the PS II complex and, hence, prevent damage by reactive oxygen species, which may be formed during disassembly and degradation of the photosynthetic apparatus during autumn (Hoch et al. 2001; Steyn et al. 2002). Our findings agree with those of Smillie and Hetherington (1999), which demonstrated in pods of the Fabacean *Bauhinia variegata* that photoinhibition by radiation centered at 460 nm is reduced by anthocyanins. Further, the presence of anthocyanins was found to lower the photoinhibitory effects of blue radiation (400–550 nm) in senescing leaves of *Cornus stolonifera* (Cornaceae) (Field et al. 2001): in the latter species, however, epidermal anthocyanins were not detected possibly due to the preparation of thin microscopic sections (15 μm), which may have ruptured epidermal cells (cf. Pfündel et al. 2006).

Clearly, the absence of significant reduction of $T_{APP}470$ in *P. tricuspidata* (Fig. 9B) or the abaxial location of anthocyanins in tropical shade plants (Fig. 10G–I) is inconsistent with a photoprotective role of anthocyanins; in these leaves, therefore, anthocyanins may primarily function as anti-oxidative agents or in plant defense mechanisms against herbivores (Close and Beadle 2003; Gould 2004; Manetas 2006).

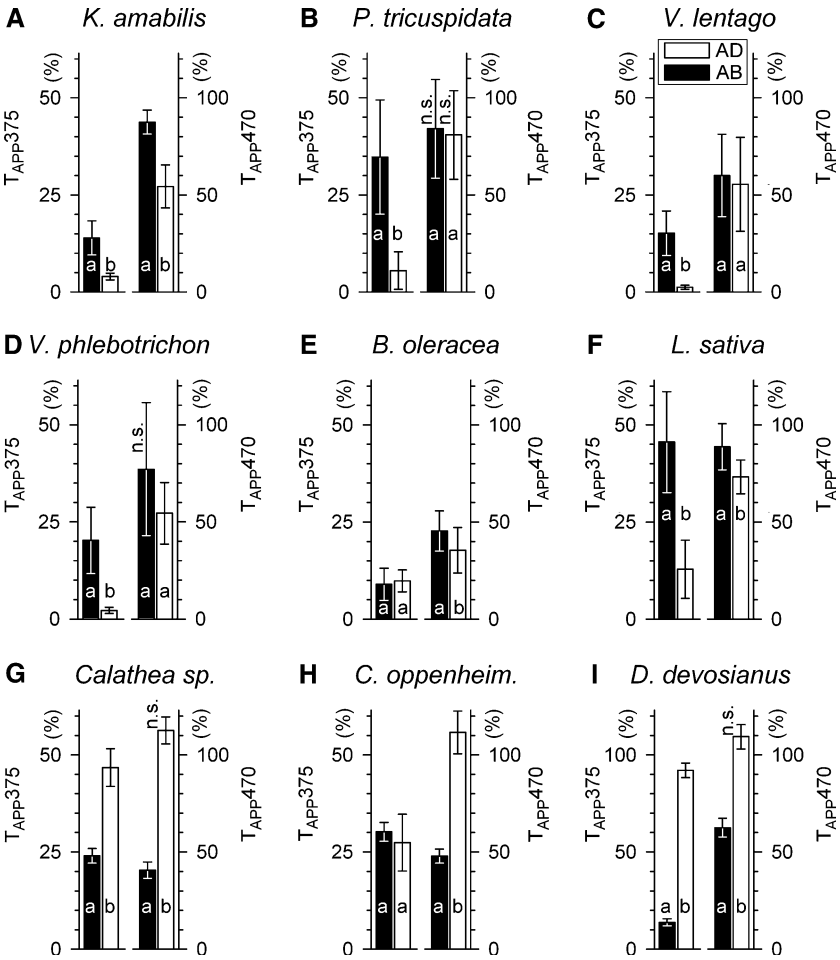

Fig. 9 Apparent transmittance of red leaves. Apparent transmittance at 375 and 470 nm ($T_{APP}375$ and $T_{APP}470$, respectively) of adaxial and abaxial surfaces (open and closed bars, respectively) for nine red-leaf species are shown. The $T_{APP}375$ was directly derived from the F375/F655 data of the Dualex fluorimeter, and the $T_{APP}470$ was calculated with UV-A-PAM data by considering the deviation of the F375/F470 of a red leaf from the theoretical F375/F470 establishes for green leaves (see text for further details). Different characters in a pair of data indicate statistically significant differences between adaxial and adaxial transmittances. Error bars signify standard deviations obtained with n = 5, 7, 17, 5, 15, 8, 5, 16, and 5 observations in panels A to I, respectively. Labeling of error bars by "n.s." indicates that mean values are not statistically significant different from 100% transmittance

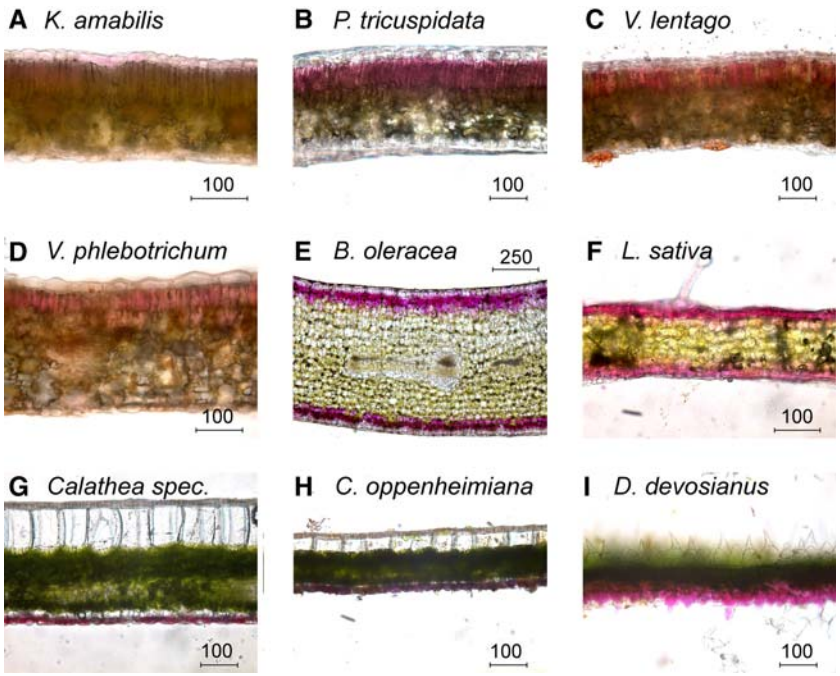

Fig. 10: Bright field microscopy of cross section of red leaves. Cross sections of nine red-leaf species are shown with adaxial leaf side up. Horizontal bars depicts intervals of 100 μm except in panel E where bar length corresponds to 250 μm

Comparison of fluorimeters using Chl *b*-less leaves

As in red leaves of *V. lentago* (Fig. 6), leaves of the Chl *b*-less mutants of *H. vulgare* exhibited much higher values of F375/F470 than of F375/F655 resulting in clear deviations from the relationship between excitation ratios established with green leaves (Fig. 11). Quantum-corrected fluorescence excitation spectra of a Chl *b*-less leaf show that fluorescence excited at 470 nm was only 20% of the value observed with the wild-type leaf (Fig. 12A); however, at the long-wavelength border of our excitation spectra, only a 50% lower value occurred in the mutant. We conclude, as for red leaves from *V. lentago*, that inefficient fluorescence excitation at 470 nm results in larger values for FER when measured by the UV-A-PAM fluorimeter rather than the Dualex. In contrast to red leaves, anthocyanin-dependent screening cannot explain the elevated F375/F470 because methanolic extracts from leaves of the Chl *b*-less mutant did not absorb significantly at wavelengths greater than 420 nm (not shown).

In the wild-type excitation spectrum, the peaks observed at 437, 475, and 485 nm almost certainly arise from absorption of Chl *a*, Chl *b*, and xanthophylls, respectively (Pfündel and Baake 1990; Marquardt and Bassi 1993). The two long-wavelength peaks (475 and 485 nm) were not apparent in the excitation spectrum of the Chl *b*-less mutant and its second derivative

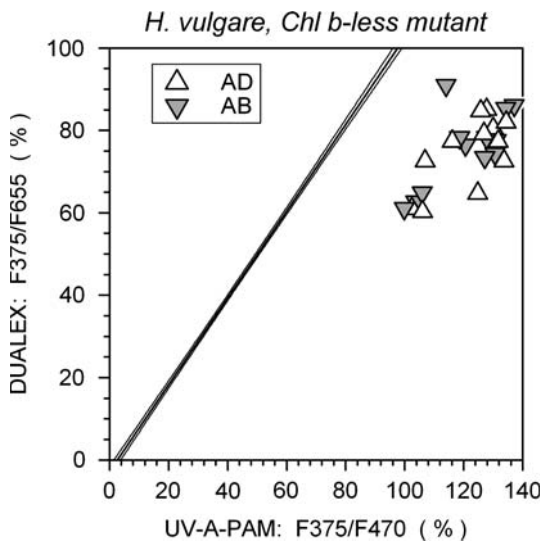

Fig. 11 Fluorescence excitation ratios (FER) measured with Chl *b*-less mutants from *H. vulgare*. FER values from the Dualex fluorimeter are plotted against those from the UV-A-PAM fluorimeter. Data from adaxial and abaxial leaf sides are drawn as open and grey-filled symbols, respectively. The regression line obtained with green leaves is depicted for comparison (see Fig. 6 for comments on regression analysis)

(Fig. 12A, B). Therefore, the inefficient excitation at 470 nm in the Chl *b*-less mutant is due to lack of absorption by Chl *b* and of xanthophylls which are both organized, in the wild-type leaf, in the light-harvesting complex II and contribute significantly to the total light absorption of photosynthesis (Siefermann-Harms 1985).

Remarkably, the clearly decreased efficiency for fluorescence excitation between 450 and 500 nm in the Chl *b*-less mutant was associated with only a minor reduction of leaf absorptance (Fig. 12C). In the latter spectral region, the mutant leaf showed an absorptance maximum at 490 nm probably corresponding to absorption by the xanthophylls (Pfündel and Baake 1990). Comparison of absorptance and fluorescence excitation spectra suggests, therefore, that chloroplasts of the Chl *b*-less mutant contain significant amounts of xanthophylls that are not involved in light harvesting.

The fluorescence excitation spectrum of the Chl *b*-less mutant exhibited a peak at 497 nm (Fig. 12A). This peak was not observed in the corresponding absorptance spectrum and its second derivative (Fig. 12C, D). The latter spectrum, however, exhibited a minimum at 508 nm. In comparison, we detected a minimum of around 510 nm in second derivatives of absorptance spectra from barley wild-type leaves (Fig. 12D) and leaves of many other plant species (data not shown). Theoretically, this minimum might arise from absorption at 510 nm by a particular fraction of the xanthophyll, lutein, which is located in trimers of the light-harvesting complex II (Ruban et al. 2000). Since light-harvesting proteins are absent in Chl *b*-less barley mutants (Bossmann et al. 1997), however, we cannot explain our 508 nm minimum by lutein absorption. Therefore, the origin of the minima around 510 nm in second derivative absorptance spectra is unclear. In any case, the spectrum of the hypothetical compound giving rise to 508 nm absorption is too much red-shifted to explain the fluorescence excitation peak at 497 nm of the mutant leaf (Fig. 12A).

Also, absorbance spectra of acetonic extracts from mutant leaves did not reveal bathochromically shifted carotenoids to explain the 497 nm peak; thus, we assume that the xanthophylls absorbing at 490 nm (Fig. 12C) compete with absorption by functional carotenoids in photosystems, and thereby reduce the efficiency of excitation of chlorophyll fluorescence. Specifically, we suggest that the peak at 497 nm arises from excitation of light-harvesting carotenoids, which, at wavelengths of 490 nm and below, are reduced due to competing xanthophyll absorption and, in the range of 500–530 nm, drops to zero owing to the known absorption properties of carotenoids. Our hypothesis is

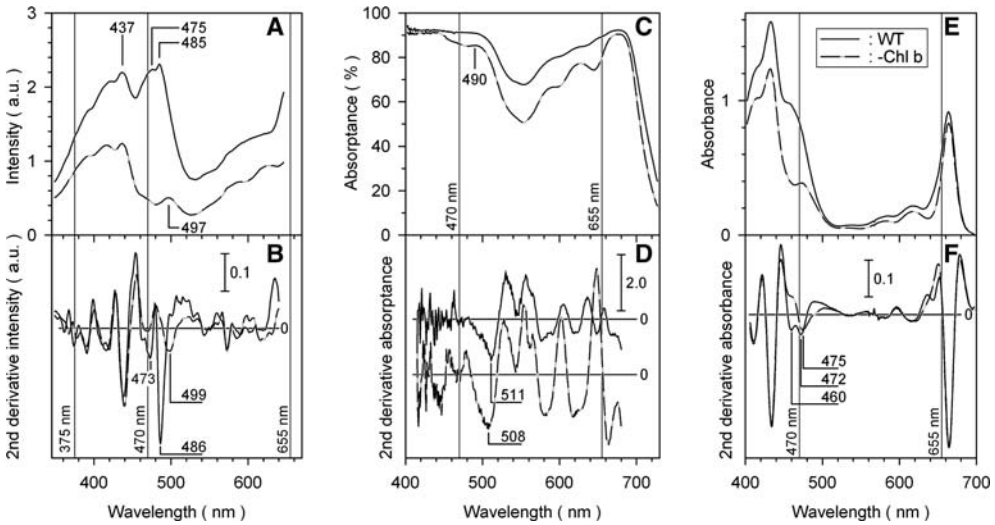

Fig. 12 Spectroscopic characteristics of leaves from wild-type and Chl *b*-less mutants of *H. vulgare*. Panels (**A**) to (**C**) show quantum-corrected fluorescence excitation spectra, absorptance spectra, and absorbance spectra of acetonic extracts, respectively, of wild-type (solid lines) and Chl *b*-less mutants (dashed lines) leaves. Second derivatives of spectra are depicted below the original spectra. Vertical lines indicate the central wavelengths of excitation in the visible by the Dualex and the UV-A-PAM fluorimeters

consistent with a quantitative model that describes fluorescence excitation spectra of Chl *b*-deficient leaves (Pfündel and Baake 1990).

We conclude from the spectral data of Fig. 12 that the high F375/F470 ratio in Chl *b*-less leaves of *H. vulgare* (Fig. 11) arise from the expected lack of absorption by Chl *b* but, in addition, from competing absorption of radiation by xanthophylls which are not functionally associated with chlorophylls. If we accept that non-photosynthetic carotenoids can reduce availability of light for photosynthesis, then a carotenoid contribution to the low values for $T_{APP}470$ observed with autumn leaves (Fig. 9) cannot be excluded. Hypothetically, combined light absorption by carotenoids and anthocyanins, could reduce intensities of radiation in the entire blue-green spectral region to relieve high-light stress in autumn leaves.

Conclusion

Using artificial filters and green leaves exhibiting various natural UV screening, we have demonstrated general agreement between data obtained with the Dualex and UV-A-PAM fluorimeters: this implies that fluorescence measurements are unaffected by the different optical paths which fluorescence takes to reach the detector (Fig. 1). The flexible light guide employed by the UV-A-PAM fluorimeter, however, allows evaluation of UV screening in fruits (Kolb et al. 2003; Hagen et al. 2006) while the Dualex leaf clip restricts its use to relatively flat samples. Refined analysis of our data suggests that lower contributions of stray radiation to the total signal are recorded by the Dualex than by the UV-A-PAM fluorimeter. The factors influencing false signals by stray light need to be investigated to optimize fluorescence detection in the next generation of portable fluorimeters.

We have shown that the two fluorimeters deviate from each other when red leaves or Chl *b*-less leaves were investigated and that these deviations arise from inefficient excitation of chlorophyll fluorescence at 470 nm, which is the excitation wavelength in the visible range of the UV-A-PAM fluorimeter. In fact, the combined results of two fluorimeters allowed us to retrieve information on screening of blue radiation by anthocyanins in addition to screening of UV radiation by phenolics, which would not have been possible by any of the two fluorimeters. To monitor filtering properties of leaf surfaces simultaneously in both the ultraviolet and visible spectral range, portable fluorimeters are required, which employ excitation at several wavelengths. Such instruments will not only aid the understanding of plant stress responses but also provide an important measuring tool for agriculture so that flavonoid and anthocyanin concentrations in foodstuff can be non-invasively monitored (Kolb et al. 2003; Goulas et al. 2004; Agati et al. 2005; Hagen et al. 2006).

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