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In vivo epidermal UV-A absorbance is induced by sunlight and protects *Soldanella alpina* leaves from photoinhibition

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This work is dedicated to Serge Aubert.

Abstract. Alpine plants like *Soldanella alpina* L. are subjected to high PAR and high UV radiation. Among the important photoprotective mechanisms that prevent photoinhibition under such conditions, passive optical barriers such as UV-absorbing compounds were investigated. In this study, temporal and spatial patterns of epidermal UV-A absorbance for *S. alpina* leaves were investigated with a combination of absorbance measurements at 375 nm and imaging methods. UV-A absorbance was highest in plants acclimated to full sunlight and was markedly stable during the leaves' lifetime. UV-A absorbance was correlated with leaf structure (leaf mass per area ratio, density of epidermal cells and stomata) and biochemical features such as chlorophyll and carotenoid content and ratio, which are characteristics of light acclimation. UV-A-absorbing compounds were mainly localised in the epidermal vacuoles and trichomes. Leaves with low UV-A absorbance were significantly more photosensitive than leaves with high UV-A absorbance. However, the epidermal UV-A absorbance increased in low-absorbance leaves under full sunlight even in the absence of UV radiation. Results suggest that high epidermal UV-A absorbance protects *S. alpina* leaves from photoinactivation, which is especially important after snowmelt, when plants are suddenly exposed to full sunlight.

Additional keywords: Alpine plants, chlorophyll fluorescence, flavonoids, light acclimation, microscopic imaging.

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Introduction

At high altitudes, the maximum solar intensity of PAR and UV-A (315–400 nm) and UV-B (280–315 nm) radiation is generally higher than at low altitudes (Blumthaler 2012). High PAR as well as UV-A and UV-B intensities can impair photosynthetic activity in nonacclimated plants. Recently, Takahashi *et al*. (2010) identified UV radiation as the most efficient radiation for inducing photoinhibition of PSII. UV-A radiation may induce photodamage of the Mn cluster of the oxygen evolving complex, thus rendering PSII more vulnerable to visible sunlight-induced inhibition (Vass *et al*. 2002; Takahashi *et al*. 2010). In addition, isolated catalase is inactivated by UV-A radiation (Aubailly *et al*. 2000; Feierabend 2005).

Under natural growing conditions, UV-absorbing compounds usually protect photosynthesis from UV-induced photoinactivation (Hakala-Yatkin *et al*. 2010). Accordingly, several alpine plant species investigated in their natural environment, including *Soldanella alpina* L., showed neither any apparent photoinhibition of PSII nor inactivation of catalase (Streb and Cornic 2012), whereas isolated thylakoids and catalase enzymes from the same species were readily photoinactivated by light (Streb *et al*. 1997). The importance of UV-absorbing compounds in mediating the phototolerance of *S. alpina* leaves is presently unknown.

Flavonoids increase with altitude, including anthocyanins and phenolics as the main UV-absorbing compounds in plants (Rozema *et al*. 1997; Filella and Penuelas 1999). Comparative measurements of UV-absorbance in Alpine and Arctic varieties of the same species showed lower UV transmittance in the alpine varieties under natural conditions but no such difference was seen when plants were grown under common laboratory conditions, suggesting that UV-absorbing compounds are induced by natural light (Nybakken *et al*. 2004). Recently, Barnes *et al*. (2013) showed that PAR radiation is more important than UV radiation for the induction of low UV transmittance and the accumulation of UV-absorbing compounds, but the presence of UV was necessary

to induce maximum protection. Furthermore, leaves developed under shade conditions adjusted their UV transmittance when transferred to sunlight, whereas sun-leaves transferred to shade conditions did not change their UV-shielding potential (Barnes *et al*. 2013).

The development of new nondestructive portable fluorescence techniques for measuring the UV absorption of the upper and lower leaf surface has facilitated the estimation of epidermal UV-absorbing compounds under field conditions (Goulas *et al*. 2004) and the imaging of UV-A absorbance in the laboratory (Meyer *et al*. 2009). In the present investigation, these techniques were used to investigate the temporal and spatial patterns of UV-A absorbance of leaves in *S. alpina.* Leaves of *S. alpina* can survive at least two vegetation periods, acclimate to various light microclimates at different altitudes (1800–2400 m) and can be cultivated in a growth chamber under cold and continuous low light conditions. The UV-A absorbance of leaves under snow and after snowmelt was investigated *in situ*. The effects of UV radiation and sun exposure on the induction of high UV-absorbance was then evaluated in mature and newly developed leaves. Finally, the impact of UV-A absorbance on photoinhibition and the UV-A absorbance pattern over the leaf surface were investigated to estimate the relationship between leaf structure and photoprotection.

Materials and methods

Plant material and sites description

Soldanella alpina L. plants were investigated at the south slope of the Lautaret and Galibier Passes in the French Alps at two growing sites.

The first site (Site 1) was located at 2100 m in altitude near the Alpine research station of the Lautaret Pass. At this site, *S. alpina* plants were collected from a slope facing north-east. Site 2 was located at 2400 m in altitude near the south route of the Galibier Pass on a slope facing south-east, which was described in Laureau *et al*. (2011).

S. alpina plants from both sites were transferred into pods with soil from the growing site and further cultivated in a growth chamber under controlled conditions (24 h light at 100 µmol m^{-2} s^{-1} PAR, mean temperature 6°C) as described in Laureau *et al*. (2013). Individual leaves of *S. alpina* at the growing site and from plants transplanted into pods were marked with tape and their UV-A absorption was followed during the growing season or during the leaf's lifetime. Leaves were marked to distinguish those that survived the preceding winter and those developing under the described conditions.

Manipulation of light conditions at the natural site

The light conditions of *S. alpina* leaves from both sites were manipulated. The UV-A absorbance of mature leaves developed at Site 1 in a shaded subset was followed during 28 days in July 2012 and 2013. In a parallel subset, surrounding vegetation shading *S. alpina* was removed and leaves were exposed to either full sunlight or to sunlight passing a UV exclusion filter (Plexiglas PVCG, Air and Eau Systèmes, Ludres, France; cutoff filter at 400 nm) as described in Bachereau *et al*. (1998). The UV exclusion filter slightly attenuated PAR by 12%. At Site 2, the UV-A absorbance of all leaves from different plants was measured after snowmelt and leaves were marked. One set of leaves (40%) was protected by the UV exclusion filter, a second set (37%) of leaves was shaded by green nylon to give a ~50% reduction in incident PAR as described in Laureau *et al*. (2013) and a third set (23%) was exposed to full sunlight. The transmission spectrum of the green nylon filter is shown in Fig. S1, available as Supplementary Material to this paper, showing an equal absorption of the radiation spectrum between 300 nm and 700 nm. The leaf temperature (°C) and the incident PAR at the leaf level were recorded every 30 s with a datalogger (CR23X, Campbell Scientific, Logan, UT, USA) on representative leaves as described in Laureau *et al*. (2011). The size of the filters was ~1 m^2 and they were placed to protect leaves completely from sunlight (shade) or UV radiation while allowing sufficient aeration of leaves.

Optical measurements of UV absorbance and leaf chlorophyll

UV absorbance was measured from both leaf sides of attached leaves with a leaf-clip device (Dualex, Force-A, Orsay, France) as described in Goulas *et al*. (2004). In plants growing at their natural site, a UV-A Dualex meter was used to measure absorbance at 375 nm (DA_{375}) by comparing the chlorophyll fluorescence yield excited at 650 nm and at 375 nm. The excitation at 375 nm lies in the absorption range of flavonoids (Goulas *et al*. 2004).

For laboratory experiments, absorbance at 322 nm was measured with a UV-B Dualex meter from the same leaf location as measured with the UV-A meter. The UV-B Dualex meter compares the fluorescence yield at 650 nm and at 322 nm and allows estimation of the hydroxycinnamic acid (HCA) content (Latouche *et al*. 2013). Since HCA estimation is modified by flavonoids, the results were corrected by the estimated flavonoids content according to Latouche *et al*. (2013). Ten readings from each Dualex meter were taken, including five from each leaf side. Results were used to estimate the total flavonoids and the total HCA content of the leaf epidermis (Goulas *et al*. 2004). Leaves were then stored overnight under humid atmosphere and low temperature conditions, and examined microscopically the following day at an imaging facility (ImagoSeine, Institut Jacques Monod, Paris, France; see fluorescence stereomicroscopy and microscopy section).

Estimation of PSII photoinhibition

PSII photoinhibition was estimated from chlorophyll fluorescence at ambient temperature either on attached leaves at the natural growing site or on detached leaves. At the growing site, leaves were measured at midday and dark-acclimated with the leaf clip (Walz, Effeltrich, Germany) for 30 min. Subsequently, F_0 and F_m values were determined with a Mini-Pam (Walz).

Detached leaves were kept in either water or a solution of 3 mM lincomycine as described in Laureau *et al*. (2013). After 1 h dark incubation, F_0 and F_m values were measured. Leaves were then exposed to direct sunlight or to sunlight passing through the UV exclusion filter at 22°C. Measurements of F_0 and F_m were taken after 30-min dark acclimation of leaves following the light period. The F_v/F_m ratio was taken as the maximum quantum yield

of PSII (Streb *et al*. 1997) and the results are shown as a percentage of untreated control leaves.

Leaf fluorescence spectroscopy

Fluorescence emission spectra were recorded with a Cary Eclipse spectrofluorimeter (Varian Inc., Les Ulis, France) as described in Bidel *et al*. (2007) and Meyer *et al*. (2009). Spectra were acquired on both leaf sides on a leaf area 6 mm in diameter under UV excitation (365 nm) at room temperature (20°C) and under dim light. Spectra were corrected and smoothed as described in Cerovic *et al*. (2002). Fluorescence intensity was expressed in quinine sulfate equivalent units (Cerovic *et al*. 2002). One quinine sulfate equivalent unit corresponds to the fluorescence of 1 pmol mL^{-1} of quinine sulfate in a 1-cm light path of 0.105 mol L^{-1} perchloric acid in water (1 pmol cm^{-2}), excited at 347.5 nm and measured at 450 nm under identical measuring conditions.

Fluorescence stereomicroscopy and microscopy

Stereomicroscopic and microscopic images were taken from the same leaf area used for spectroscopy. The whole leaf was placed under a stereomicroscope (SteREO Lumar ver.12, Zeiss, Göttingen, Germany) equipped with a colour-cooled Charge-Coupled Device camera (AxioCam HRc, Zeiss) and a 100-W mercury vapour excitation lamp (HBO, Zeiss). Leaf fluorescence was imaged on both leaf sides using a 330WB80 excitation filter (Omega, Battleboro, VT, USA) and a LP400 emission filter (Omega). Chlorophyll fluorescence was imaged using UV-A (357/44, Semrock, Rochester, NY, USA) or green (535/50, Semrock) excitation filters and an orange glass emission filter (OG590, Schott, Vanves, France). The 42-bit colour images of 1388 × 1040 pixel size corresponded to an area of 0.42 cm^2 at a 12× magnification (NeoLumar S 1.5× quartz objectives, Zeiss).

The fluorescence microscopy method followed Meyer *et al*. (2009). The samples were mounted in water with Tween 20 (0.5%, v/v) (Sigma, St Louis, MO, USA). Fluorescence images were also acquired on transverse leaf sections. Sections were cut manually with a razor blade. Images of fluorescence were made with an inverted epifluorescence microscope (Axiovert 200, Zeiss) equipped with a monochrome-cooled CCD camera (AxioCam MRm, Zeiss), a colour-cooled CCD camera (AxioCam HRc, Zeiss) and a 100-W mercury vapour excitation lamp (HBO, Zeiss). Visible leaf fluorescence was imaged using a 330WB80 excitation filter (Omega), a long-pass dichroic beam splitter (DCLP400, Omega) and a LP400 emission filter (Omega). Leaf blue fluorescence was imaged using a 330WB80 excitation filter, a long pass dichroic beam splitter (390DRLP02, Omega) and a 450WB80 emission filter (Omega). The 12-bit monochrome and 42-bit colour images of 1388 × 1040 pixel size corresponded to an area of 1.7 × 1.3 and 0.22 × 0.17 mm^2 at 50× and 400× magnification (Plan-Neofluar 5× and Plan-Apochomatic 40× quartz objectives, Zeiss), respectively.

Stereomicroscopic and microscopic images of epidermal UV absorbance at 365 nm were analysed according to the logFER (logarithm of the chlorophyll Fluorescence Excitation Ratio) method (Cerovic *et al*. 2002) and compared with DA_{375} values as described in Meyer *et al*. (2009). The trichome density was determined by counting trichomes on stereomicroscopic images of UV-excited visible fluorescence or UV-A absorbance. Ground epidermal cells and stomata density were determined by counting the cells on UV-excited blue fluorescence images obtained by microscopy.

For stereomicroscopy and microscopy, experiments were automated using Axio Vision software (AxioVision ver. 4.4, Zeiss), and images were processed using ImageJ 1.36b (National Institutes of Health, Bethesda, MD, USA).

Leaf mass per area

The whole leaf or three leaf discs (0.7 mm in diameter) corresponding to the Dualex measurements were sampled and oven-dried for 72 h at 60°C before being weighed. Leaf mass per area (LMA) was measured as the ratio of leaf DW to area (g m^{-2}). For the light manipulation experiments at Sites 1 and 2, at least three values were averaged per treatment.

Biochemical measurements

For all biochemical measurements, leaves were collected in the morning and extracted in 50 mM KH_2PO_4/K_2HPO_4 (pH 7.5) as described in Laureau *et al*. (2011). Measurements of catalase activity (EC 1.11.1.6), glycolate oxidase (EC 1.1.3.1), cytoplasmic ascorbate peroxidase (EC 1.11.1.11) and total protein content were carried out as previously described (Streb *et al*. 1997).

The chlorophyll and carotenoid content was measured from 80% acetone extracts as described in Laureau *et al*. (2013).

Pigment contents and enzyme activities were also measured from leaves incubated in the presence of 35.5 µM cycloheximide for 6 h in either sunlight or behind a UV exclusion filter.

Data analysis

Statistical differences were analysed with the software package Statistica ver. 6.1 (StatSoft Inc., Maison-Alfort, France) and Sigma Plot (Systat Software, Inc., USA). Differences between means were assessed using Student's *t*-test at the 0.05 significance level. Since more than two different treatments were performed, all treatments were compared in a pairwise fashion. Significant differences were indicated by different letters. In cases where normality was violated, the data were log-transformed. The relationships between DA_{375} and LMA, DA_{375} and absorbance at 322 nm (DA_{322}) and between either DA_{375} or DA_{322} and epidermal cell density were tested from a regression analysis.

Results

Leaf UV-A absorbance of S. alpina *in the field during the leaves' lifetime*

In the field, the highest total leaf UV-A absorbance was measured in sun leaves of *S. alpina* at Site 2 (Table 1), whereas total DA_{375} values were markedly lower in shaded leaves found at Site 1 (1.9 ± 0.2, $n = 16$). Furthermore, total DA_{375} values varying from 1.2 to 2.8 were determined in different leaves from the same plant. Hence, *S. alpina* leaves with varying UV-A absorbance were found *in situ*, often in the same plant, depending on local growing conditions. We therefore classified leaves as having high absorbance (HA, total DA_{375} value higher than 2.5), intermediate absorbance (IA, total DA_{375} between 1.5 and 2.5) and low absorbance (LA, total DA_{375} value lower than 1.5).

Table 1. Leaf UV-A absorption of *Soldanella alpina* at 2400 m altitude (Site 2) measured in June in green and in senescent leaves
Some of the measurements were performed on the previous year's leaves that were still covered by snow. Leaves transferred to a growing chamber for up to 1 year (old leaves) and leaves that developed newly in the growing chamber (new leaves) were also measured. Values are mean ± s.e. Different letters indicate a statistically significant difference at the 5% level, tested by comparing every single leaf type with each other forming pairs for the *t*-test. DA_{375}, absoption as measured by absorbance at 375 nm

Site	Leaves	*n*	Abaxial DA_{375}	Adaxial DA_{375}	Total DA_{375}
Site 2	Green leaves in snow	8	1.10 ± 0.07^{a}	1.82 ± 0.05^{a}	2.92 ± 0.10^{a}
	Green leaves, snowfree	118	1.31 ± 0.03^{a}	1.80 ± 0.02^{a}	3.11 ± 0.05^{a}
	Senescent leaves, snowfree	7	1.47 ± 0.17^{a}	$1.78 \pm 0\ 0.16^{ab}$	3.26 ± 0.33^{a}
Growth chamber	Old leaves	25	1.22 ± 0.26^{a}	1.67 ± 0.17^{b}	2.89 ± 0.35^{a}
	New leaves	23	0.25 ± 0.05^{b}	0.63 ± 0.14^{c}	0.88 ± 0.09^{b}

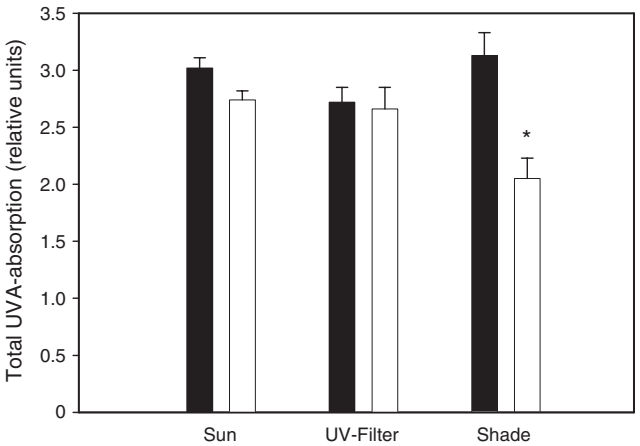

Fig. 1. Total leaf UV-A absorption of *Soldanella alpina*. Leaves were marked in June (black) and compared with leaves that were newly developed (white) during a period of 1 month, under different treatments at Site 2. Leaves were either left under natural conditions (Sun), protected by an UV-exclusion filter (UV Filter) or shaded by green nylon, which reduced the incident PAR by 50% (Shade). Values shown are the means and s.e. of at least six independent leaves. Significant differences at the 5% level are marked with an asterisk.

The UV-A absorbance of *S. alpina* leaves was investigated over the course of their life cycle at Site 2. Leaves surviving the winter (leaves under snow) had nearly the same UV-A absorbance as they did during the first 2 weeks following natural snowmelt (Table 1). There were small variations in leaf absorbance (by 10%, data not shown) during the vegetation period; however, UV-A absorbance remained unchanged when leaves began to senesce. Leaves transferred to a growth chamber with low PAR and low temperature conditions showed no major variation in total DA_{375} compared with leaves measured at the natural site in June (Table 1, old leaves). In contrast, leaves newly developed in the growth chamber had markedly lower total DA_{375} compared with those developed under all other conditions (Table 1).

Induction of high leaf UV-A absorbance by sunlight

In order to differentiate whether UV radiation or high PAR induces the formation of UV-A-absorbing compounds, *S. alpina* leaves at Site 2 were marked after snowmelt and either left under sunlight, protected by a UV-exclusion filter or covered with green nylon to reduce PAR by ~50% (Fig. 1). After 1 month, newly developed leaves had the same total UV-A absorbance as marked leaves, except for those artificially shaded (Fig. 1). This suggests that the formation of UV-A absorbers is induced independently of UV radiation but requires high PAR. Furthermore, the slight reduction of PAR by the UV exclusion filter did not significantly affect UV-A absorbance.

The induction of high UV-A absorbance was investigated in more detail at Site 1. *S. alpina* plants were completely covered by the surrounding vegetation at the end of June and included leaves with very low and intermediate UV-A absorbance. After 28 days, the DA_{375} of LA and IA control leaves covered by the natural vegetation increased only slightly (Fig. 2*a*, *b*). However, after the surrounding vegetation was removed, the DA_{375} of LA and IA leaves increased strongly to a final value of ~2.6. The same increase was induced 2 weeks later on control leaves (Fig. 2*a*, *b*). Leaves exposed to sunlight but protected by the UV filter increased their DA_{375} similar to leaves without the filter, although this increase was retarded and final DA_{375} values were slightly lower than those in direct sunlight (Fig. 2*a*, *b*). Nevertheless, the formation of UV-absorbing compounds was clearly induced in the absence of UV radiation but not in the shade. The different treatments slightly changed the mean daytime leaf temperature. In sun-exposed leaves, the temperature varied between 14°C and 16°C; in leaves shaded by vegetation, the leaf temperature was 18−19°C; in leaves protected by the UV filter, it was 19−21°C (data not shown).

Photosensitivity of leaves with different UV-A absorbance levels

LA leaves exposed to direct sunlight at Site 1 showed a strong decline in F_v/F_m ratios, suggesting photoinhibition. This was observed neither in leaves protected from UV radiation nor in control leaves covered by vegetation (Fig. 2*c*).

Therefore, the photosensitivity of selected HA and LA leaves was investigated (Table 2). As suggested, HA leaves showed characteristic high light acclimation, as indicated by their lower total chlorophyll content, lower chlorophyll : carotenoid ratio, higher chl *a*/*b* ratio and higher ascorbate peroxidase activity compared with LA leaves. HA leaves also showed a trend for higher glycolate oxidase activity; catalase activity was similar to levels found in LA leaves (Table 2, control).

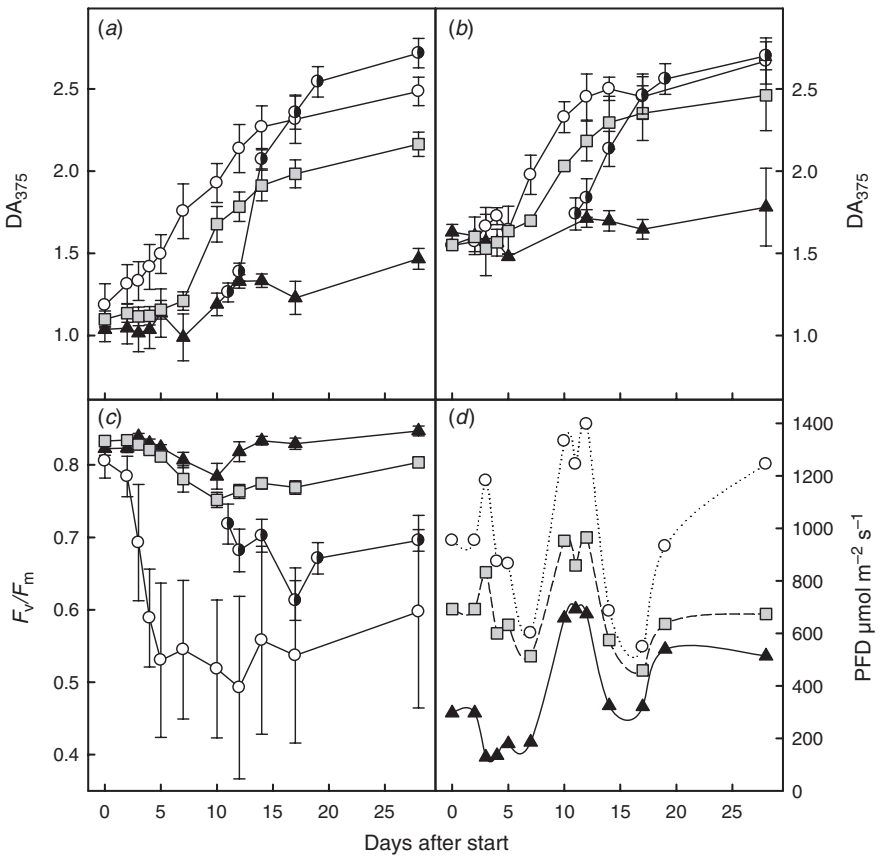

Fig. 2. (*a*, *b*) Induction of total UV-A absorption as measured by absorbance at 375 nm (DA_{375}) by natural sunlight, (*c*) changes in the F_v/F_m ratio and (*d*) mean day (8–18 h) PAR during the observation time. *Soldanella alpina* leaves growing at 2100 m above sea level (Site 1) were selected by their total DA_{375} value and divided into (*a*, *c*) leaves with low absorbance (LA) up to a total DA_{375} index of 1.5 and (*b*) leaves with intermediate absorbance (IA) higher than a total DA_{375} index of 1.5 and lower than 2.5. The vegetation covering the leaves was removed after the first 2 days and leaves were exposed to natural sunlight (white circle) (LA and IA $n \geq 3$) for a period of 26 days (the control was kept under vegetation (black triangle); LA $n \geq 5$, IA $n \geq 4$). One part of the leaves was protected from direct sunlight by an UV-exclusion filter (grey square) (LA $n \geq 19$; IA $n \geq 3$). A second set of leaves was exposed to direct sunlight on Day 11 (black and white circles) (LA $n \geq 11$; IA $n \geq 8$). Shown are mean values and the s.e.

Table 2. Mean PAR during 6 h sunlight treatment, total UV-A absorption as measured by absorbance at 375 nm (DA_{375}), pigment contents (chlorophyll a and b (chl*a+b*)) and ratios (chl *a/b* and total chlorophyll : total carotenoid (chl : car)) and several enzyme activities (catalase, glycolate oxidase (GO) and ascorbate peroxidase (Apx)) of *Soldanella alpina* leaves with low UV-A absorption (LA) and with high UV-A absorption (HA) before (0 h) and after 6 h illumination with full sunlight (Sun) or behind a UV exclusion filter (Sun-UV)

Leaves treated for 6 h were incubated in a solution containing 35.5 µM cycloheximide. Values are means ± s.e. ($n = 5$). Different letters indicates a statistically significant difference at the 5% level, separately comparing each treatment to each other by the *t*-test.

	LA leaves			HA leaves		
	Control	Sun	Sun-UV	Control	Sun	Sun-UV
Mean PAR (µmol m^{-2} s^{-1})		1600	1400		1600	1400
Total DA_{375}	1.33 ± 0.06^{a}			3.13 ± 0.05^{b}		
Chla+b (mg g^{-1} FW)	2.2 ± 0.2^{a}	1.6 ± 0.1^{b}	1.9 ± 0.1^{ab}	1.0 ± 0.04^{c}	0.97 ± 0.05^{c}	0.80 ± 0.04^{d}
Chl*a/b*	3.2 ± 0.1^{a}	3.5 ± 0.08^{a}	3.4 ± 0.1^{a}	4.8 ± 0.1^{b}	4.9 ± 0.2^{b}	4.9 ± 0.2^{b}
Chl : Car	5.3 ± 0.08^{a}	4.8 ± 0.08^{b}	4.6 ± 0.03^{b}	2.9 ± 0.2^{c}	2.7 ± 0.2^{c}	2.7 ± 0.2^{c}
Catalase (µkat g^{-1} FW)	43.1 ± 1.7^{a}	28.9 ± 2.7^{b}	34.3 ± 4.8^{ab}	42.3 ± 6.6^{ab}	31.2 ± 4.7^{b}	29.1 ± 2.2^{b}
GO (nkat g^{-1} FW)	7.8 ± 1.1^{a}	10.2 ± 1.1^{a}	8.4 ± 0.4^{a}	10.1 ± 2.5^{a}	10.6 ± 1.3^{a}	10.4 ± 1.7^{a}
Apx (nkat g^{-1} FW)	84.7 ± 11.5^{a}	103 ± 10.2^{acd}	94.4 ± 9.0^{ac}	132 ± 5.7^{b}	126 ± 11.1^{bc}	147.1 ± 22.8^{bd}

Furthermore, LMA and total DA_{375} were strongly positively correlated in randomly collected leaves (Fig. S2).

To test whether the presence of UV-absorbing compounds in HA compared with LA leaves protects them from light inactivation of catalase and PSII, detached leaves were incubated in the presence of either cycloheximide (Table 2) or lincomycine (Fig. 3). Cylcoheximide blocks cytoplasmic protein synthesis, whereas lincomycine blocks chloroplastic protein synthesis. In the absence of protein synthesis inhibitors, inactivated catalase or PSII activity may be readily compensated by new protein synthesis (Streb *et al*. 1997), thus masking the possible protective effect of UV-absorbing compounds. In LA leaves incubated in cycloheximide and illuminated in sunlight, total chlorophyll content and catalase activity declined significantly compared with leaves measured before the treatment, while UV filtration provided partial protection (Table 2). Although catalase activity also declined in HA leaves, this change was not significant. Surprisingly, the total chlorophyll content decreased in HA leaves protected by the UV filter but not in the absence of the filter.

This result was confirmed by the resulting F_v/F_m ratios in lincomycin-treated leaves illuminated with sunlight. The F_v/F_m ratios of LA leaves declined significantly more than those of HA leaves, while this decline was significantly less severe in LA leaves protected by the UV filter (Fig. 3). In HA leaves, the presence or absence of UV radiation did not significantly impact the decline of the F_v/F_m ratio but leaves in the absence of UV radiation appeared to be slightly more inactivated (Fig. 3). This, together with the results shown in Table 2 (HA leaves: chlorophyll and catalase), indicates that the slight decrease in PAR caused by UV exclusion does not affect the magnitude of photoinactivation. Furthermore, the F_v/F_m ratios of light-acclimated leaves (HA) compared with shade-acclimated leaves (LA) were similar when illuminated in the absence of UV, suggesting no additional protective effect of light acclimation on photoinhibition by visible radiation.

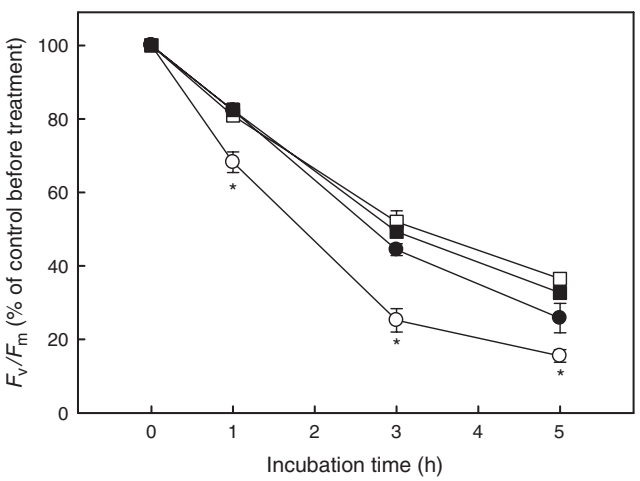

Fig. 3. Photoinhibition of *Soldanella alpina* leaves as measured by the F_v/F_m ratio as a percentage of the initial value. Leaves of *S. alpina* were separated into groups with high (HA, squares) and low (LA, circles) UV-A absorption as measured by absorbance at 375 nm (DA_{375}) (see Table 2). Leaves were dark-incubated in the presence of 3 mM lincomycine for 1 h and the F_v/F_m ratio was measured (0 h). Subsequently, leaves were exposed to direct sunlight at 22°C (white circles and squares) at a mean PAR of 1600 µmol m^{-2} s^{-1} or behind a UV-exclusion filter (black circles and squares) at a mean PAR of 1400 µmol m^{-2} s^{-1}. Significant difference at the 5% level is marked with an asterisk by comparing pairs of different treatments with Student's *t*-test. Results are means with s.e. of three independent experiments with at least three different leaves for each experiment.

Imaging of leaves with high and low UV-A absorbance

Leaves with significant differences in UV-A absorption were further used to investigate a possible relationship between UV-A absorbance and leaf structure. Here, leaves newly developed in the growth chamber are referred to as 'GC leaves'.

Fluorescence emission spectra excited by UV-A radiation showed a low and wide emission peak in the blue-green region, which could result from cuticle and epidermal HCA, and two major peaks in the red (685 nm) and far-red (740 nm) regions corresponding to chlorophyll fluorescence (Fig. 4*a*). According to the DA_{375} values, UV-A was clearly less absorbed in GC and IA leaves than in the HA leaves for both leaf surfaces. This is also shown by the variation of red to blue-green leaf surface fluorescence from GC to HA leaves (Fig. 4*b*). In GC leaves, UV radiation was less absorbed by the epidermis and excited primarily red chlorophyll fluorescence emanating from the mesophyll. In IA, and more in the adaxial side of the HA leaves, UV radiation induced the emission of blue-green fluorescence in the epidermis, including cuticle, whereas chloroplasts in the mesophyll were shielded from UV excitation (Fig. 4*b*). This blue-green fluorescence was shown more by images than by the emission spectra because of the wider band pass of the excitation filter used for imaging. In addition, the structure of leaves investigated in Table 1 (HA, GC old leaves) and Table 2 (HA, LA) depended on light acclimation. The LMA was the highest in HA leaves and similar in LA, IA and GC leaves (Table 3). This result agreed with the mesophyll size of HA leaves being the thickest but the mesophyll sizes of LA, IA and GC leaves being similar (data not shown). The stomatal density was similar in HA, IA and LA leaves but was significantly higher than in GC leaves (Table 3). This is also visible in the density of bright spots in Fig. 4*b*. However, the stomatal density was not reflected by the fluorescence emission spectra, since abaxial blue fluorescence was lower in HA than in GC leaves (Fig. 4*a*). The mixed origin of abaxial blue fluorescence and the complex optic of this leaf side prevent the images from being directly related to the emission spectra in the blue domain. The adaxial trichome density was the highest in HA leaves, and higher in IA and LA leaves than in GC leaves, whereas the abaxial trichome density did not differ significantly among leaves (Table 3). Adaxial epidermal ground cell density was higher in HA than in IA, LA and GC leaves, and was similar between IA, LA and GC leaves (Table 3), which is clearly visible in Figs 4*b* and 5*a*, *b*.

The higher UV-A absorbance of HA compared with GC leaves was also due to the higher UV-A absorbance of epidermal cells and trichomes (Fig. 5*a–d*). On both leaf sides, UV-A was absorbed by soluble flavonoids in the vacuole of epidermal cells, as shown by the low fluorescence intensity and the high absorbance of this cell compartment (Fig. 5*e–h*). UV absorption of the cell wall, adaxial cuticle and abaxial stomatal ostiole was

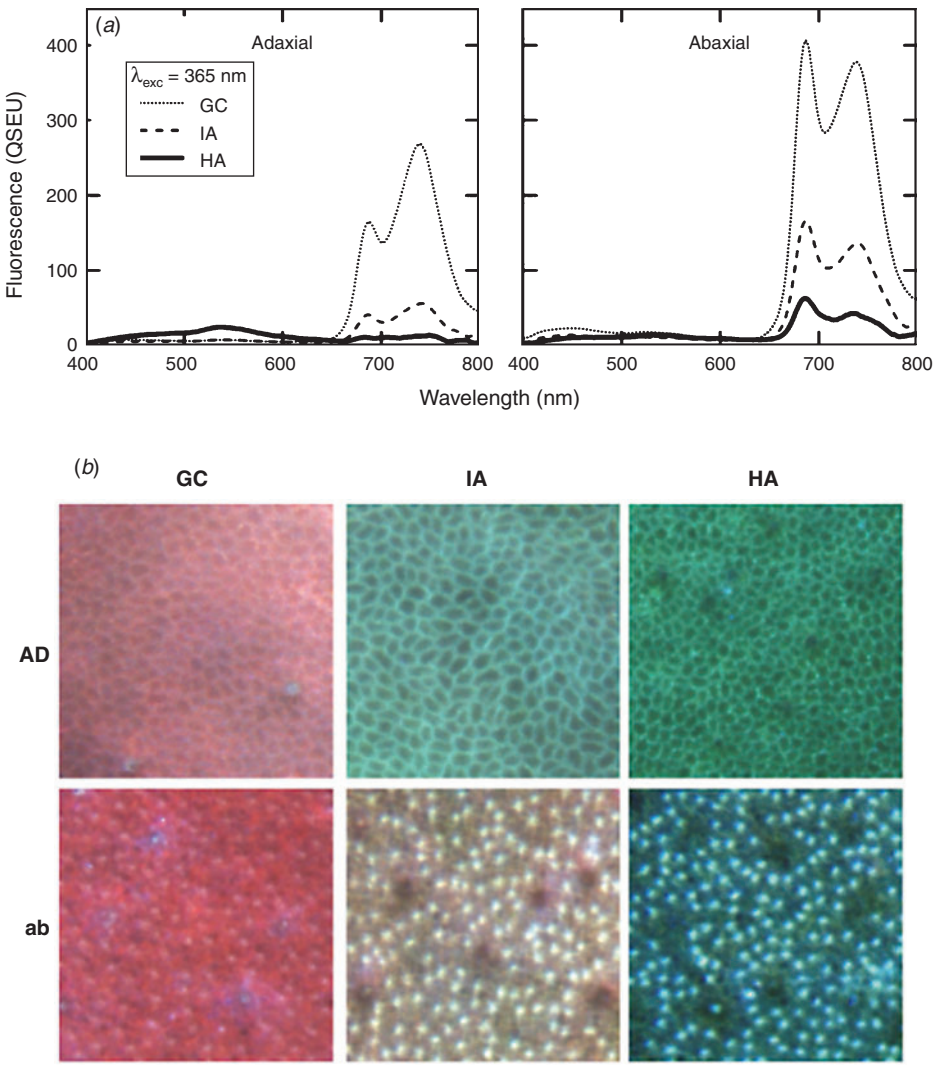

Fig. 4. (*a*) Fluorescence emission spectra and (*b*) colour fluorescence images of adaxial (AD) and abaxial (ab) sides of high absorbance (HA), intermediate absorbance (IA) and growth chamber (GC) leaves or *Soldanella alpina*. In (*a*), the fluorescence was excited at 365 nm. In (*b*), images were obtained using a 330WB80 excitation filter and a LP400 emission filter in order to localise the UV-B-induced blue fluorescence emitted by hydroxycinnamic acid (HCA) and the UV-A absorbance by flavonoids. Images were taken with the same exposure time using a 12× magnification and correspond to 1 mm^2 leaf area. In (*b*), the large dark spots in HA and abaxial IA leaves and the blue spots over GC leaves are trichomes. On the abaxial side, the bright spots are stomata. In (b), total absorption as measured by absorbance at 375 nm (DA_{375}) values were 0.8, 2.2 and 3.4 in GC, IA and HA leaves, respectively. QSEU, quinine sulfate equivalent units.

Table 3. Mean values of leaf mass per area (LMA), and stomatal, trichome and adaxial epidermal ground cells densities of *Soldanella alpina* leaves grown in a growth chamber (GC) and grown in the Alps under intermediate to low (LA + IA) and high (HA) light conditions
Values are mean ± s.e. and the sample size is indicated in parentheses. Different letters indicate statistically significant differences among GC, LA + IA and HA leaves at the 5% level by separately comparing each leaf type to each other

		GC	LA + IA	HA
LMA (g m^{-2})		$50.0^{bc} \pm 2.4$ (12)	$58.0^{ac} \pm 4.7$ (16)	$66.3^{a} \pm 3.4$ (14)
Stomatal density (stomata mm^{-2})		$116^{b} \pm 7.1$ (7)	$153^{a} \pm 11.9$ (14)	$170^{a} \pm 5.5$ (10)
Trichome density, (trichomes mm^{-2})	Adaxial	$1.6^{c} \pm 0.4$ (5)	$3.4^{b} \pm 0.1$ (6)	$4.5^{a} \pm 0.4$ (10)
	Abaxial	$6.4^{a} \pm 1.1$ (4)	$8.5^{a} \pm 2.0$ (8)	$12.1^{a} \pm 1.8$ (9)
Adaxial ground epidermal cell density per mm^2		$254^{b} \pm 15.1$ (7)	$250^{b} \pm 6.9$ (11)	$355^{a} \pm 25.6$ (10)

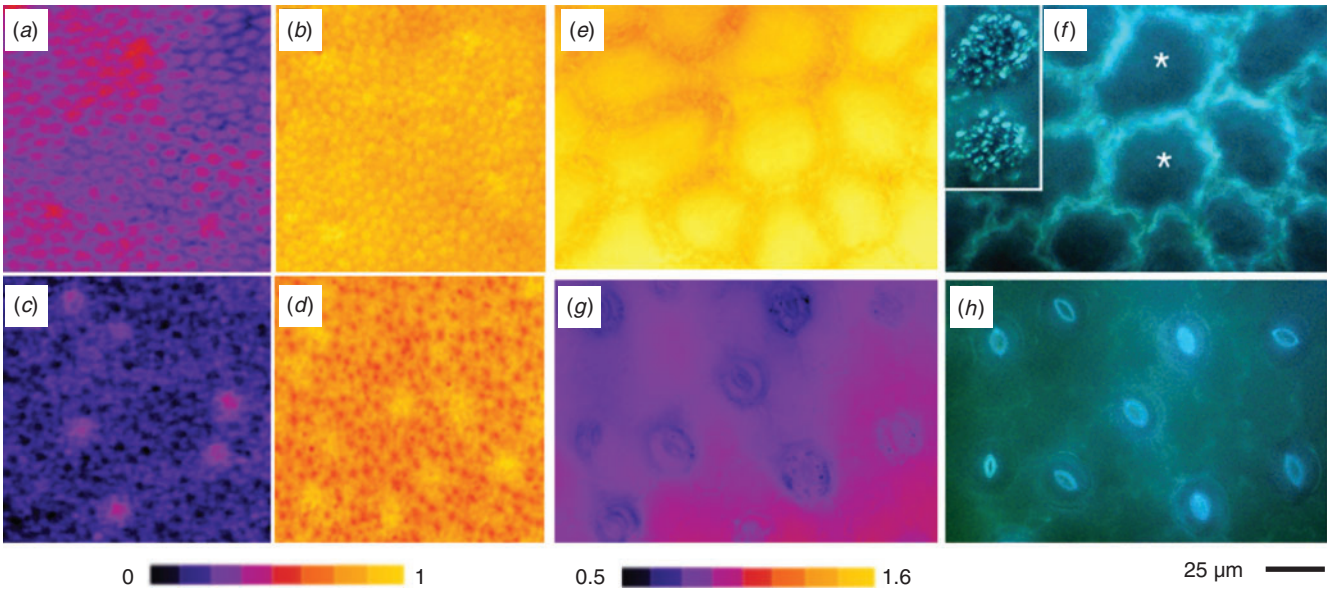

Fig. 5. UV-A absorbance images of (*a*, *b*, *e*) adaxial and (*c*, *d*, *g*) abaxial sides of (*a*, *c*) growth chamber (GC) and (*b*, *d*, *e*, *g*) high absorbance (HA) leaves of *Soldanella alpina*, and colour fluorescence images of the (*f*) adaxial and (*h*) abaxial sides of HA leaves. Images were taken with (*a–d*) a 12× magnification using a stereomicroscope and correspond to a 1-mm^2 leaf area and (*e–h*) with a 400× magnification using an epifluorescence microscope. In (*f*) and (*h*), images were obtained using a 330WB80 excitation filter and a LP400 emission filter in order to localise the UV-B-induced blue fluorescence emitted by hydroxycinnamic acid and the UV-A absorbance by flavonoids. The insert in (*f*) shows the fluorescence from the cuticle surface of the two adaxial epidermal cells marked with an asterisk. (*a–d*) Bright spots show trichomes, and (*c*, *d*) dark small spots show stomata and (*g*) chloroplasts in the guard cells. The three-bit colour scale corresponds to a UV-A absorbance scale (*a–d*) from 0 to 1 and (*e*, *g*) from 0.5 to 1.6.

mainly attributed to HCA, which emitted a blue fluorescence under UV-excitation (Fig. 5*f, h*). Furthermore, UV-absorbing compounds were present in trichomes (Fig. 5*a–d*), but blue fluorescence was only emitted by GC leaves (Fig. 4*b*), suggesting the presence of HCA in trichomes in GC leaves only.

In GC, LA and IA leaves, adaxial DA_{375} increased strongly and linearly with epidermal cell density. In HA leaves, this increase was less sharp (Fig. 6*a*). Conversely, DA_{322} values declined (Fig. 6*a*), meaning the ratio between DA_{375} and DA_{322} values increased linearly with the epidermal ground cell density (Fig. 6*b*). The negative relationship between total DA_{375} and total DA_{322} values (Fig. S3) showed that UV-B absorbance by HCA decreased and UV-A absorbance by flavonoids increased from GC to LA, IA to HA leaves depending on the leaf acclimation to light.

Discussion

The UV-A absorbance of *S. alpina* leaves depends on growth light conditions with a total UV-A absorbance of up to 3.7 in sun leaves and 0.9 in shade and GC leaves. The characteristic shade and sun acclimation (Walters 2005) of LA and HA leaves was confirmed by a higher LMA, a higher chl *a*/*b* ratio, a lower total chlorophyll content, a lower chlorophyll : carotenoid ratio and higher antioxidant enzyme activity in HA compared with LA leaves. Furthermore, in shade leaves, the stomatal and epidermal ground cell densities were lower than in sun leaves, as reported for non-Alpine species (Lake *et al*. 2001; Cookson and Garnier 2006).

High epidermal UV absorbance is suggested to be induced by UV-B radiation (Lois 1994; Bidel *et al*. 2007; Morales *et al*. 2013), probably mediated by the UV-B photoreceptor UVR8 (Morales *et al*. 2013). However, PAR radiation was also shown to induce the synthesis of UV-absorbing compounds in various species (Barnes *et al*. 2013; Morales *et al*. 2013), as was observed here in *S. alpina* leaves. Control leaves, which were kept under the vegetation during the exposure period, did not show any significant variation of UV-A absorbance.

After transfer to natural high sunlight by removing the vegetation, UV-A absorption increased strongly in LA and IA leaves, irrespective of the presence or absence of UV radiation. This was confirmed by comparing the UV-A absorbance of leaves already present with those developed after installation of an UV filter. Both old and developed leaves had the same UV-A absorbance, whereas UV-A absorbance remained low in leaves developed under artificial shading.

Leaves of *S. alpina* are hypostomatous with a bifacial anatomy including a palisade and spongy parenchyma in the mesophyll, and with UV-A absorbance being higher in the upper than in the lower epidermis. Flavonoids in epidermal vacuoles and trichomes were the main UV-A absorbers. According to Harborne (1968), quercetin and kaempferol are the dominant flavonoids in most Primulaceae; this is especially true for *S. alpina* leaves, which also contain a large amount of HCA esters and lack anthocyanins. The inverse relationship between DA_{375} and DA_{322} suggests a balance between flavonoids and HCA concentrations, suggesting HCA's availability in the limitation of flavonoid accumulation. This was reported for some other species acclimating to light and UV (Bidel *et al*. 2007; Agati and Tattini 2010), and is supported by the analysis of secondary metabolic turnover (Kleiner *et al*. 1999). The saturation of adaxial UV-A absorbance despite increasing

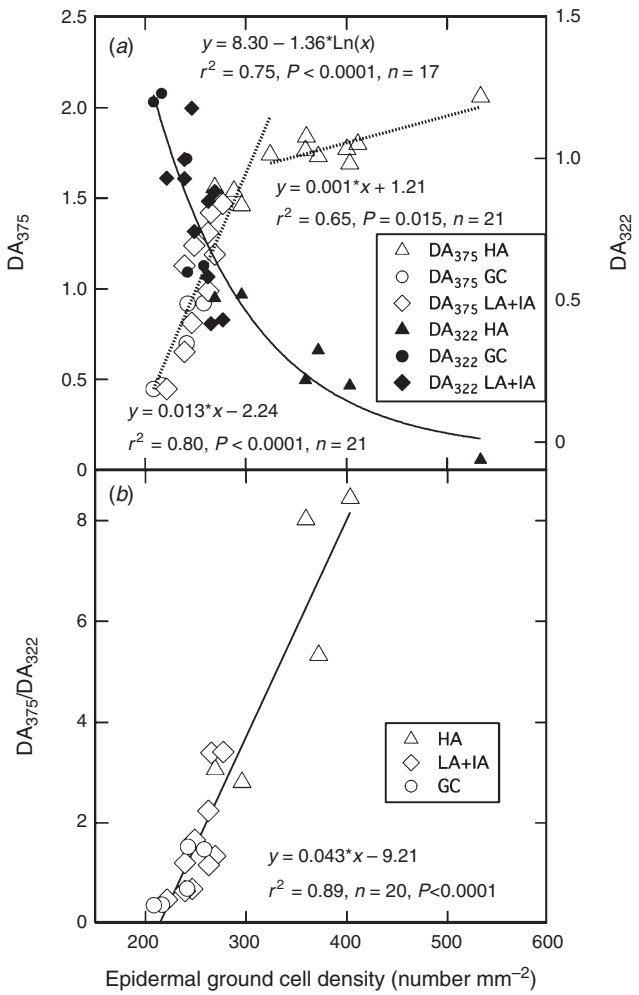

Fig. 6. (*a*) Relationship between absorption as measured by absorbance at 375 nm (DA_{375}) and absorption as measured by absorbance at 322 nm (DA_{322}), and (*b*) the DA_{375} : DA_{322} ratio and the density of epidermal ground cells on the adaxial side of high absorbance (HA), low and intermediate absorbance (LA + IA) and growth chamber (GC) leaves of *Soldanella alpina*. Regression equations and the correlation coefficient are indicated.

cell density might be explained by either a balance between flavonoids and HCA concentration, which could limit the increase of flavonoids or by flavonoid polymerisation to condensed tannins, which are not detected by the Dualex. Further imaging of the leaf cuticle is required to investigate if its structure and UV-excited blue fluorescence could be related to the difference in DA_{322} among leaves. In addition, in transverse sections of HA leaves (data not shown), low fluorescence of the upper layer of the palisade parenchyma was observed, suggesting an accumulation of flavonoids in mesophyll cells. A similar localisation of chlorophyll and flavonoids is known to prevent the full detection of flavonoids by the Dualex (Bengtsson *et al.* 2006).

In *S. alpina* leaves, the UV-A absorbers were highly stable. Sun leaves kept their UV-A absorption high with only small variations during the vegetation period and the leaf lifetime, even after prolonged transfer to GC conditions at low light, in senescent leaves or in pre-snowmelt leaves from the previous year. The high stability of low epidermal UV transmittance was also reported by Barnes *et al.* (2013) in sun leaves of *Populus tremuloides* Michx., which remained unaffected by shade treatment. Since epidermal UV-A absorbance is proportional to LMA, as reported in other species (Goulas *et al.* 2004; Meyer *et al.* 2006), and is correlated with epidermal cell density, it is expected to be a stable leaf trait correlating to the light environment during leaf development. Because the HA and LA leaf structures are determined during early leaf development (Cookson and Garnier 2006), it is improbable that inducible increases of leaf UV-A absorbance and respective flavonoid contents correlate to changes in leaf anatomy. As in other species (Lois 1994; Kleiner *et al.* 1999; Louis *et al.* 2009; Agati and Tattini 2010), there might be two pools of flavonoids in *S. alpina* leaves, one constitutively developed during leaf formation and another flexible pool induced in fully developed leaves.

Since UV-A radiation can induce photoinactivation of PSII (Vass *et al.* 2002; Hakala-Yatkin *et al.* 2010; Takahashi *et al.* 2010) and of catalase (Aubailly *et al.* 2000), it can be expected that HA plants are more tolerant to UV and high light than LA plants. This is clearly shown when the maximum inactivation rates were measured after blocking new protein synthesis in the chloroplast with lincomycine. The decline of the F_v/F_m ratio was higher in LA compared with HA leaves in full sunlight, whereas the presence of a UV exclusion filter protected LA leaves, similar to UV-A absorbers in HA leaves. Furthermore, *in situ* shading by the surrounding vegetation and UV filtering protected LA leaves from photoinhibition. Also, total chlorophyll contents and catalase activity declined in illuminated LA leaves, whereas this was less pronounced in HA leaves when protein synthesis in the cytoplasm was blocked by cycloheximide. However, according to Takahashi *et al.* (2010), the efficiency of photoinhibition increases sharply at the shortest UV-A wavelengths, whereas the decline of DA_{322} and the proportional increase in DA_{375} values might indicate higher sensitivity to short UV-A wavelengths. Since DA_{322} estimates the HCA content and DA_{375} values estimates the flavonoids content, this result suggests that flavonoids replace HCA in sufficiently shielding *S. alpina* leaves from short UV wavelengths-induced photoinhibition.

In conclusion, high epidermal UV-A absorbance of *S. alpina* leaves is induced by sunlight, independent of UV radiation, and is largely irreversible. This high UV-A absorbance protects *S. alpina* leaves from photoinactivation of PSII, catalase and chlorophyll degradation in sunlight. This feature may be particularly important in *S. alpina* leaves surviving the winter for tolerating high PAR after snowmelt in the following year.

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