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Ultraviolet and photosynthetically active radiation can both induce photoprotective capacity allowing barley to overcome high radiation stress

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Abstract

The main objective of this study was to determine the effects of acclimation to ultraviolet (UV) and photosynthetically active radiation (PAR) on photoprotective mechanisms in barley leaves. Barley plants were acclimated for 7 days under three combinations of high or low UV and PAR treatments ([UV–PAR–], [UV–PAR+], [UV+PAR+]). Subsequently, plants were exposed to short-term high radiation stress (HRS; defined by high intensities of PAR – 1000 μmol m^{-2} s^{-1}, UV-A – 10 W m^{-2} and UV-B 2 W m^{-2} for 4 hours), to test their photoprotective capacity. The barley variety sensitive to photooxidative stress (Barke) had low constitutive flavonoid content compared to the resistant variety (Bonus) under low UV and PAR intensities. The accumulation of lutonarin and 3-feruloylquinic acid, but not of saponarin, was greatly enhanced by high PAR and further increased by UV exposure. Acclimation of plants to both high UV and PAR intensities also increased the total pool of xanthophyll-cycle pigments (VAZ). Subsequent exposure to HRS revealed that prior acclimation to UV and PAR was able to ameliorate the negative consequences of HRS on photosynthesis. Both total contents of epidermal flavonols and the total pool of VAZ were closely correlated with small reductions in light-saturated CO_2 assimilation and maximum quantum yield of photosystem II photochemistry caused by HRS. Based on these results, we conclude that growth under high PAR can substantially increase the photoprotective capacity of barley plants compared with plants grown under low PAR. However, additional UV radiation is necessary to fully induce photoprotective mechanisms in the variety Barke. This study demonstrates that UV-exposure can lead to enhanced photoprotective capacity and can contribute to the induction of tolerance to high radiation stress in barley.

Highlights

- acclimation to high PAR and UV increases the photoprotective capacity of barley
- UV is necessary for efficient photoprotection in a constitutively-sensitive variety
- acclimation to high PAR and UV induces accumulation of lutonarin and ferulic acid
- accumulation of xanthophylls and flavonols correlates with enhanced photoprotection

Keywords

barley genotype, photoinhibition, photoprotection, polyphenols, xanthophylls

Abbreviations

A_{max}, light-saturated rate of CO_2 assimilation; Cars, total content of carotenoids; DEPS, de-epoxidation state of the xanthophyll-cycle pigments; F_V/F_M, maximum quantum yield of photosystem II photochemistry; HRS, high radiation stress; Chls, total content of chlorophylls; PAR, photosynthetically active radiation; PS II, photosystem II; UV, ultraviolet radiation; UV-B_{BE}, daily biologically effective dose of ultraviolet B radiation; VAZ, total pool of xanthophyll-cycle pigments

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1. Introduction

Elevated doses of ultraviolet-B radiation (UV-B; 280-315 nm) has the potential to negatively affect growth, development (e.g. Ballaré et al., 2011; Wargent et al., 2009; Gruber et al., 2010) and carbon assimilation (e.g. Guidi et al., 2011; Klem et al., 2012, Urban et al., 2006) due to their ability to damage DNA and cause production of reactive oxygen species (Hakala-Yatkin et al., 2010; Hideg et al., 2013). However, susceptibility to UV-B radiation is dependent on the complex interplay between protection, repair and damage, and as a consequence, plant responses vary depending on dose, UV-spectral composition, acclimation time, genotype and other co-occurring environmental factors (Jordan, 2002; Kataria et al., 2014).

Many studies have identified the photosynthetic machinery as a major target of elevated UV-B radiation, including reports on the degradation of light-harvesting complexes, inactivation of photosystem (PS) II function, accelerated degradation of the D1 and D2 proteins of PSII, disruption of thylakoid membrane integrity, and the degradation of Rubisco causing reduced carboxylation efficiency (reviewed in Bornman, 1989; Kateria et al., 2014; Takahashi et al., 2010). UV-B has been also reported to cause alterations in leaf morphology (reviewed in Jansen, 2002; Robson et al., 2014) and stomatal function (Nogues et al., 1999; Urban et al., 2006); changes which may indirectly reduce photosynthetic rates through decreases in light interception and availability of CO_2, respectively. However, there is now growing evidence that photosynthetic rates can be sustained under realistic doses of UV radiation, thanks to the induction of enhanced photoprotection (Bolink et al., 2001; Hakala-Yatkin et al., 2010; Wargent et al., 2011) or the recovery of photochemical quantum yield (Xu & Gao 2010).

Under natural conditions, high irradiance of UV-B usually occurs simultaneously with high irradiance of photosynthetically active radiation (PAR; 400–700 nm) (Brown et al.,

94 1994). However, the UV:PAR ratio in the canopy varies enormously, between sunflecks that
95 are relatively depleted in UV-B (UV:PAR < 1) to shaded understorey where UV-B is strongly
96 enriched (UV:PAR up to 5) at midday (reviewed in Robson et al., 2014). High PAR can cause
97 photoinhibition, a decrease in the photochemical performance of PS II. It has been reported
98 that photodamage to PS II under sunlight is primarily associated with UV rather than PAR
99 (Hakala-Yatkin et al., 2010; Takahashi et al., 2010). Plants have evolved a range of
100 mechanisms to decrease damaging effects of either UV or PAR absorbed by leaves. These
101 include mechanisms minimizing absorption of excessive light (leaf/chloroplast movements
102 and reduction in the light-harvesting complexes), accumulation of photoprotective pigments
103 and antioxidants, induction of repair mechanisms, and/or changes in leaf morphology (Ballaré
104 et al., 2011; Heijde and Ulm, 2012; Jordan, 2002; Kataria et al., 2014). The main
105 photoprotective responses associated with acclimation to UV and PAR are respectively, the
106 accumulation of flavonoids and an increase of the xanthophyll-cycle carotenoid pool.
107 However, the crosstalk between these two responses is poorly understood. UV and PAR both
108 induce flavonoids, albeit of slightly different classes (Christie and Jenkins, 1996; Kolb et al.,
109 2001). Flavonoids contribute in general to the photoprotection of photosynthesis through UV-
110 screening (Bassman, 2004; Jordan, 2002) but especially by scavenging free radicals and/or
111 reactive oxygen species (Umeda and Shibamoto, 2008). Excessive light energy is dissipated
112 by non-photochemical quenching (Baker, 2008), particularly *via* the carotenoids of the
113 xanthophyll-cycle (Demmig-Adams and Adams, 2006). Flavonoids, e.g. anthocyanins, also
114 contribute to energy dissipation (Gould, 2004). In addition, Hernández and Van Breusegem
115 (2010) hypothesized that the biosynthesis of flavonoids might act as an escape valve for
116 excess energy by consuming triose phosphate, ATP and NADPH and thus creating a sink for
117 reduced carbon. Therefore, UV-induced accumulation of flavonoids and upregulation of PS II
118 repair capacity (Sicora et al., 2003; Xu and Gao, 2010) may have the potential to increase the

119 photoprotective capacity of plants exposed to excessive radiation. Several studies have indeed
120 implicated UV radiation in the induction of enhanced photoprotection against both high PAR
121 and UV (Bolink et al., 2001; Hakala-Yattkin et al., 2010; Wargent et al., 2011).
122 In an earlier study (Klem et al., 2012), we demonstrated that high PAR caused a
123 different flavonol accumulation pattern in two barley varieties with distinct tolerance to UV.
124 Differences in flavonol induction were associated with differences in amelioration of the
125 negative effects of UV radiation on photochemistry and carbon assimilation. There are
126 mechanistic similarities in the photodamage of PS II induced by UV-B radiation and by PAR
127 (reviewed in Vass, 2012). PAR and UV-B are also known to interact during photorepair
128 (Sicora et al., 2003). Therefore, we hypothesized that cross-tolerance to photodamage induced
129 by either UV or PAR is likely. In this study, we examined whether UV-B irradiance can also
130 induce protection against subsequent high radiation stress (HRS) defined by high irradiance of
131 UV and PAR. The following specific hypotheses were tested in a field experiment: (1)
132 acclimation to PAR and supplemental UV will enhance the protective capacity of plants, and
133 in doing so reduce photoinhibition under subsequent HRS, and (2) PAR- and particularly UV-
134 induced flavonoids and xanthophyll-cycle pigments represent the main photoprotective
135 mechanisms against high radiation. The hypotheses were tested using two barley varieties
136 differing in their sensitivity to UV-B radiation: Barke (sensitive) and Bonus (tolerant).
137
138 **2. Material and methods**
139 *2.1. Plant material*
140 The pre-cultivation of barley plants, described in detail by Klem et al. (2012), was done
141 in August 2010, in the garden of the Global Change Research Centre ASCR (Brno, CZ). The
142 seeds of both varieties (sensitive Barke and tolerant Bonus) were germinated at room
143 temperature on wet filter paper for 48 hours. Seeds of both varieties were provided by the

barley gene bank of the Agricultural Research Institute Kroměříž Ltd., Czech Republic. Only germinating seeds were then transplanted into small pots (5 cm in diameter) filled with a mixture (1:1) of horticultural substrate and a substrate for pot-plants (Agro CS, Ceska Skalice, CZ). Three seeds were transplanted into each pot in a triangular spatial distribution to avoid mutual shading of plants during early growth. Uniform watering was ensured through capillary action from plastic trays.

The plants were pre-cultivated under conditions of low PAR and exclusion of UV-A and UV-B in open-sided chambers (area 1 m^2, height 50 cm). Neutral density filters 0.6ND (Lee Filters, Hampshire, UK) were used to reduce the PAR irradiance to 25% of natural sunlight. Clear plastic Lee U.V. 226 filters (Lee Filters, UK) were used for UV-A and UV-B exclusion. The latter filters also caused a small reduction (up to 10%) in PAR (see www.leefilters.com for detailed spectral filter characteristics). The UV and PAR filters covered the top and upper part of the side walls (20 cm down from the top) to attenuate direct solar radiation. The actual intensities of PAR, UV-A and UV-B radiation were monitored during this period using Li-190SA (Li-Cor, Lincoln, Nebraska, USA), SKU 420 (Skye Instruments Ltd, Powys, UK) and SKU 430 (Skye Instruments Ltd, UK) sensors, respectively. After 14 days of pre-cultivation, the barley plants were transferred to individual PAR and UV acclimation treatment plots. The 2^{nd} leaf, which was used for all measurements and analyses, had already been fully formed at the beginning of the UV/PAR treatments.

2.2. Acclimation to UV and PAR

Sixteen pots of each variety (48 plants per treatment) were exposed to the following UV/PAR acclimation treatments: [UV–PAR–] representing UV exclusion and reduction of PAR to approximately 25%; [UV–PAR+] which represents UV exclusion and ambient PAR; and [UV+PAR+] which represents supplemental UV radiation and ambient PAR. The lamp

output in [UV+] was continuously (every 10s) modulated to provide double the solar UV irradiance. The relative proportion of UV-A and UV-B was the same as under ambient UV conditions. Barley plants were acclimated to the individual UV/PAR regimes for 7 days. Changes in weather conditions, from cloudy (up to a daily maximum PAR of 500 µmol m^{-2} s^{-1} and sum of 2.3 MJ m^{-2} day^{-1}) to clear skies (daily maximum PAR of up to 1500 µmol m^{-2} s^{-1} and sum of 5 MJ m^{-2} day^{-1}), led to changes in the ambient UV-B doses. Daily biologically effective UV-B doses ($UV\text{-}B_{BE}$) were taken as zero under [UV−] treatments, while $UV\text{-}B_{BE}$ amounted to 14.7–26.1, 11.6–20.5 and 2.8–4.9 kJ m^{-2} day^{-1} under [UV+] treatment, calculated from action spectra for flavonoid accumulation (Ibdah et al., 2002), plant growth inhibition (Flint and Caldwell, 2003) and Green's formulation of the generalized plant action spectrum (Green et al., 1974), respectively. A spectroradiometer SM 9000 (PSI, Brno, CZ) was used to measure the emission spectrum of the UV lamps in the range 200–980 nm. See Klem et al. (2012) for details.

Individual acclimation treatments were provided by open-sided chambers (area 1 m^2, height 50 cm) covered by UV and PAR filters (Lee U.V. 226 and Lee 0.6ND filters; Lee Filters, UK). The UV and PAR filters covered the top and upper part of the side walls (20 cm down from the top). A modulated UV lamp system (Konel, Zlín, CZ), placed into the chambers, was used to achieve enhanced UV intensities. The system consists of two UV-A (TL 20 W/10 SLV; Philips) and three UV-B (TL 20 W/12 RS SLV; Philips) fluorescent lamps. The system monitors incident UV-B and UV-B irradiance under the lamp-bank, and adjusts lamp output to a specified dose using a feedback and amplification circuit. To avoid transmission of UV-C radiation (< 280 nm), the UV fluorescent lamps were wrapped in pre-solarised (8h) 0.13 mm thick cellulose diacetate film. Li-190SA (Li-Cor, Lincoln, Nebraska, USA), SKU 420 (Skye Instruments Ltd, Powys, UK) and SKU 430 (Skye Instruments Ltd, UK) sensors were used to monitor PAR, UV-A and UV-B radiation, respectively, in each

194 acclimation treatment. Outputs from all radiation sensors were recorded using a data logger
195 DL2e (Delta-T Devices Ltd., Cambridge, UK).
196
197 *2.3. Short-term high radiation stress*
198 After 7 days of acclimation to different UV/PAR treatments, 32 barley plants of each
199 variety per treatment, were transferred into darkness for 12 hours. After dark adaptation, half
200 the samples were used for analyses of UV-screening compounds, photosynthetic pigments
201 and xanthophyll-cycle pigments. The other half of the plants was placed into controlled
202 conditions in a growth chamber (Bio-Line HB 1014, Heraeus Vötsch - Industrietechnik, D)
203 and exposed to high radiation stress (HRS). The irradiation module consisted of krypton and
204 halogen lamps with additional UV-B fluorescent lamps (TL 20W/12 RS SLV; Philips) and
205 plants were exposed to HRS defined by continuous high intensities of PAR (1000 μmol m^{-2} s^{-1}),
206 UV-A (10 W m^{-2}) and UV-B (2 W m^{-2}) for 4 hours. UV-B_{BE} amounted to 17.5, 13.7 and
207 3.3 kJ m^{-2} day^{-1} under the [UV+] treatment, calculated according to the action spectra for
208 flavonoid accumulation (Ibdah et al., 2002), plant growth inhibition (Flint and Caldwell,
209 2003) and Green's formulation of the generalized plant action spectrum (Green et al., 1974),
210 respectively.
211 A constant air temperature of 25°C and relative humidity 65% was maintained during
212 the high light stress treatment.
213
214 *2.4. Physiological measurements*
215 An open gas-exchange system Li-6400 (Li-Cor, Lincoln, NE, USA) was used to
216 estimate the light-saturated (1200 μmol photons m^{-2} s^{-1}) CO_2 assimilation rate (A_{max}). All
217 measurements were performed on the intact leaves (2^{nd} leaf) of five plants per treatment under
218 constant microclimatic conditions (leaf temperature: 25±1°C, relative air humidity: 55±3%)

and under ambient CO_2 concentration (385±5 μmol CO_2 mol^{-1}). Simultaneously, measurements of maximum quantum yield of chlorophyll fluorescence (F_V/F_M) in dark-adapted (25 min) leaves were made using a FluorPen FP 100 (PSI, CZ). Physiological measurements were done during the last day of UV/PAR acclimation and again immediately after the application of HRS stress.

2.5. LC-MS analysis of phenolic compounds

Samples for HPLC-DAD analyses were prepared as follows; the leaf central segments (100 mg of fresh weight) were sampled after 12 hours of dark adaptation following each UV/PAR acclimation treatment. The leaf area of the sample was measured using a flat-bed scanner, then the sample was homogenized in a grinding bowl in 3 ml of 40% methanol, ultrasonicated (Ultrasonic compact cleaner UC 006 DM1, Tesla, CZ) for 5 min, and then centrifuged (6000 RPM, 3 min; EBA 20 Hettich Zentrifugen, D). One ml of the supernatant was filtered through a 0.2 μm filter (Premium Syringe Filters, Agilent, USA) and used for HPLC-DAD analysis.

Analyses were performed according Kolb and Pfündel (2005) using a TSP HPLC system (TSP Analytical, USA) equipped with a diode array detector (DAD). Separation was done on a LiChrospher chromatographic column (RP-18, 250x4 mm, 5 μm). A gradient of two mobile phases was used during the analysis. Mobile phase A consisted of water and phosphoric acid (850 ml H_2O + 100 μl H_3PO_4). Mobile phase B consisted of acidified methanol solution (765 ml CH_3CH_2OH + 85 ml H_2O + 100 μl H_3PO_4). The flow of the mobile phase during analyses was constant (1 ml min^{-1}). Chromatograms were recorded at the absorption wavelengths of 220, 314 and 440 nm. Absorption spectra were collected in spectral region 220–500 nm. For detection and quantification of unbound phenolic compounds, the signal obtained from the 314 nm detector was used (detection at 220, 440 nm

offered us other supportive information). Lutonarin and saponarin were identified by authentic standards (Sigma-Aldrich).

LC-MS analyses for identification of unknown compounds, were performed using an LCQ Accela Fleet (Thermo Fisher Scientific, San Jose, CA, USA) equipped with electro-spray (ESI), atmospheric pressure chemical (APCI) and atmospheric pressure photo (APPI) ionization sources and a photodiode array detector. A Luna C18, 150 x 2 mm, 3 μm, column (Phenomenex, Torrance, CA, USA) was used with water-acetonitrile-formic acid as a mobile phase. Mobile phase A used 5% of acetonitrile + 0.1% of formic acid; mobile phase B used 80% of acetonitrile + 0.1% of formic acid. The gradient was increased from 5 % of B to 35 % of B in 55 min and then held up for 10 min. The injection volume was 10 μL and the flow rate was 0.250 mL min^{-1}. APCI capillary temperature was 275 °C, APCI vaporizer temperature 400 °C, sheath gas flow 58 L min^{-1}, auxiliary gas flow 10 L min^{-1}, source voltage 6 kV, source current 5 μA, and capillary voltage 10 V. The 3-feruloylquinic acid was identified based on MS/MS data in the negative ion mode (see Kuhnert et al., 2010 for details). The base peak at *m/z* 193 and fragmentation peaks at *m/z* 173 and *m/z* 143 were used for unequivocal identification.

Peak areas were manually integrated. For the inter-sample comparison of the relative quantities of saponarin (isovitexin-7-O-glucoside), lutonarin (isoorientin-7-O-glucoside) and 3-feruloylquinic acid, peak areas were normalised against leaf area ((peak area [mAU s^{-1}]/leaf projected area [cm^2]) 10^{-6}), and means and SD were calculated (n=5).

2.6. Analysis of photosynthetic pigments

To assess the effect of acclimation to the different UV/PAR treatments, dark-adapted leaves (12h) were used for the quantification of xanthophyll-cycle pigments (violaxanthin, antheraxanthin, zeaxanthin), chlorophylls and total carotenoids. The photosynthetic pigments

were extracted from leaves using 100% acetone with a small amount of $MgCO_3$. After centrifugation at 3500 *g* for 3 min, the supernatant was diluted to 80% acetone and measured using a spectrophotometer UV/VIS 550 (Unicam, UK). The contents of total chlorophylls (Chls) and total carotenoids (Cars), Chl *a/b* and Chls/Cars ratios were calculated using the equations of Lichtenthaler (1987). The contents of individual carotenoids, including the pool of xanthophyll-cycle pigments (violaxanthin + antheraxanthin + zeaxanthin; VAZ) expressed on a Chl *a*+*b* basis was estimated by gradient reversed-phase HPLC (TSP Analytical, USA) according to Štroch et al. (2008). The de-epoxidation state of the xanthophyll-cycle pigments (DEPS) in dark-adapted leaves was calculated as (Z+A)/(V+A+Z).

2.7. Data analysis

Before the analysis of variance (ANOVA), the normality of data for individual parameters was tested using a Kolgomorov-Smirnov test and homogeneity of variances was tested using a Levene test. For UV and barley variety effects, the data were analysed using a two-way fixed-effect ANOVA model.

A multiple range test was performed to investigate the effects of UV/PAR treatments and barley variety on physiological parameters and contents of pigments and flavonoids. Tukey's post-hoc ($p = 0.05$) test was used to detect significant differences between treatments.

Paired Student's t-test was used to compare differences in A_{max} and F_V/F_M before and after HRS application within individual UV/PAR acclimation treatments. All statistical tests were done in Statistica 9 software (StatSoft, Tulsa, USA).

3. Results

3.1. Effect of UV/PAR acclimation on epidermal UV-screening

Following 7 days of acclimation to the distinct UV/PAR treatments, there were markedly pronounced changes in *in vivo* flavonol contents (Fig. 1 and Table 1). Constitutive levels of UV-screening flavonols, determined from the [UV–PAR–] treatment, were higher (by 31%) in the UV-tolerant variety Bonus than the sensitive variety Barke. The exposure of plants to [UV–PAR+] treatment led to a significant increase in flavonols in both varieties compared to [UV–PAR–] treatment (by 119 and 140% in Bonus and Barke, respectively; Fig. 1). The [UV+PAR+] treatment, as compared to [UV–PAR+], resulted in a significant increase in flavonol content only in variety Barke, but not in variety Bonus.

3.2. Changes in flavonoid contents in response to UV/PAR acclimation

Several flavonoid compounds were identified by HPLC (Fig. 2). The flavonoids present at the highest concentration were saponarin and lutonarin, irrespective of the barley variety. Lutonarin content was consistently higher in the UV-tolerant variety Bonus than in sensitive Barke under each radiation treatment (Fig. 3 A and Table 1). The difference between barley varieties was highest (7-times higher in Bonus) under the constitutive, low radiation conditions ([UV–PAR–]). Yet, UV/PAR-induced lutonarin accumulation was relatively greater in variety Barke. An increase in the content of lutonarin was induced in both varieties following acclimation to [UV+PAR+] (by 172 and 816% in Bonus and Barke, respectively). Increases in lutonarin content under [UV-PAR+] were intermediate (by 54 and 361% in Bonus and Barke, respectively), indicating that acclimation to PAR, and further acclimation to UV, were both partially able to induce this response (Fig 3A; Table 1).

317 The content of saponarin was slightly higher in variety Barke as compared with variety
318 Bonus (Fig. 3B; Table 1), but there was no effect of light treatment on saponarin
319 accumulation (Table 1).
320 The 3-feruloylquinic acid (a chlorogenic acid derivative) content in both barley varieties
321 was induced by UV rather than high PAR, as evident from its high content in the [UV+PAR+]
322 treatment, but not the [UV–PAR+], when compared with [UV–PAR–] (Fig. 3 C).
323
324 *3.3. Effect of UV/PAR acclimation treatments on photosynthetic pigments*
325 Acclimation to high UV/PAR treatments led to a decrease in total chlorophyll (Chls)
326 content, which was particularly evident in variety Barke (Table 2). The ratio of total
327 chlorophylls to carotenoids (Chls/Cars) significantly decreased in response to [UV–PAR+]
328 and [UV+PAR+] treatments, while the ratio of Chl *a*/*b* remained unaffected by the high
329 UV/PAR treatments (Table 2). This result indicates an increasingly important photoprotective
330 role for carotenoids in plants acclimated to high PAR/UV conditions.
331 Compared to the [UV–PAR–] treatment, high PAR led to an increase in the total content
332 of xanthophyll pigments (VAZ) by 40 and 36% in [UV–PAR+]; in Bonus and Barke,
333 respectively (Fig. 4 A). Additional high UV produced a further increase in VAZ of 48 and
334 58% in [UV+PAR+] compared with [UV–PAR–], in Bonus and Barke, respectively (Fig. 4
335 A). The nominal level of DEPS after night-time relaxation was slightly higher for plants
336 grown under [UV+PAR+] when compared with [UV–PAR–], however, these differences
337 were significant only in variety Barke (Fig. 4 B).
338 The relationship of Chls/Cars to total *in vivo* flavonols was analysed to evaluate the
339 trade-off between photosynthetic pigments and flavonols. The Chls/Cars ratio decreased
340 linearly with increasing total flavonol content, and this relationship was consistent for both
341 barley varieties (Fig. 5).

342

343 *3.4. Response of photosynthetic parameters to short-term HRS*

344 Changes in light-saturated CO_2 assimilation rate (A_{max}) and maximum quantum yield of
345 photosystem II (F_V/F_M) following exposure to short-term HRS are presented in Fig. 6. The
346 greatest statistically significant reduction in A_{max} was in plants acclimated to [UV–PAR–]
347 conditions, and was true of both barley varieties (66 and 74% in variety Bonus and Barke,
348 respectively). When plants were acclimated to the [UV–PAR+] treatment, there was only a
349 significant reduction in A_{max} in the sensitive variety Barke (43%). Changes in A_{max} were
350 statistically non-significant in [UV+PAR+] acclimated plants, irrespective of variety. The
351 effects of HRS on F_V/F_M were similar to those on A_{max}, but relatively less pronounced (Fig. 6
352 C,D).

353 The relative reductions in A_{max} and F_V/F_M following HRS application were inversely
354 proportional to the content of flavonols (Fig. 7 A, C) and xanthophyll-cycle pigments (Fig. 7
355 B, D) accumulated in leaves. There was a consistent linear increase in leaf flavonols and VAZ
356 content that was associated with a smaller decrease of both A_{max} and F_V/F_M for both varieties.
357 Although all relationships had high coefficients of determination ($p \leq 0.01$), the reduction in
358 F_V/F_M was relatively small.

359

360 **4. Discussion**

361 *4.1. Light induced accumulation of flavonoids*

362 To test the effectiveness of UV and PAR in inducing barley photoprotective capacity to
363 high radiation stress (HRS), we used two varieties Barke and Bonus that are, respectively,
364 sensitive and tolerant to light-induced oxidative stress (Klem et al., 2012; Wu and von
365 Tiedemann, 2004). This enabled us to differentiate the importance of constitutive and induced
366 accumulation of flavonoids, as well as the contribution of the PAR and UV portions of the

367 solar spectrum towards induced tolerance. The sensitivity of Barke was reflected in its lower
368 constitutive total flavonoids content, particularly of lutonarin and 3-feruloylquinic acid, as
369 compared to Bonus. The results however also show that the increase in these compounds
370 induced by high PAR and particularly UV intensities was more pronounced in the sensitive
371 variety Barke (Figs. 1, 3). Other authors also report that both UV and high PAR can induce
372 flavonoid accumulation (Götz et al., 2010; Kaffarnik et al., 2006). Thus, our results reinforce
373 the importance of taking both PAR and UV acclimation into consideration when considering
374 plant responses to UV-B radiation (Krizek, 2004; Götz et al., 2010).
375
376 *4.2. Accumulation of specific flavonoid compounds*
377 To obtain a better understanding of the responses to PAR and UV radiation at the
378 metabolite scale, we quantified several individual flavonoids. In agreement with Ferreres et
379 al. (2008), we report that saponarin and lutonarin derivatives constitute the major part of the
380 phenolic pool in barley leaf extracts. In our study, the lutonarin content was highly responsive
381 to UV/PAR acclimation treatments, whereas saponarin remained almost unaffected by
382 UV/PAR exposure (Fig. 3). Similarly, Reuber et al. (1996) reported that supplementary UV-
383 B radiation increased the concentration of saponarin in primary barley leaves by only 30%,
384 while the increase in lutonarin content was approximately 500%. Also, Schmitz-Hoerner and
385 Weissenböck (2003) reported only small increases in saponarin under supplemental UV-B,
386 whereas lutonarin increased five-fold. In contrast, Liu et al. (1995) reported that barley leaves
387 accumulated high levels of both lutonarin and saponarin when grown in a greenhouse under
388 high PAR intensities. A possible explanation for such discrepancies could be that different
389 phenolics accumulate in different parts of the leaf. Saponarin accumulates mostly in
390 epidermal cells and in the outermost cell layers of the mesophyll (Kaspar et al., 2010).
391 Therefore, by adjusting its leaf anatomical structure a plant may modulate the functionality of

392 individual flavonoid compounds and their content per leaf area. As the specific leaf area of
393 barley is reduced by high UV and PAR (Klem et al., 2012), any increase in saponarin
394 concentration in epidermal cells may be masked when calculated per leaf area unit.
395 The location of saponarin in and just below the epidermal cell layer (Kaspar et al.,
396 2010), suggests that it plays a major role in UV protection, either through screening or
397 through its antioxidative activities. Saponarin is also implicated in prolonging antioxidative
398 function in barley leaves by inhibiting β-carotene degradation (Umeda and Shibamoto, 2008).
399 However, the relatively high saponarin content of the sensitive variety Barke in our
400 experiment, as compared to the tolerant variety Bonus, and its small response to the UV/PAR
401 treatments, implies that it has only a limited role in protection against HRS. In contrast,
402 lutonarin accumulation was particularly induced by our UV/PAR acclimation treatments, and
403 its accumulation is not limited to a specific tissue or cell type (Schmitz-Hoerner and
404 Weissenböck, 2003), suggesting that this compound is of more general importance for
405 scavenging of reactive oxygen species. The flavonoid 3-feruloylquinic acid is present in
406 barley leaves at lower concentrations than saponarin and lutonarin (Fig. 3). Yet, this
407 compound is strongly induced by UV/PAR, suggesting that it also has a protective role. In
408 *Coffea canephora*, feruloylquinic acid is particularly accumulated in juvenile leaves where it
409 is closely associated with chloroplasts (Mondolot et al., 2006). The association with
410 chloroplasts suggests a protective role against photooxidative damage.
411 Differential regulation of flavonoids prompts questions about the functional role of
412 individual phenolic compounds, as well as about the control of their biosynthesis. Work by
413 Ryan et al. (2002) showed that UV-B induced a higher rate of production of dihydroxylated
414 flavonols than of their mono-hydroxylated equivalents in wild-type *Petunia* leaves. It is
415 thought that hydroxylation increases the antioxidant capacity of these compounds, without
416 affecting their UV-absorbing properties. Consistently, Götz et al. (2010) reported that PAR-

induced accumulation of di-hydroxylated quercetin in *Arabidopsis* leads to basic UV protection that is further increased by additional UV-B radiation. In contrast, the accumulation of mono-hydroxylated kaempferol derivatives and sinapoyl glucose was less pronounced under additional UV-B. Therefore, it is tempting to interpret the strong induction of the di-hydroxylated compound lutonarin by both UV and PAR that we report in this study as up-regulation of antioxidant activity. In contrast, the mono-hydroxylated compound saponarin was not induced in this study.

4.3. Flavonoids and protection against HRS

The application of short-term HRS was designed to test the hypothesis that accumulation of photoprotective phenolic compounds and xanthophyll-cycle pigments induced following UV/PAR acclimation protects barley plants against photooxidative stress. In our study, increased accumulation of flavonols under [UV–PAR+] and [UV+PAR+] treatments was positively correlated with the maintenance of the both stages of photosynthesis associated with the quantum yield of PS II (F_V/F_M; Fig. 6 C,D and Fig. 7 C) and with CO_2 assimilation (A_{max}; Fig. 6 A,B and Fig. 7 A). The results show that both PAR and UV radiation represent important ecological factors that control the photoprotective capacity of plants. This result agrees with previous findings that UV may induce enhanced photoprotection against high-light stress or long-wave UV radiation (Bolink et al., 2001; Hakala-Yatkin et al., 2010). The general role of optical screening by epidermal UV-absorbing pigments, presumably flavonoids, in photoprotection was confirmed (e.g. Adamse and Britz, 1996; Bolink et al., 2001; Kataria et al., 2014).

Our study also highlights the importance of genotype in determining photoprotective capacity and inducible response. In barley plants acclimated to the [UV–PAR+] treatment, reductions in F_V/F_M and A_{max} were minor in resistant variety Bonus, whereas the effect of

short-term radiation stress was pronounced in the sensitive variety Barke. On the other hand, there was almost no difference between the two varieties in the reduction of photosynthetic activities when acclimated to [UV+PAR+] conditions. Thus, inducible protective mechanisms can compensate for the differences in constitutive protection between barley genotypes under these conditions.

4.4. Xanthophyll-cycle pigments and protection against HRS

A pronounced increase in the xanthophyll pool (VAZ) was revealed in plants acclimated to [UV–PAR+] (by ca 40%) and [UV+PAR+] (by ca 60%) in comparison with [UV–PAR–] (Fig. 4A). Such increases in VAZ have been associated with enhanced protection of the photosynthetic apparatus against photooxidative stress (Demmig-Adams and Adams, 2006; Jahns and Holzwarth, 2012). Previously, it was shown that growth under a high PAR produced an up-to three-fold increase in the VAZ pool in barley (Kurasová et al., 2002). In this study, the effective acclimation of both barley varieties to increased PAR and UV was further evident from a low DEPS estimated in plants that were dark adapted overnight (Fig. 4 B). Radiation stress is typically accompanied by markedly increased pre-dawn DEPS (Demmig-Adams and Adams, 2006; Kurasová et al., 2002; Štroch et al., 2008). Moreover, the reduced Chls/Cars ratio is a further indicator that high UV and PAR stimulated the photoprotective role of carotenoids. These protective responses are effective because there is no selective destruction of Chl *a* (demonstrated as only slight changes in the Chl *a*/*b* ratio), not even under the [UV+PAR+] treatment (Table 2).

Similarly to flavonols, there was a positive relationship between VAZ and protection of both F_V/F_M and A_{max} (Fig. 7 B, D). Therefore, it is attractive to consider some form of coupling between these protective mechanisms, whether at the level of receptors, signalling pathways, or even at the level of metabolite biosynthesis. The published literature yields

equivocal information concerning the effects of UV- radiation on VAZ size and xanthophyll-cycle activity; both significant increases in VAZ size (Láposi et al., 2009) and decreases together with impairment of violaxanthin de-epoxidation have been reported (Lidon and Ramalho, 2011; Pfündel et al., 1992). However, the latter response is usually a result of acute oxidative stress induced by high UV-B irradiance (Lidon et al., 2012). In contrast, successful acclimation to UV-A and UV- B appears to be associated with enhanced VAZ size and unaffected xanthophyll-cycle activity (Láposi et al., 2009). VAZ accumulation together with a slight increase of Chl *a*/*b* indicate that in our experiment UV-radiation mainly induced the accumulation of xanthophyll-cycle pigments that were not bound to pigment protein complexes, i.e. an effective acclimation response. Induction of VAZ by PAR/UV acclimation treatments broadly correlates with the induction of flavonols. This may be an effective protection response, whereby two pathways leading to antioxidative protection are simultaneously induced. However, more research is required to determine whether these protective responses are truly linked, or rather whether these are separate but co-occurring phenomena.

Based on the photoinhibition hypothesis, a trade-off should be expected between secondary metabolism and photosynthetic pigments (Close and McArthur, 2002). In accordance with this hypothesis, we report a negative correlation between the flavonol content and the Chls/Cars ratio (Fig. 5), which means that the accumulation of flavonoids is accompanied by a decrease in chlorophyll content. Similarly, Close et al. (2003) reported a negative correlation between leaf phenolics and total chlorophyll content in *Eucalyptus nitens* seedlings.

5. Conclusions

Based on the results of our study, we conclude that acclimation to PAR and UV radiation substantially increases the photoprotective capacity of barley plants, especially in variety Barke. As a consequence, subsequent high radiation events cause less photooxidative damage, with decreased harmful effects on both stages of photosynthesis associated with photochemical quantum yield and CO_2 assimilation. Our results reveal that the accumulation of xanthophyll-cycle pigments and flavonoids (mainly flavonols) during acclimation to PAR and/or UV, correlates with protection against photoinhibitory damage to the photosynthetic apparatus. While high PAR intensity itself induces sufficient photoprotective capacity against HRS in variety Bonus, additional acclimation to UV is necessary to induce adequate protection in sensitive variety Barke. These data demonstrate the importance of UV-acclimation, showing that UV exposure can contribute to the induction of tolerance to high radiation stress in barley.

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659

660 **Table 1** Summary of significance levels (p-values of the two-way ANOVA) for the effects of
661 UV/PAR treatments (treatment) and barley variety (variety) on the total content of epidermal
662 flavonols determined by *in vivo* fluorescence and content of individual flavonoids determined
663 by HPLC. Significant effects and interactions ($p \leq 0.05$) are indicated in bold.
664

	Lutonarin	Saponarin	3-ferulolyl quinic acid	Flavonols *in vivo*
Variety	**<0.001**	**0.017**	**<0.001**	0.502
Treatment	**<0.001**	0.066	**<0.001**	**<0.001**
Variety x Treatment	0.119	0.926	0.739	**0.011**

665
666
667

668 **Table 2** Effect of UV/PAR treatments and barley variety on total chlorophyll content (Chls)
669 and total content of carotenoids (Cars) per unit leaf area, chlorophyll a/b ratio (Chl *a/b*), and
670 ratio of total chlorophylls to carotenoids (Chls/Cars). Means and standard deviations (SD) are
671 reported (n≥5). Different letters denote statistically significant differences between
672 acclimation treatments and varieties using Tukey's post hoc test (p≤0.05).
673

Variety	**Treatment**	**Chls**	**Cars**	**Chl *a/b***	**Chls/Cars**
		mg m^{-2}	mg m^{-2}	dimensionless	dimensionless
Barke	[UV–PAR–]	237 ± 23^{b}	50.0 ± 4.3^{ab}	2.80 ± 0.08^{a}	4.74 ± 0.12^{c}
	[UV–PAR+]	219 ± 14^{ab}	52.3 ± 2.9^{b}	2.93 ± 0.04^{bc}	4.19 ± 0.17^{b}
	[UV+PAR+]	206 ± 4^{a}	51.7 ± 2.0^{b}	2.88 ± 0.04^{ab}	3.98 ± 0.10^{ab}
Bonus	[UV–PAR-]	214 ± 12^{ab}	46.3 ± 2.9^{a}	3.00 ± 0.06^{cd}	4.62 ± 0.06^{c}
	[UV–PAR+]	209 ± 8^{a}	50.5 ± 2.3^{ab}	3.12 ± 0.04^{e}	4.15 ± 0.08^{ab}
	[UV+PAR+]	200 ± 12^{a}	50.4 ± 2.4^{ab}	3.07 ± 0.07^{de}	3.97 ± 0.15^{a}

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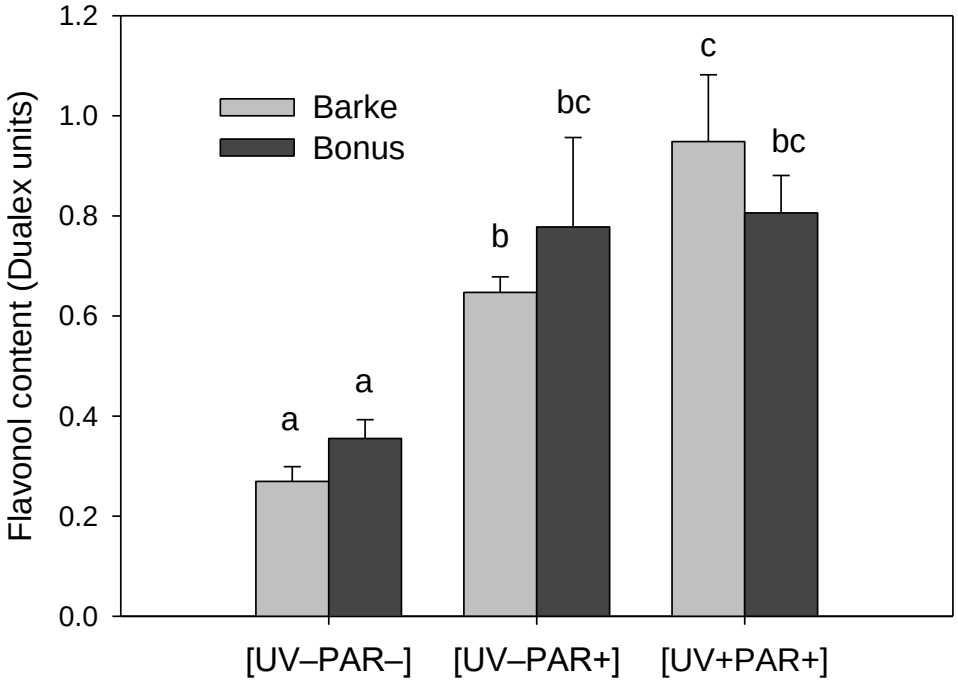

677

678 **Fig. 1** Flavonol content in barley leaves measured *in vivo* using the instrument Dualex 4 Flav
679 after 7-day acclimation to individual UV/PAR treatments. Data are presented for tolerant
680 (Bonus; dark gray) and sensitive (Barke; light gray) barley varieties. Means (columns) and
681 standard deviations (error bars) are presented ($n \geq 5$). Different letters denote statistically
682 significant differences ($p \leq 0.05$) between acclimation treatments and individual varieties using
683 Tukey's ANOVA post-hoc test.

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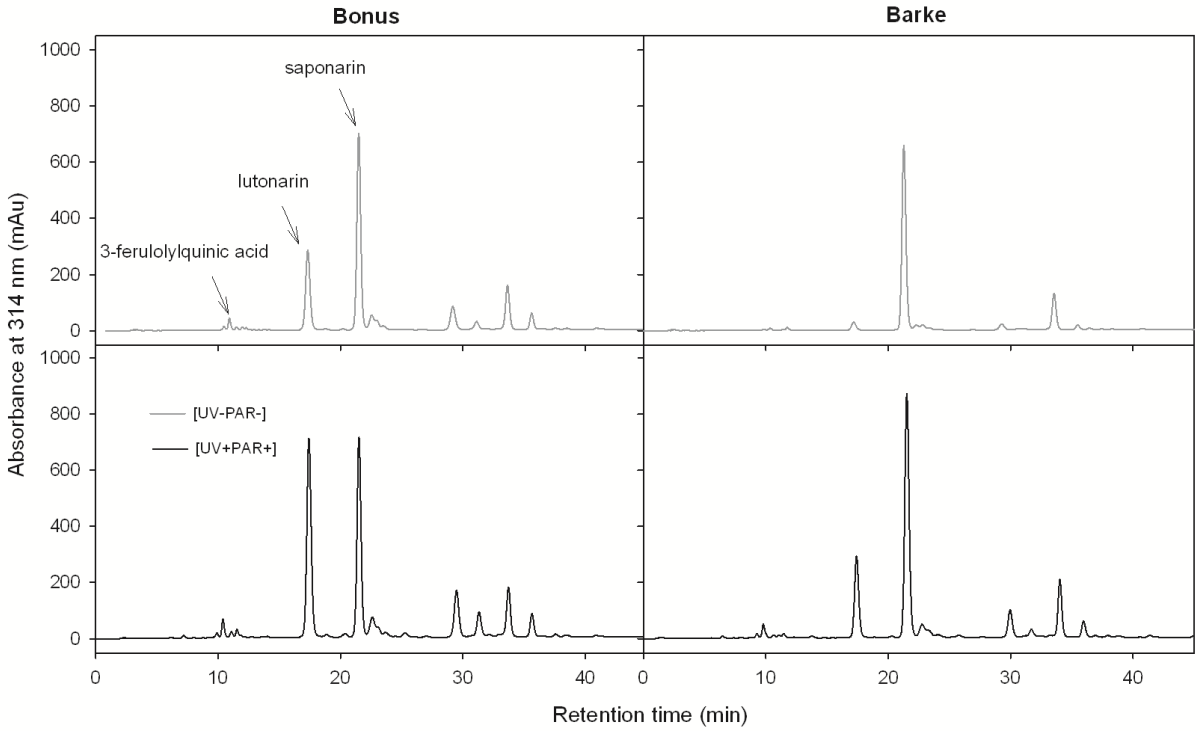

685
686 **Fig. 2** Typical chromatograms of phenolic compounds from the 2nd leaf of varieties Barke
687 and Bonus. The absorbance at 314 nm of methanolic extracts from [UV–PAR–] (grey; upper
688 panels) and [UV+PAR+] treatments (black; lower panels) are shown.

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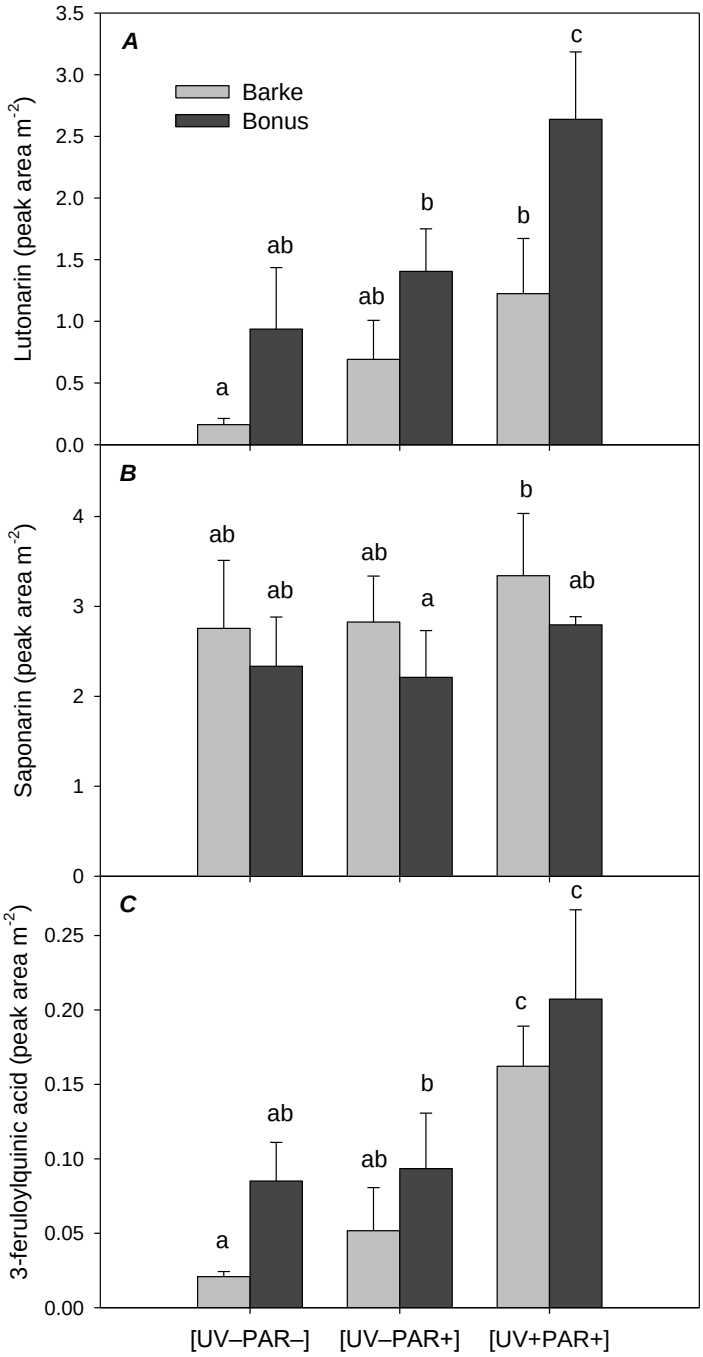
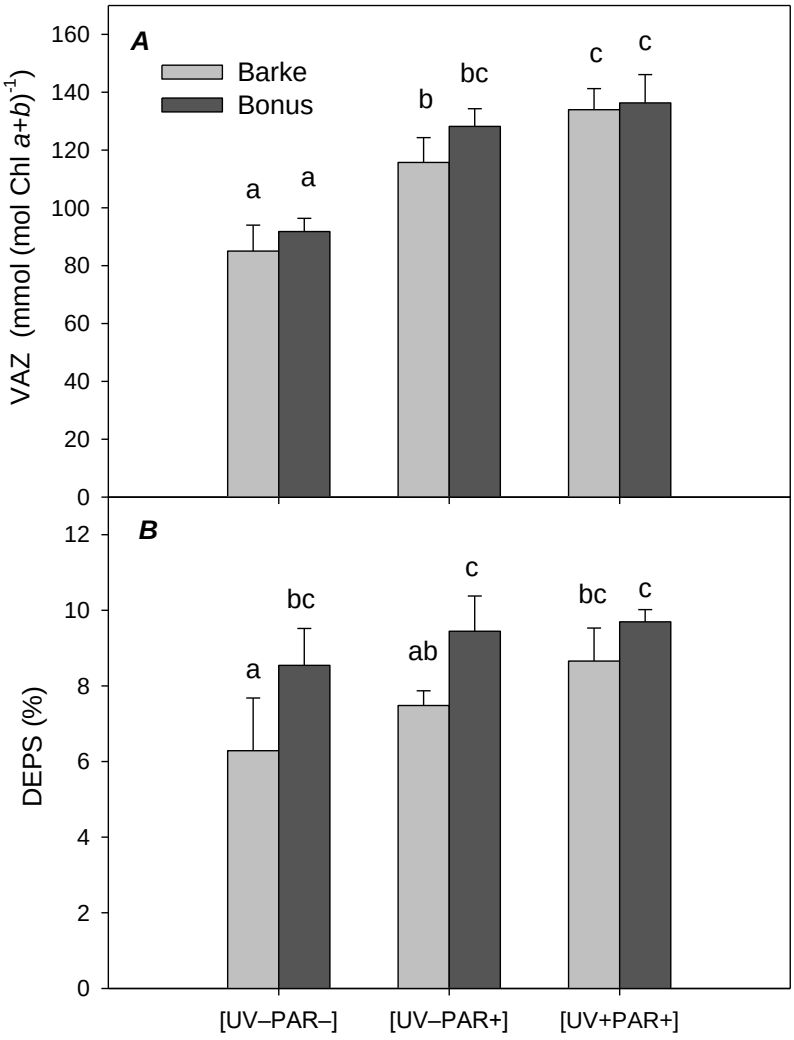


691

692 **Fig. 3** Content of lutonarin (A), saponarin (B) and 3-feruloylquinic acid (C) per unit leaf area
693 of barley leaves after 7-day acclimation to individual UV/PAR treatments in UV-tolerant
694 (Bonus; dark grey) and sensitive (Barke; light grey) barley varieties. Means (columns) and
695 standard deviations (error bars) are presented ($n \geq 5$). Different letters denote statistically
696 significant differences ($p \leq 0.05$) between acclimation treatments and leaves within individual
697 varieties.



698

699 **Fig. 4** Effect of UV/PAR acclimation on the total content of xanthophyll-cycle pigments
700 (VAZ; A) and the nominal de-epoxidation state (DEPS) of the xanthophyll-cycle pigments
701 (B) estimated in dark adapted leaves after 7-day acclimation to individual UV/PAR
702 treatments. Data are presented separately for the barley variety tolerant to UV radiation
703 (Bonus; dark grey) and the variety sensitive to UV radiation (Barke; light grey). Means
704 (columns) and standard deviations (error bars) are presented ($n \geq 5$). Different letters denote
705 statistically significant differences ($p \leq 0.05$) between acclimation treatments and individual
706 varieties using Tukey's ANOVA post-hoc test.

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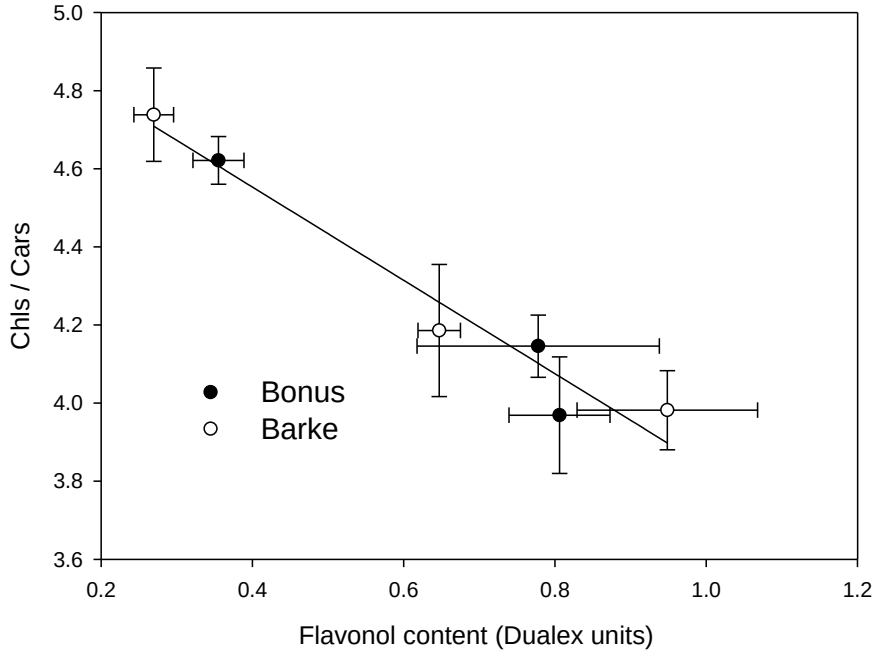

708

709 **Fig. 5** The relationship between flavonol content measured *in vivo* using the instrument

710 Dualex 4 Flav and the ratio of total chlorophylls to carotenoids (Chls/Cars) at the end of

711 UV/PAR treatments. A linear function (y=5.03-1.195x) was fitted to the data of both barley

712 varieties together (R^2=0.953; p≤0.01). Means (points) and standard deviations (vertical and

713 horizontal error bars) are presented (n≥5).

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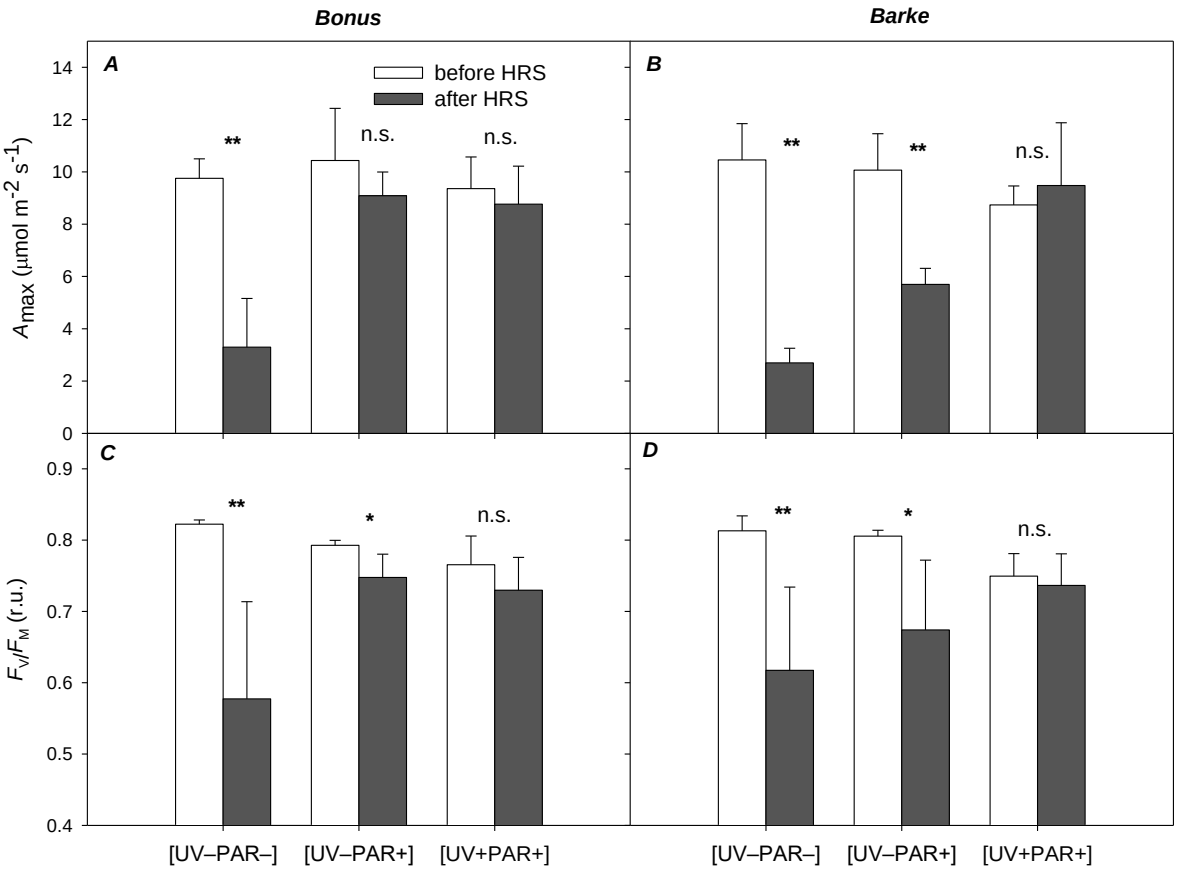

Fig. 6 Changes in the light-saturated CO_2 assimilation rate (A_{max}; A, B) and maximum quantum yield of photosystem II photochemistry (F_V/F_M; C, D) before (clear columns) and after (opaque columns) the application of short-term (4 hours) high radiation stress (HRS). The UV-tolerant (Bonus; A, C) and UV-sensitive (Barke; B, D) barley varieties were acclimated for 7 days to individual UV/PAR treatments before the application of HRS. Means (columns) and standard deviations (error bars) are presented (n≥5). Significant differences between means before and after HRS were tested using paired Student's t-test for independent samples (* significant at p≤0.05; ** significant at p≤0.01; n.s. non-significant).

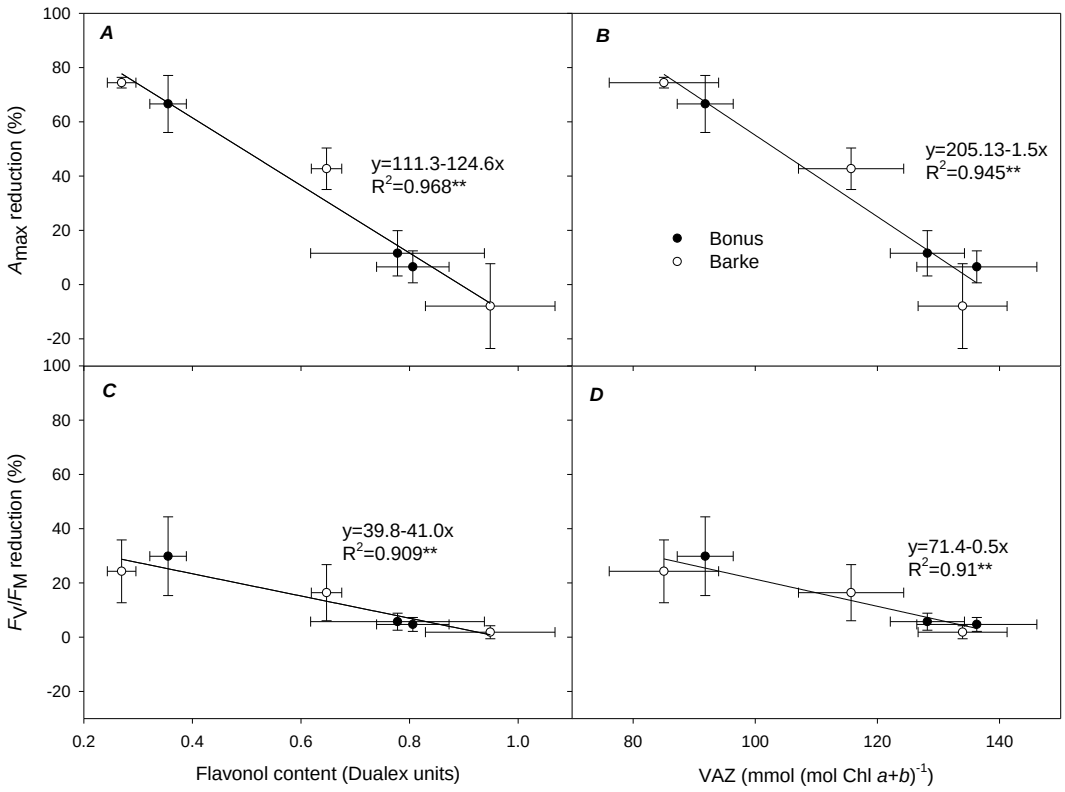

Fig. 7 Relationships between *in vivo* flavonol content (A, C), total content of xanthophyll-cycle pigments (B, D) and the relative reduction of photosynthetic parameters: light-saturated CO_2 assimilation rate A_{max} (A, B) and maximum quantum yield of photosystem II photochemistry F_V/F_M (C, D) after the application of short-term high radiation stress. Linear functions were fitted to the data of both barley varieties together ($p \leq 0.01$). Means (points) and standard deviations (vertical and horizontal error bars) are presented ($n \geq 5$).