Research article

Induction of phenolic compounds by UV and PAR is modulated by leaf ontogeny and barley genotype☆

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ABSTRACT

We investigated the effect of leaf ontogeny and barley genotype on the accumulation of phenolic compounds (PhCs) induced by ultraviolet (UV) and photosynthetically active radiation (PAR). We hypothesized that different groups of PhCs are induced in leaves differing in ontogeny, and that this has consequences for protective functions and the need for other protection mechanisms. Generally, lower constitutive contents of PhCs (under conditions of UV exclusion and reduced PAR) were found in a UV-sensitive genotype (Barke) compared to a tolerant genotype (Bonus). However, UV and PAR induced accumulation of PhCs exceeded the constitutive amounts several fold. Specifically, lutonarin, 3-feruloylquinic acid, unidentified hydroxycinnamic acid and luteolin derivatives were markedly enhanced by high PAR and UV irradiances. Leaves developed during UV and PAR treatments had higher PhCs contents than mature leaves already fully developed at the onset of the UV and PAR treatment. UV and PAR treatments had, however a minor effect on saponarin and unidentified apigenin derivatives which occur particularly in mature leaves of the tolerant genotype Bonus. In addition, high UV and PAR intensities increased the total content of xanthophylls (VAZ), while chlorophyll content was reduced, particularly in developing leaves. A redundancy analysis revealed positive associations between most of PhCs and VAZ and a negative association between total chlorophylls and carotenoids. Non-linear relationships between VAZ and lutonarin and other PhCs indicate that VAZ accumulation can compensate for the insufficient efficiency of anti-oxidative protection mediated by PhCs. Accordingly, we conclude that UV and PAR-induced accumulation of PhCs is affected by leaf ontogeny, however, this effect is compound-specific.

1. Introduction

Phenolic compounds (PhCs) represent one of the most important groups of secondary metabolites in plants, where these compounds perform a range of protective functions (Treutter, 2006). Particularly flavonoids, representing the largest group of naturally occurring PhCs in plants, are known as potential *in vitro* antioxidants. However, the precise *in vivo* significance of flavonoids within the overall complex network of antioxidant defenses remains unclear. Flavonoids, amongst others, provide protection against ultraviolet (UV) radiation. These compounds have absorption maxima predominantly in the range of 240–390 nm (Cerovic et al., 2002) and act as sunscreens in the leaf epidermis protecting the inner cells from potentially harmful short-wavelength UV radiation (Jordan, 2002; Bassman, 2004), while photosynthetically active radiation (PAR) is transmitted to the mesophyll cells.

In addition to constitutive production, which is species- and genotype-specific, bio-synthesis of PhCs is induced by a wide range of

environmental stimuli including light irradiance and spectral quality (particularly proportion of UV and blue light; Bian et al., 2015), drought (Hernández et al., 2004), nitrogen and/or phosphorus deficiency (Stewart et al., 2001), pathogen attack (Dixon and Paiva, 1995), and low temperature (Leyva et al., 1995). There is extensive literature describing the effect of light quality on PhC accumulation and the expression of chalcone synthase and other PhC biosynthesis genes in a range of plant species (reviewed by Dixon and Paiva, 1995 and Mol et al., 1996). It has been shown that the expression of chalcone synthase, the first enzyme in the flavonoid-specific branch of the phenylpropanoid biosynthesis pathway, is stimulated mainly by UV and blue light (Adamse et al., 1994). Photoreceptors of UV-B, UV-A/blue, and red/far-red wavelengths are all involved in the induction of PhCs (Ballaré et al., 1992; Beggs and Wellmann, 1994; Barnes et al., 1996; Hemm et al., 2004). UV and PAR influence the biosynthesis of specific flavonoids (Klem et al., 2015), but there are also interactive effects when plants are exposed to both UV and PAR. For example, Götz et al. (2010) reported that accumulation of UV-B induced PhCs is modulated by PAR. Furthermore, it was found that PAR and UV-B have an additive effect on the accumulation of UV-absorbing compounds (Meijkamp et al., 2001). Thus, there is ample crosstalk between induction responses triggered by different wavelengths.

Plants have evolved different mechanisms to dissipate and/or decrease exposure to excessive solar energy and to prevent photo-oxidative damage. Mechanisms include the accumulation of photoprotective pigments and antioxidants, and chloroplast movements. The carotenoids of the xanthophyll cycle mediate non-photochemical energy dissipation which is thought to be the major photoprotective mechanism by which excessive light energy is dissipated as heat (Demmig-Adams and Adams, 2000). PhCs represent a group of compounds with diverse protective functions including epidermal UV-screening and/or scavenging of free radicals and reactive oxygen species. In addition, some PhCs like anthocyanins absorb high-energy quanta of PAR and thus protect chloroplasts from photoinhibition and photooxidative injury by lowering exposure to solar radiation (Gould, 2004). PhCs are found in cell walls as well as various cellular compartments including the chloroplast, vacuole, and nucleus. In contrast to PhCs, the xanthophyll cycle pigments function mainly to protect photosynthesis against sudden increases in PAR irradiance. Hernández and Van Breusegem (2010) assumed that the accumulation of PhCs might act as an excess energy escape valve through substantial consumption of formed energy (ATP and NADPH) and as a sink for carbon. Therefore, biosynthesis of PhCs is tightly connected with the CO_2 assimilation rate and excess light (Tattini et al., 2004).

In our previous studies (Klem et al., 2012, 2015), we demonstrated that UV radiation can reduce the photosynthetic activity of barley leaves, but that the extent of the reduction is different in mature compared to young leaves. Furthermore, high PAR intensities triggered photoprotective mechanisms which partially ameliorated negative UV effects on photochemistry and carbon assimilation. PAR contributed mainly to photoprotection by enhancing the epidermal UV-screening in young leaves (Klem et al., 2012). In mature leaves, epidermal UV-screening was primarily induced by UV radiation (Klem et al., 2012). However, these studies did not provide an answer on questions how the constitutive and UV or PAR induced accumulation of individual PhCs is affected by leaf age and how their accumulation is linked to other photoprotective mechanisms, such as xanthophyll cycle pigments accumulation (VAZ), which was investigated in this study. Morales et al. (2011) have also shown that the induction of epidermal UV-screening occurs more rapidly in young leaves at the top of the plant, compared to mature leaves. Reduced accumulation of PhCs with increasing leaf age was also reported in pear leaves (Andreotti et al., 2006). The reason for such a decline may be an altered activity of phenylalanine ammonia-lyase (PAL) since PAL content decreases with increasing leaf age (Fünfgelder et al., 1993).

The objective of our study was to explore the effects of PAR and UV radiation on the accumulation of individual PhCs in barley genotypes differing in their sensitivity to photo-oxidative stress – UV sensitive

Barke versus UV tolerant Bonus. The following hypotheses were tested under field conditions: (1) UV and PAR treatment induces different groups of PhCs in young developing compared to mature non-senescent leaves; (2) the biosynthesis of individual PhCs is genotype-specific and also depends on the UV:PAR ratio, (3) accumulation of PhCs is orchestrated with accumulation of xanthophyll cycle pigments and their de-epoxidation state to provide balanced protective functions in plants, (4) accumulated epidermal PhCs are stable in time and persist in leaves even during subsequent exposure to low PAR and UV intensities.

2. Material and methods

2.1. Plant material

The pre-treatment of barley plants was described in detail by Klem et al. (2012). Two barley genotypes differing in sensitivity to UV radiation were studied: Barke (sensitive) and Bonus (tolerant). All seeds were pre-germinated on wetted filter paper for 48 h at room temperature, and germinating seeds were subsequently used. Three seeds per pot (5 cm in diameter) were transplanted with a triangular pattern to avoid mutual shading of plants. The pots were filled with a mixture (1:1) of horticulture substrate and a substrate for pot houseplants (Agro CS, Ceska Skalice, CZ). Plastic trays woth capillary matting ensured uniform watering.

The plants were grown under conditions of low PAR irradiances (up to 400 μmol photons m^{-2} s^{-1}) and UV-A and UV-B exclusion for 14 days (full development of the second leaf) in the garden of Global Change Research Institute CAS in Brno (49.187˚N; 16.592˚E) from August 13, 2010. Neutral density filters 0.6ND (Lee Filters, Hampshire, UK) were used to reduce PAR to 25% of natural irradiance. The transparent plastic filter Lee U.V. 226 (Lee Filters, UK; cutting-off wavelength ca. 390 nm) was used for UV-A and UV-B exclusion. Subsequently, the barley plants were transferred to individual PAR and UV treatments.

2.2. Acclimation to UV and PAR

Individual UV and PAR treatments were provided using outdoor chambers with tops and the upper half of sides covered by UV and PAR filters (Lee U.V. 226 in all treatments and Lee 0.6ND filters in [UV–PAR–] treatment only; Lee Filters, UK). A modulated UV lamp system (Konel, Zlín, CZ) was used to provide required UV intensities. The system used UV-A (TL 20 W/10 SLV; Philips) and UV-B (TL 20 W/12 RS SLV; Philips) fluorescent lamps. See Klem et al. (2012) for a detailed description of the chambers and modulated lamp system. To block residual UV-C radiation the lamps were covered by cellulose diacetate film (thickness of 0.13 mm). Li-190SA (Li-Cor, Lincoln, USA), SKU 420 and SKU 430 (Skye Instruments, Powys, UK) radiation sensors were used outside and inside chambers to continuously measure intensities of PAR, UV-A, and UV-B, respectively, and for feedback regulation of the UV lamp output. A data logger DL2e (Delta-T Devices, Cambridge, UK) was used to record the readings of all sensors.

Sixteen replicates (pots) of each genotype (i.e. 48 plants per treatment) were grown in following UV and PAR conditions: [UV–PAR–] with excluded UV and reduced PAR to ca. 25% of ambient irradiance, [UV–PAR+] with excluded UV but retained ambient PAR irradiance, and [UV+PAR+] representing enhanced UV and ambient PAR irradiance. The [UV–PAR–] treatment was in this study used to define the constitutive accumulation level of phenolic compounds, while [UV–PAR+] and [UV+PAR+] treatments enabled determination of PAR-induced and PAR and UV-induced accumulation, respectively. The UV irradiance of fluorescent lamps was adjusted to provide ca. 200% of the actual ambient UV irradiance. The ratio of UV-A to UV-B was kept the same as under ambient (sunlight) conditions. The barley plants were exposed to specific UV and PAR conditions for seven days. The first three leaves were fully developed at the end of the seven days

exposure experiment. The 2nd leaf (hereinafter referred to as mature) was already fully developed at the beginning of the UV and PAR treatments, whereas the 3rd (hereinafter referred to as young) leaf developed during the UV and PAR treatments. Differences between varieties in ontogeny were minimal with just one day delay of new leaf development in variety Bonus.

Changes in sky conditions, from cloudy to sunny (daily sum of PAR up to 2.3 and 5 MJ m^{-2} day^{-1}, respectively) affected daily maxima of ambient UV irradiances. UV-B doses ranged from 0.35 to 1.4 W m^{-2} and UV-A from 5.8 to 20.5 W m^{-2} during cloudy and sunny days, respectively. While daily sums of biologically effective UV-B doses (UV-B$_{BE}$) can be considered as zero under [UV−] conditions, UV-B$_{BE}$ amounted to 14.7–26.1, 11.5–16.1 and 20.5–28.6 kJ m^{-2} day^{-1} under [UV+] using the action spectrum for flavonoid accumulation (Ibdah et al., 2002), plant growth inhibition (Flint and Caldwell, 2003) and generalized plant action spectrum (Thimijan et al., 1978), respectively. Such doses are roughly equivalent to full-summer maxima under extremely reduced stratospheric ozone layer conditions. UV-B$_{BE}$ was determined on the basis of the emission spectrum of the UV lamps determined by a spectroradiometer SM 9000 (PSI, Brno, CZ). The air temperature was measured at the height of plants using EMS33 sensor (EMS, Brno, CZ). The mean daily temperature ranged between 12.2 and 19.4 °C. Due to open walls of the chambers, continuous air flow was possible, and the temperature at the level of plants was not increased more than 1.5 °C compared to ambient conditions.

2.3. Induction and stability of flavonol index

Induction of UV-screening capacity by epidermal flavonols during UV and PAR treatments was measured in 2nd and 3rd leaves every day between 12:00–14:00 (CET). Screening capacity was measured as the flavonol index using the instrument Dualex 4 Flav (Force-A, Orsay, F). Measurements centred on the middle part of the leaves. To determine the stability of the flavonol index, six replicates acclimated to each specific UV and PAR treatment were placed back under [UV–PAR–] conditions and the flavonol index was measured immediately after the transition to [UV–PAR–] and again after 4, 7, and 10 days.

2.4. HPLC analyses of phenolic compounds

Central leaf segments (ca. 100 mg of fresh weight) were sampled for HPLC analyses after 12 h of dark adaptation at the end of UV and PAR treatments. The area of leaf segments was determined immediately using a flat-bed scanner and calculated by Cernota software (Kalina and Slovák, 2004). The sample was subsequently homogenized in a grinding bowl together with 3 ml of 40% methanol, ultrasonicated (UC 006 DM1, Tesla Vráble, CZ) for 5 min and centrifuged at 6000 RPM for 3 min (EBA 20, Hettich Zentrifugen, Tuttlingen, DE). The supernatant (ca. 1 ml) was filtered through a filter with 0.2 μm porosity (Premium Syringe Filters, Agilent, Santa Clara, USA) and then used for HPLC analysis.

Analyses were performed according to Kolb and Pfündel (2005) with a slight modification for an HPLC system (TSP Analytical, USA) equipped with a diode array detector. A LiChrospher chromatographic column was used to separate metabolites (RP-18, 250 × 4 mm, 5 μm). A gradient of two mobile phases (A: 850 ml H$_2$O + 100 μl H$_3$PO$_4$ and B: 765 ml CH$_3$CH$_2$OH + 85 ml H$_2$O + 100 μl H$_3$PO$_4$) was applied. The gradient elution started with 80% of mobile phase A, its content was decreasing for 7 min to 66% followed by isocratic elution for another 5 min. After that, the content of mobile phase A was reduced to 60% for 6 min and subsequently, 5 min long isocratic elution took place. At 23rd min of separation, a slow decrease of mobile phase A was applied (for 25 min from 60 to 35%). Then the content of mobile phase A was steeply reduced to 0% during 1 min, and the separation column was flushed for another 5 min. by 100% of mobile phase B. During the last two minutes of the analysis the content of mobile phase A was increased to the initial state (80%). Between each analysis, the 7 min long

equilibration was performed.

Chromatograms were recorded using absorption wavelengths of 220, 314 or 440 nm. Absorption spectra were measured in the spectral range between 220 and 500 nm. Detection and quantification of PhCs were mostly based on the signal at 314 nm. The chromatograms recorded on absorption wavelengths 220 and 440 nm were used to test the possible contamination with other organic molecules or photosynthetic pigments, respectively. Integration of peak areas was done manually, and peak area was subsequently normalised to leaf area. Compounds with retention times of 7.76 and 10.44 min were on the basis of absorption spectra classified as hydroxycinnamic acid derivatives and referred to as HCA$_1$ and HCA$_2$ hereinafter. Identification of 3-feruloylquinic acid (FQA) was performed using LC/MS and MS/MS fragmentation as described by Klem et al. (2015). Lutonarin and saponarin were identified on the basis of comparison of retention times and absorption spectra with standards. Compounds with a retention time of 31.54 and 33.26 min were on the basis of chromatograms and absorption spectra classified as luteolin derivatives referred to as LD$_1$ and LD$_2$ hereinafter. A compound with a retention time of 35.53 min was on the basis of absorption spectra identified as an apigenin derivative and referred to as AD hereinafter.

2.5. Analysis of photosynthetic pigments

The dark adapted leaves (12h) were used for analysis of xanthophyll cycle pigments (violaxanthin, antheraxanthin, zeaxanthin), chlorophylls and total carotenoids. The photosynthetic pigments (chlorophylls and carotenoids) were extracted from leaves with 100% acetone with the addition of a small amount of MgCO$_3$. The supernatant, obtained after centrifugation, was subsequently diluted in 80% acetone and used for a spectrophotometric measurements by UV/VIS Unicam 550 (Thermo Spectronic, Cambridge, UK). The contents of total chlorophylls (Chl $a+b$; Chls) and total carotenoids (Car $x + c$; Cars), Chl a/b and Chls/Cars ratios were calculated according to Lichtenthaler (1987). Chls and Cars contents were expressed per unit of leaf area.

The contents of individual xanthophylls together with the total pool of xanthophyll pigments (violaxanthin + antheraxanthin + zeaxanthin; VAZ) were estimated by gradient reversed-phase HPLC (TSP Analytical, USA) and subsequently expressed per unit of Chl according to Štroch et al. (2008). The nominal de-epoxidation state of the xanthophyll cycle pigments (DEPS) in dark-adapted leaves was calculated as $(Z + A)/(V + A + Z)$.

2.6. Data analysis

Before analysis of variance (ANOVA), the normality of data for individual parameters was tested using a Kolgomorov-Smirnov test. For the general analysis of UV, PAR, and leaf-age effects, the data were separately analysed within the individual barley genotypes using a two-way fixed-effect ANOVA model. The homogeneity of variances was tested using a Levene test, and where necessary a square root or reciprocal transformation was used to improve the homogeneity of variances. Pearson two-tailed correlation analyses were performed between selected parameters to determine whether these parameters were related ($n = 30$).

A multiple range test (Tukey's post-hoc; $p = 0.05$) was used to evaluate statistically significant differences between means for individual UV and PAR treatments and leaf-age classes within each barley variety separately. All statistical tests were performed in Statistica 12 software (StatSoft, Tulsa, USA). Linear and non-linear relationships were analysed using SigmaPlot 11 software (Systat Software, Chicago, USA).

To explore the associations between the observed effects of leaf ontogeny, PAR, and UV-exposure, a multivariate redundancy analysis (RDA) based on all measured traits was performed individually for both barley genotypes using Canoco 5 software (Microcomputer Power, Ithaca, USA).

Table 1

Summary of significance levels (*p*-values of the two-way ANOVA) for the effects of leaf age, UV and PAR treatments and their interactions on the flavonol index (Flav. index), the content of individual flavonoids determined by HPLC (HCA$_1$ and HCA$_2$ – unidentified hydroxycinnamic acids, FQA – 3-feruloylquinic acid, lutonarin, saponarin, LD$_1$ and LD$_2$ – unidentified luteolin derivatives, AD – apigenin derivative), the nominal de-epoxidation state (DEPS) of the xanthophyll-cycle pigments, the total content of xanthophyll-cycle pigments (VAZ), the contents of pigments (total chlorophyll; Chls and carotenoids; Cars), and their ratios. Significant effects ($p \leq 0.05$) are indicated in bold.

| Effects	Barke			Bonus		
	Leaf age	UV and PAR	Interaction	Leaf age	UV and PAR	Interaction
Flav. index	**≤ 0.001**	**≤ 0.001**	0.070	**≤ 0.001**	**≤ 0.001**	0.919
HCA$_1$	**≤ 0.001**	**≤ 0.001**	**0.047**	**≤ 0.001**	**≤ 0.001**	**0.013**
HCA$_2$	**≤ 0.001**	**≤ 0.001**	0.906	**≤ 0.001**	**≤ 0.001**	**0.022**
FQA	**≤ 0.001**	**≤ 0.001**	0.081	**≤ 0.001**	**0.001**	**0.009**
Lutonarin	**≤ 0.001**	**≤ 0.001**	**0.021**	**≤ 0.001**	**≤ 0.001**	**0.022**
Saponarin	0.484	0.425	0.299	**≤ 0.001**	0.091	0.292
LD1	**0.001**	**≤ 0.001**	**≤ 0.001**	**≤ 0.001**	**≤ 0.001**	**0.017**
LD2	**≤ 0.001**	**≤ 0.001**	**0.002**	**≤ 0.001**	**≤ 0.001**	**≤ 0.001**
AD	**≤ 0.001**	**0.035**	**≤ 0.001**	**≤ 0.001**	**0.002**	0.067
DEPS	**0.023**	**≤ 0.001**	0.330	**≤ 0.001**	**≤ 0.001**	**0.035**
VAZ	**≤ 0.001**	**≤ 0.001**	0.184	**≤ 0.001**	**≤ 0.001**	0.868
Chls	**≤ 0.001**	**≤ 0.001**	**0.010**	**≤ 0.001**	**0.003**	**0.009**
Cars	**≤ 0.001**	**0.025**	**0.002**	**≤ 0.001**	0.297	**0.002**
Chl *a/b*	**≤ 0.001**	**≤ 0.001**	**0.047**	**≤ 0.001**	0.447	**0.003**
Chls/Cars	**≤ 0.001**	**≤ 0.001**	**0.017**	**≤ 0.001**	**≤ 0.001**	**≤ 0.001**
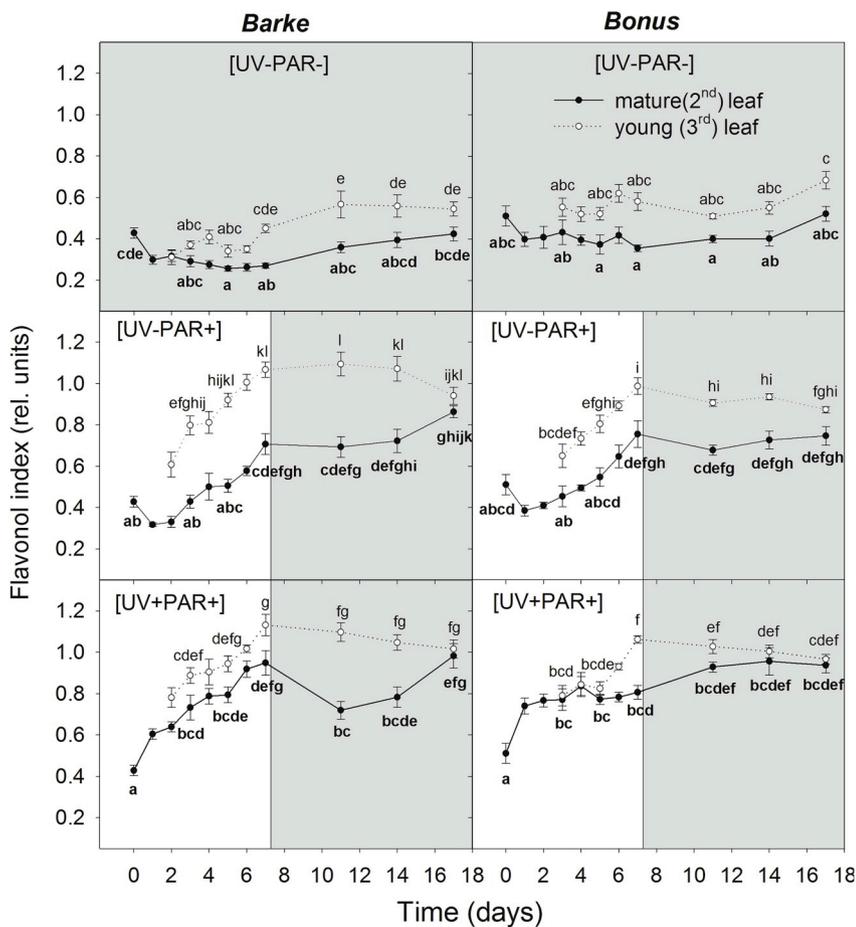
Fig. 1. Flavonol index in mature (2nd) and young (3rd) barley leaves measured *in vivo* using the instrument Dualex 4 Flav during both 7-day acclimation to different UV and PAR treatments (white area) and during the following relaxation [UV–PAR–] treatment (indicated as a shaded grey area). Data are presented for UV-sensitive (Barke) and UV-tolerant (Bonus) barley genotypes. Means and standard errors are presented ($n \geq 5$). Different letters denote statistically significant differences ($p \leq 0.05$) between dates of measurement and leaves within individual genotypes and UV and PAR treatments (results for day 0, 3, 5, 7, 11, 14 and 17 are presented only).

3. Results

3.1. Effect of UV and PAR acclimation on flavonol index

Significant changes in the flavonol index after 7-days acclimation to individual UV and PAR treatments were confirmed by ANOVA analysis (Table 1).

The dynamics of flavonol index changes is shown in Fig. 1. A higher flavonol index, measured under [UV–PAR–] treatment, was found in Bonus as compared to Barke 7 days after the beginning of treatment. The flavonol index was 31 and 29% higher in, respectively, the 2nd and 3rd leaf of Bonus when compared with Barke. The exposure of plants to [UV–PAR+] treatment led to a significant increase in flavonol index 7 days after the beginning of treatment in mature (2nd) leaves of both genotypes as compared to [UV–PAR–] treatment. The increase was 119 and 140% in Bonus and Barke, respectively. The [UV+PAR+]
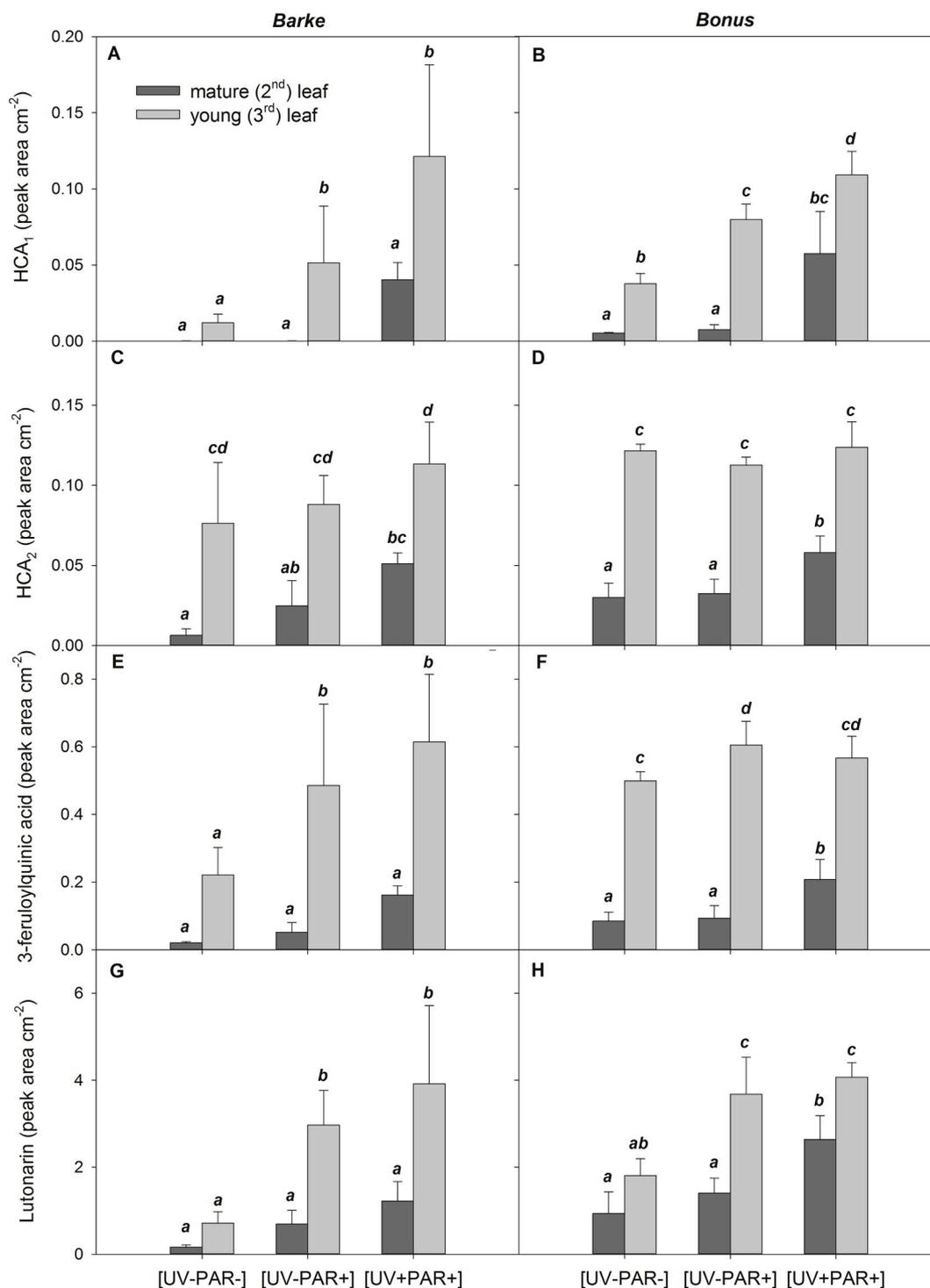
Fig. 2. Content of unidentified hydroxycinnamic acids HCA$_1$ (A, B) and HCA$_2$ (C, D), 3-feruloylquinic acid (E, F) and lutonarin (G, H) per unit leaf area of young (3rd; light grey) and mature (2nd; dark grey) barley leaves after 7-day acclimation to individual UV and PAR treatments in UV-tolerant (Bonus) and UV-sensitive (Barke) barley genotypes. Means (columns) and standard deviations (error bars) are presented ($n \geq 5$). Different letters denote statistically significant differences ($p \leq 0.05$) between acclimation treatments and leaves within individual genotypes.

treatment, as compared to [UV–PAR +], resulted in a further significant increase of the flavonol index 7 days after the beginning of the treatment in mature (2nd) leaves of Barke (by 47%), whereas a negligible increase (4%) was found in Bonus. The changes in young (3rd) leaves caused by UV and PAR treatments were similar as compared to mature (2nd) leaves. However, the responses to PAR treatment were more pronounced in genotype Barke (Fig. 1).

The flavonol index after 7 days of UV and PAR treatments was significantly higher in young (3rd) as compared to mature (2nd) leaves with differences ranging from 16 to 40% irrespective of barley genotype and radiation treatment. The highest relative differences between mature and young leaves (39 and 40% in Bonus and Barke, respectively) were observed under [UV–PAR–] treatment, demonstrating the important role of leaf age in controlling the constitutive accumulation of epidermal flavonols. However, UV and PAR treatments altered the differences between leaf age classes and induced significant, additional
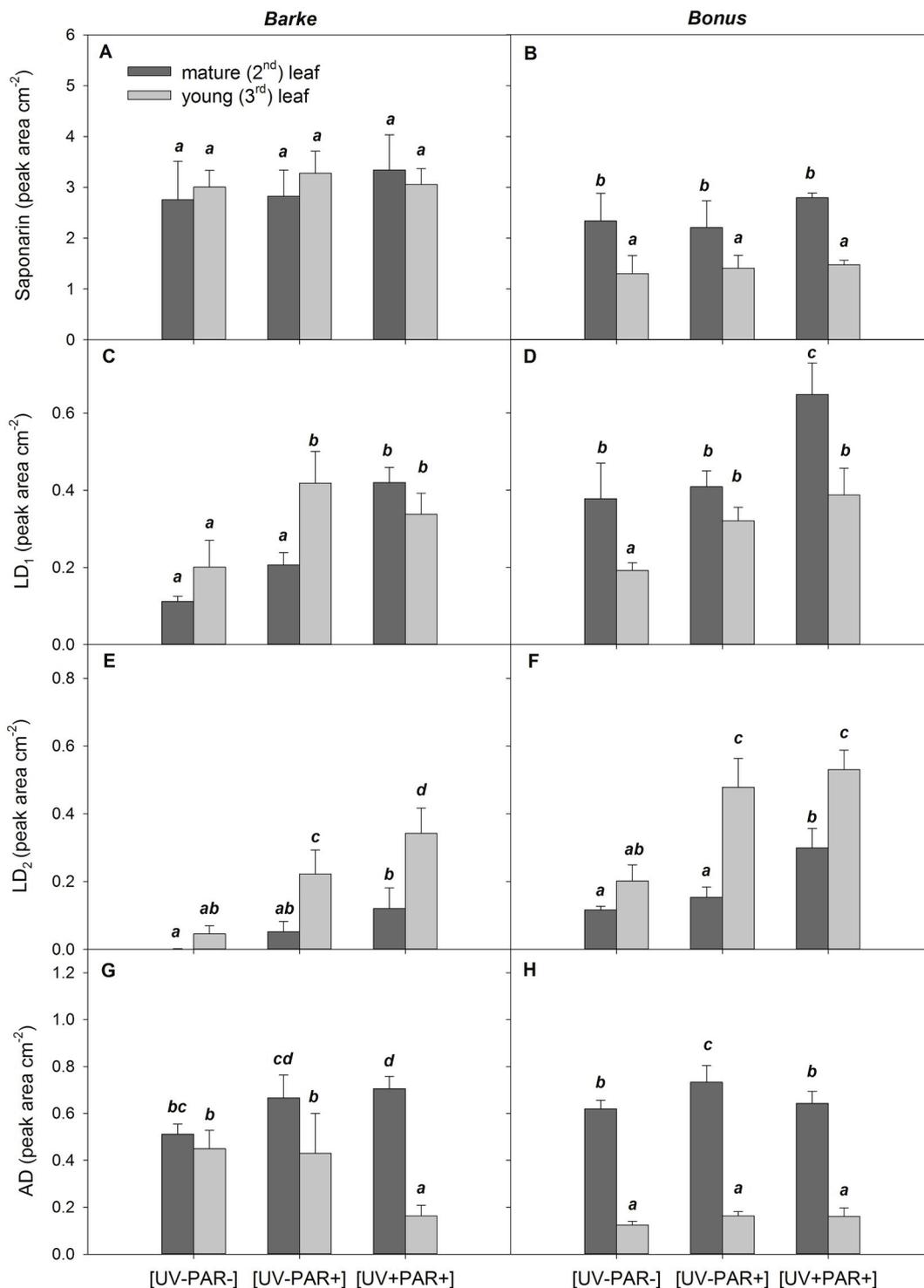
Fig. 3. Content of saponarin (A, B), unidentified luteolin derivatives LD_1 (C, D) and LD_2 (E, F) and apigenin derivative AD (G, H) per unit leaf area of barley young (3rd; light grey) and mature (2nd; dark grey) leaves after 7-day acclimation to individual UV and PAR treatments in UV-tolerant (Bonus) and UV-sensitive (Barke) barley genotypes. Means (columns) and standard deviations (error bars) are presented ($n \geq 5$). Different letters denote statistically significant differences ($p \leq 0.05$) between acclimation treatments and leaves within individual genotypes.

differences between genotypes studied. While the tolerant genotype Bonus showed only a 23% difference of flavonol index between young and mature leaves under [UV–PAR +] treatment, this amounted to 39% in the sensitive genotype Barke. The difference between mature (2nd) and young (3rd) leaves remained in genotype Bonus almost unchanged (24%) under [UV + PAR +]. However, the differential decreased to just 16% in genotype Barke (Fig. 1).

The flavonol index was found to be stable during the 10-day period after the transition of both barley genotypes from UV and PAR treatments to [UV–PAR–] conditions (Fig. 1). Generally, the differences between 2nd and 3rd leaves decreased with time due to minor increases in flavonol index in mature (2nd) leaves and reductions in young (3rd) leaves.
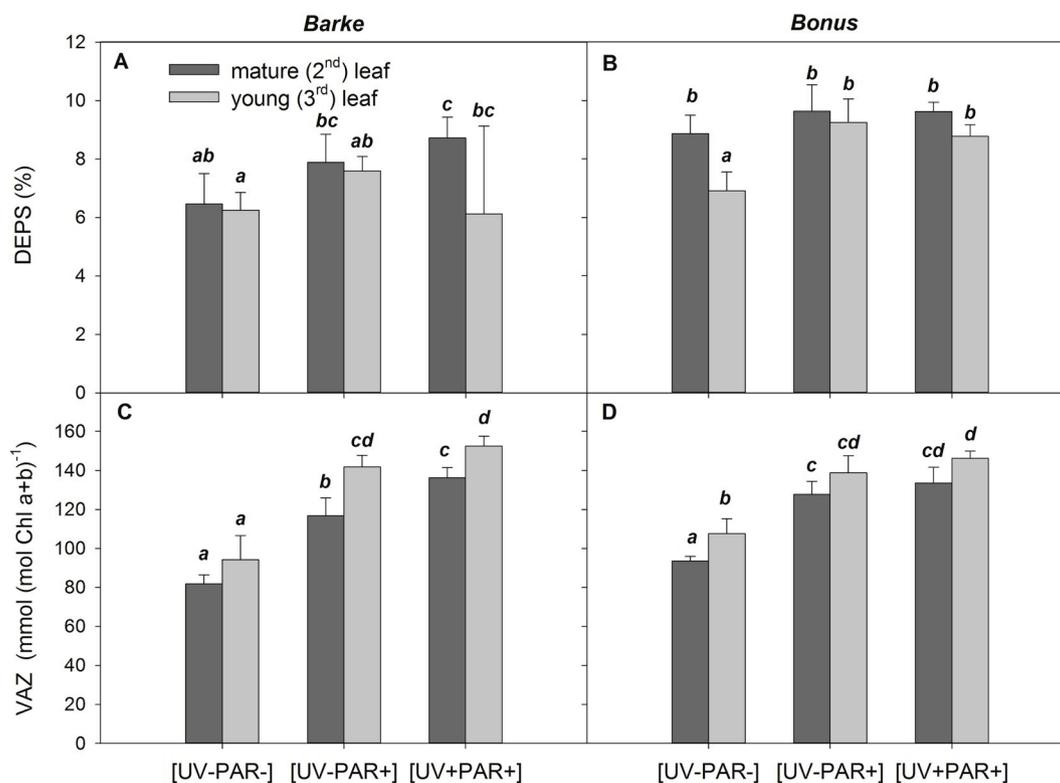
Fig. 4. Nominal de-epoxidation state (DEPS) of the xanthophyll-cycle pigments (A, B), and the total content of xanthophyll-cycle pigments (VAZ; C, D) estimated in dark-adapted young (3rd; light grey) and mature (2nd; dark grey) leaves after 7-day acclimation to individual UV and PAR treatments. Data are presented separately for the UV-tolerant (Bonus) and UV-sensitive (Barke) barley genotypes. Means (columns) and standard deviations (error bars) are presented ($n \geq 5$). Different letters denote statistically significant differences ($p \leq 0.05$) between acclimation treatments and individual genotypes using Tukey's ANOVA posthoc test.

3.2. Changes of individual PhCs in response to UV and PAR treatments

ANOVA's did show that all PhCs determined by HPLC, except saponarin, were significantly affected by both leaf age and UV and PAR treatment (Table 1). Generally, the content of HCA_1, HCA_2, FQA, lutonarin and LD_2 was significantly higher in young (3rd) compared to mature (2nd) leaves (Figs. 2 and 3). In contrast, higher AD content was found in mature (2nd) relative to young (3rd) leaves (Fig. 3). The response of saponarin and LD_1 to leaf ontogeny was genotype-specific. Higher contents of both compounds were found in mature, compared to young, Bonus leaves. In contrast, similar levels of saponarin and LD_1 were found in mature and young leaves of variety Barke, with the exception of slightly higher LD_1 levels in young leaves in the [UV–PAR +] treatment (Fig. 3). Generally, LD_2 accumulated strongest in Bonus, while saponarin accumulated more in Barke. The effect of PAR treatment on most of PhCs was not statistically significant in mature (2nd) leaves, while in young (3rd) leaves it was. On the other hand, UV treatment affected the accumulation of most PhCs similarly in both young (3rd) and mature (2nd) leaves. The greatest differences between barley genotypes were found in plants acclimated to [UV–PAR–] conditions representing the different constitutive accumulation of PhCs. The effect of UV and PAR treatment on the accumulation of PhCs was generally more pronounced in Barke, particularly for HCA_2, FQA and lutonarin. The effects of PAR and UV on the accumulation of PhCs, particularly HCA_1, FQA, lutonarin, and LD_2, were similar in young and mature Barke leaves. However, in Bonus, the accumulation of PhCs in young leaves was strongest induced by PAR but in mature leaves by UV.

Accumulation of saponarin and AD was less affected by PAR and UV, but in Bonus accumulation of these compounds was significantly affected by leaf age, with higher accumulation in mature leaves. In contrast, in Barke, the accumulation of saponarin was influenced neither by leaf age nor by UV and PAR treatment. UV and PAR treatment led to reduced AD content in young Barke leaves, while a slight positive effect was observed in mature leaves. Significantly higher contents of LD_1 and mainly AD were found in mature (2nd) as compared to young (3rd) leaves of both genotypes irrespective of UV and PAR treatment, except LD_1 in genotype Barke (Fig. 3).

3.3. Effect of UV and PAR treatments on chlorophylls and carotenoids

ANOVA's revealed that chlorophylls and carotenoids contents, and their ratios, were significantly affected by both leaf age and UV and PAR treatment in variety Barke, while in variety Bonus the effect of UV and PAR on total carotenoids and chlorophyll ratio (Chl a/b) was not significant (Table 1).

In comparison to [UV–PAR–], the treatments with higher UV and PAR led to an increase in total content of xanthophyll pigments (VAZ) in mature (2nd) leaves by 40 and 36% under [UV–PAR +], and by 48 and 58% under [UV + PAR +] in Bonus and Barke, respectively (Fig. 4C and D). The VAZ content was slightly higher in young (3rd) as compared to mature (2nd) leaves, irrespective of barley genotype, however, these changes were mostly statistically insignificant. The nominal level of violaxanthin de-epoxidation (DEPS) after dark-adaptation was not significantly influenced by the UV and PAR treatment or the leaf age (Fig. 4A and B). Acclimation to [UV–PAR +] and [UV + PAR +] treatments led to a slight decrease in total chlorophylls (Chls) content in mature (2nd) leaves of both genotypes as compared to [UV–PAR–] treatment (Fig. 5A and B). These reductions were significant only in genotype Barke and [UV + PAR +] treatment. The reductions of Chls content caused by UV and PAR treatments in Barke genotype were more pronounced in young (3rd) leaves. The Chls content was lower in young (3rd) than in mature (2nd) leaves in both genotypes studied. These differences between leaf age classes were statistically significant in genotype Bonus under all UV and PAR treatments (Fig. 5 A, B). The Chl
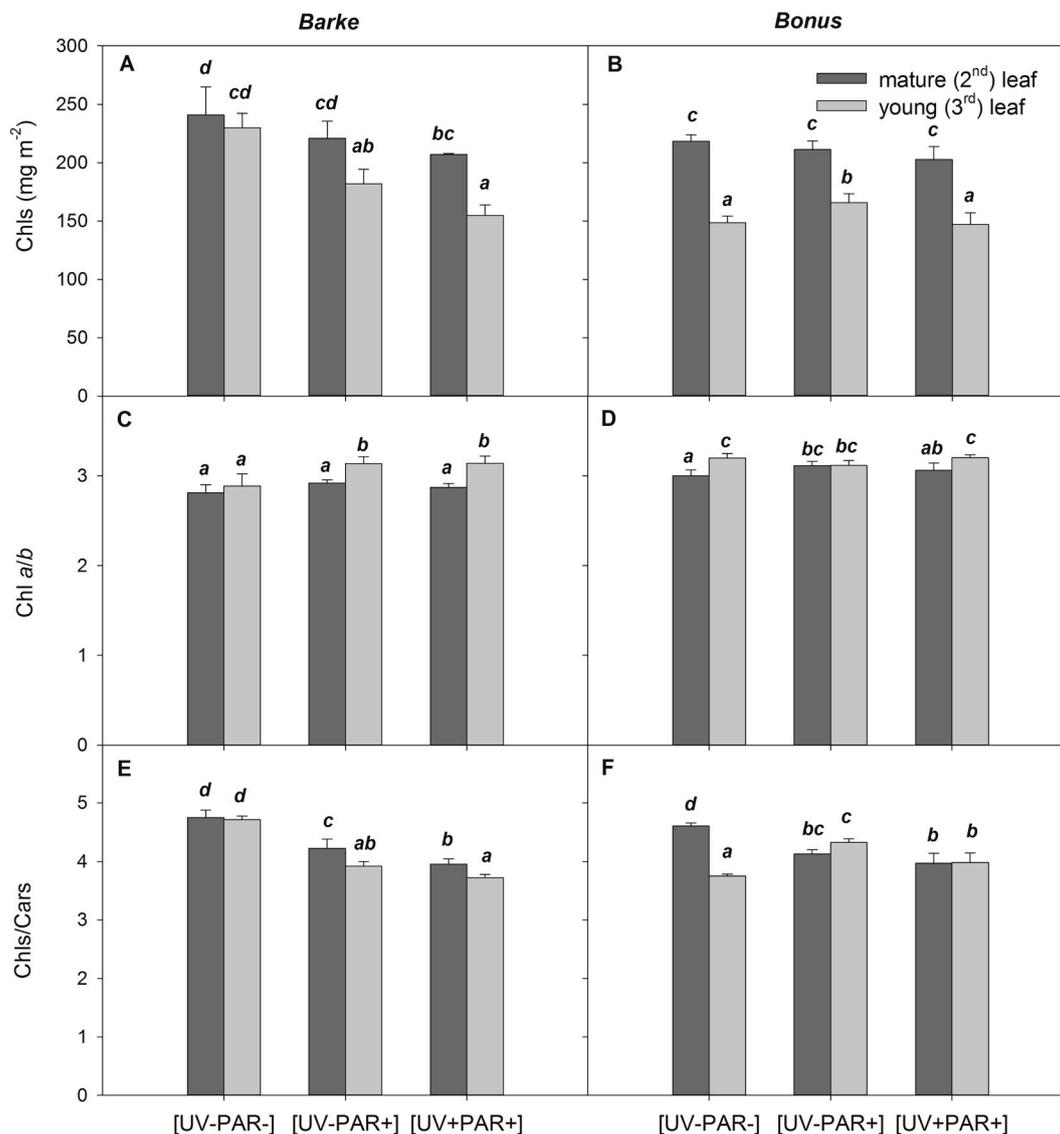
Fig. 5. Total chlorophylls content (Chls; A, B), chlorophyll *a* to chlorophyll *b* ratio (Chl *a/b*; C, D) and ratio between total chlorophylls and total carotenoids (Chls/Cars; E, F) estimated in dark-adapted young (3rd; light grey) and mature (2nd; dark grey) leaves after 7-day acclimation to individual UV and PAR treatments. Data are presented separately for the UV-tolerant (Bonus) and UV-sensitive (Barke) barley genotypes. Means (columns) and standard deviations (error bars) are presented ($n \geq 5$). Different letters denote statistically significant differences ($p \leq 0.05$) between acclimation treatments and individual genotypes using Tukey's ANOVA posthoc test.

a/b ratio was significantly higher in young (3rd) as compared to mature (2nd) leaves under [UV–PAR +] and [UV + PAR +] treatment in genotype Barke, while in genotype Bonus these differences were statistically significant only under [UV–PAR–] and [UV + PAR +] treatments (Fig. 5 C, D). The Chls/Cars ratio was reduced in response to enhanced UV and ambient PAR irradiances in both leaves and both genotypes, except in the case of the young (3rd) leaf of genotype Bonus (Fig. 5 E, F). Chls/Cars ratio was significantly higher in mature (2nd) than in young (3rd) leaves under [UV–PAR +] and [UV + PAR +] treatments in Barke, and under the [UV–PAR–] treatment in Bonus.

3.4. Associations between PhCs, pigments, UV and PAR treatment and leaf age

For both genotypes, RDA revealed that accumulation of AD is strongly, and positively related to leaf age (Fig. 6). A strong antagonistic relationship with leaf age was observed for HCA_2 and FQA in both barley genotypes. In contrast to genotype Barke, the genotype

Bonus also showed positive associations between leaf age, saponarin, and total contents of chlorophylls and carotenoids (Fig. 6).

In contrast to the total content of chlorophylls and carotenoids, Chl *a/b* showed a negative association with leaf age (Fig. 6). In addition, RDA revealed in both genotypes positive associations of lutonarin with VAZ, DEPS, and Chl *a/b*. The relationships between lutonarin and VAZ and between lutonarin and DEPS are shown in Fig. 7. While for VAZ the rectangular hyperbola relationship to lutonarin was found in both genotypes irrespective of leaf age, for DEPS the linear relationships were separated by leaf age. In general, these relationships are shifted to higher DEPS values in mature leaves. Relationships between Chls and lutonarin and between Chls and flavonol index (Fig. 8) show a significant inverse linear course. However, the data were grouped by leaf age, with lower values of flavonol index and lutonarin content and higher Chls values in mature leaves.
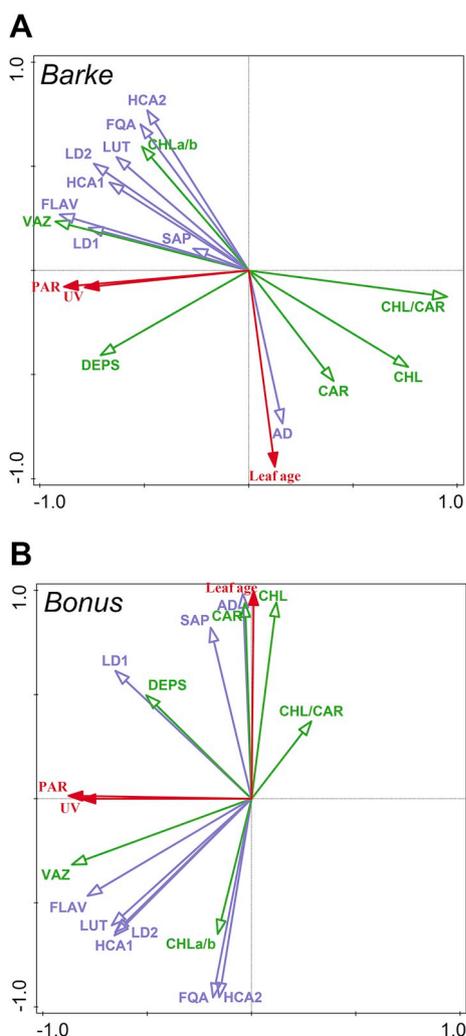
Fig. 6. Biplot of the redundancy analysis (RDA) results for effects of PAR, UV and leaf age on individual phenolic compounds and leaf pigments separately for genotypes Barke and Bonus. CHL – total chlorophylls content (Chl $a+b$; Chls), CHLa/b – chlorophyll a to chlorophyll b ratio (Chl a/b), CAR – total carotenoids (Cars), CHL/CAR – ratio between total chlorophylls and total carotenoids (Chls/Cars), DEPS – nominal de-epoxidation state of the xanthophyll-cycle pigments, VAZ – total content of xanthophyll-cycle pigments, HCA1 and HCA2 – hydroxycinnamic acid derivatives, FQA – feruloylquinic acid, LUT – lutonarin, SAP – saponarin, LD1 and LD2 – luteolin derivatives, AD – apigenin derivative, FLAV – flavonol index.

4. Discussion

4.1. Constitutive and inducible accumulation of PhCs

Barley genotypes with distinct sensitivity to photo-oxidative stress (Wu and von Tiedemann, 2004; Klem et al., 2012), i.e., sensitive Barke and tolerant Bonus, were investigated to test the hypothesis that constitutive PhC content is higher in the tolerant genotype, and that accumulation of both constitutive and inducible PhCs is further modulated by leaf ontogeny. Indeed, we found lower flavonol index (Fig. 1) and PhC contents (Figs. 3 and 4) in Barke compared to Bonus under [UV–PAR–] treatment. In addition, a higher constitutive flavonol index and higher constitutive levels of PhCs were found in young compared to mature leaves (Figs. 1–3). Sun et al. (2010) have also reported higher constitutive as well as UV-induced contents of flavonoids in young compared to mature leaves of *Ginkgo biloba*. Similarly, Andreotti et al. (2006) found a decreasing content of total PhCs, particularly flavonol glycosides and cinnamic acids, with increasing leaf age of pear leaves.

Reduced contents of flavonol glycosides and derivatives of ferulic acid are likely associated with an altered total PAL activity since the enzyme concentration decreases during ongoing leaf ontogeny (Fünfgelder et al., 1993). Although higher total PhCs content was found in young blackcurrant leaves, specific PhCs may increase with leaf ontogeny (Vagiri et al., 2015). In our study, AD, and also in Bonus saponarin and LD_1, accumulated to higher levels in mature, compared to young leaves.

Lower constitutive contents of saponarin and AD in young leaves of tolerant genotype Bonus were found as compared to sensitive genotype Barke. Noticeably, accumulation of these two compounds was induced neither by higher PAR nor by UV radiation. On the contrary, Kaspar et al. (2010) identified saponarin in barley plants as a major PhC induced by UV-B radiation. Since saponarin primarily accumulates in epidermal vacuoles, the presence of a central vacuole is crucial for saponarin biosynthesis from naringenin (Marinova et al., 2007). Accordingly, an increase of saponarin content with leaf age could be associated with increasing vacuole capacity during leaf ontogeny (Fleming, 2006). Contrary to saponarin and AD, all other PhCs showed higher constitutive accumulation in young leaves. In our study, FQA and HCA_2 (Fig. 3) were found to be the most distinct constitutively accumulated PhCs in young leaves. In accordance with our results, Mondolot et al. (2006) found that FQA is localized mainly in chloroplasts of juvenile leaves having a likely photo-protective role. In mature leaves, FQA was particularly found in the vascular system suggesting its translocation via phloem and involvement in lignification processes. These processes may be a reason for reduced content of FQA in mature leaves.

4.2. UV- and PAR-induced accumulations of PhCs

Chromatographic analysis of major PhCs in barley leaves revealed different responses of individual PhCs to radiation conditions and also different interactions with leaf age (Fig. 3). Accumulation of HCA_1, HCA_2, FQA, lutonarin and LD_2 was typically induced by both UV and PAR in young (3rd) leaves, while in mature (2nd) leaves UV-induced accumulation was more pronounced. In contrast, the effect of UV and PAR treatments on saponarin and AD accumulation was negligible. Similarly, Reuber et al. (1996) found a much stronger effect of UV-B radiation on lutonarin than on saponarin concentrations. However, substantial induction of lutonarin accumulation by higher PAR intensities was also reported (Klem et al., 2015; Nezval et al., 2017). In accordance with our findings, the accumulation of PhCs during UV exposure has been reported to be stronger in young as compared to mature leaves of several other crops (Kakani et al., 2004; Reifenrath and Müller, 2007) and tree species (Ibañez et al., 2008; Sun et al., 2010). While high PAR intensities induced accumulation of PhCs particularly in young, developing leaves, UV-B induction of PhCs was effective in both mature (2nd) and young (3rd) leaves. This effect could be partially explained by the prolongation of PAL activity under enhanced UV-B radiation (Liu and McClure, 1995).

Since no significant differences in the flavonol index (Fig. 1) were found between [UV–PAR+] and [UV+PAR+] treatments in young leaves, we assume that higher PAR intensities are sufficient to saturate epidermal PhCs accumulation in young barley leaves. On the other hand, UV radiation was necessary to achieve maxima of the flavonol index in mature leaves, particularly in genotype Barke. It is generally assumed that young leaves require more efficient protection against photo-oxidative stress, since these are more important for plant growth than mature leaves (Reifenrath and Müller, 2007). The association between higher PAR and the flavonol index and PhCs accumulation is probably due to high energy demand for PhCs biosynthesis, particularly the shikimate pathway (Hernández and Van Breusegem, 2010). Approximately 20% of the fixed carbon is estimated to flow through the shikimate pathway and hence the accumulation of PhCs is strongly coupled with photosynthetic carbon fixation (Rippert et al., 2004).

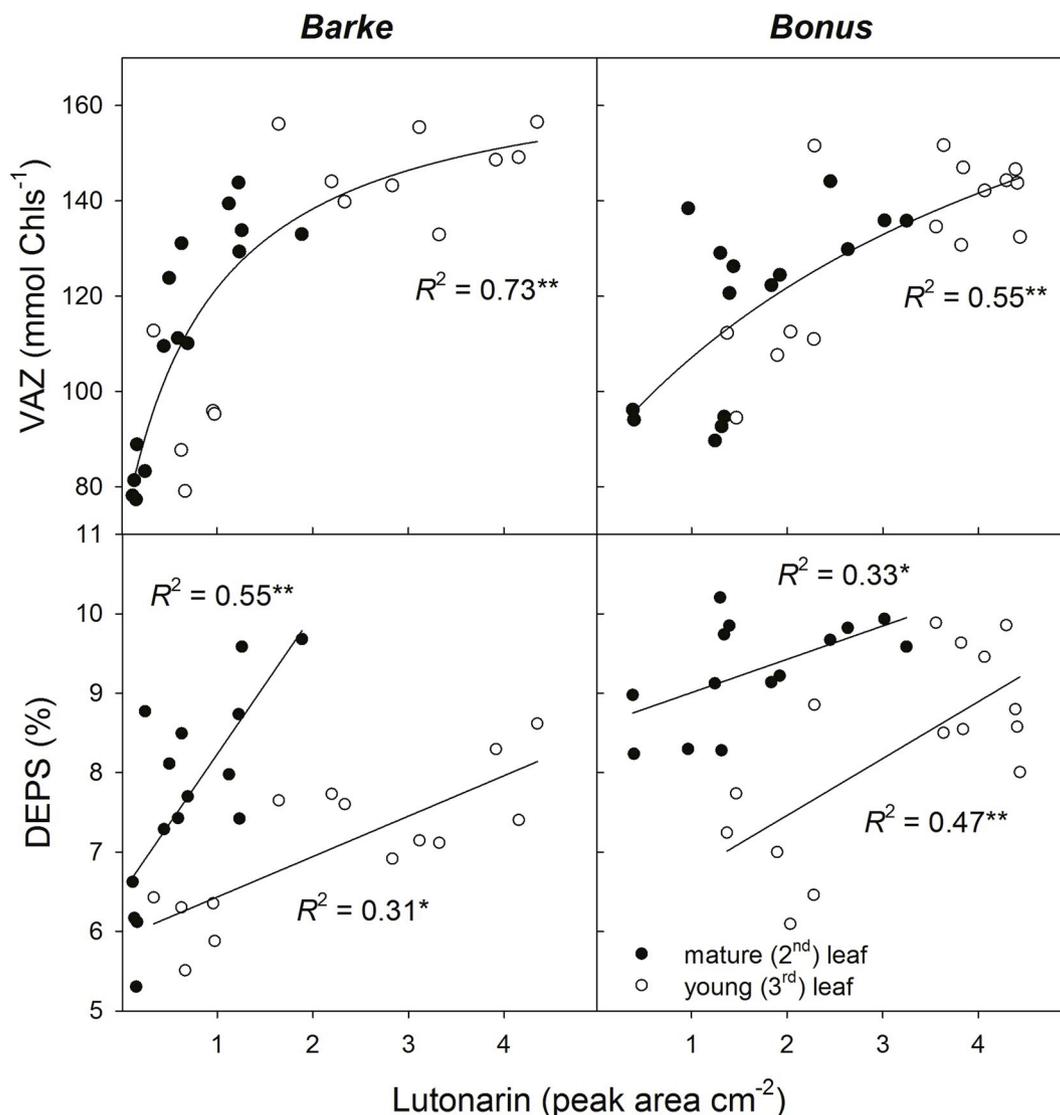Under natural solar conditions, the ratio of UV to PAR strongly

Fig. 7. The relationship between the total content of xanthophyll-cycle pigments (VAZ), the nominal de-epoxidation state of the xanthophyll-cycle pigments (DEPS) and lutonarin content at the end of the UV and PAR treatments. A hyperbolic function was fitted to the data of both mature and young leaves together for VAZ, and a linear function was fitted to the data of mature and young leaves separately for DEPS. Points represent individual replicates. Coefficients of determination (R^2) and significance levels (*$p \leq 0.05$ and **$p \leq 0.01$) are shown.

depends on time-of-day, season, altitude, latitude and is also changed by penetration through the canopy (Brown et al., 1994; Jansen et al., 2017). The importance of PAR and UV interactions in understanding plant responses was recently noted (Götz et al., 2010). It is assumed that the interactive effect of PAR and UV radiation on the accumulation of PhCs is mediated by phytochrome and its regulation of PAL bio-synthetic pathway. Accordingly, Sreelakshmi and Sharma (2008) found that red light stimulates PAL activity in tomato seedlings via phytochrome-mediated induction of the enzyme synthesis.

4.3. Associations between the accumulation of PhCs and xanthophyll cycle pigments

A pronounced increase in the pool of the xanthophyll cycle pigments (VAZ) in plants acclimated to a higher level of PAR [UV–PAR+] in comparison with [UV–PAR–] (Fig. 4), is a crucial response associated with enhanced protection of photosynthetic apparatus against photo-oxidative stress (Demmig-Adams and Adams, 2006; Jahns and Holzwarth, 2012). In our study, the VAZ pool was generally greater in young than in mature barley leaves, even though the role of leaf

ontogeny on VAZ accumulation was less pronounced as compared to PhCs. A bigger VAZ pool indicates higher demands for photoprotection in young leaves. Increases in the VAZ pool in young leaves under high-light stress were also documented by Havaux et al. (2000). Similarly, Manetas et al. (2002) demonstrated higher VAZ accumulation in young leaves of *Rosa* and *Ricinus* if expressed per leaf area unit, but not if expressed on a chlorophyll basis. This difference is based on opposite effects of leaf age on total chlorophylls and VAZ accumulation.

The effective acclimation of both barley genotypes to increased PAR and UV was documented by low DEPS levels in plants that were dark-adapted overnight (Fig. 4). Radiation stress is usually associated with increased DEPS (Kurasová et al., 2002; Demmig-Adams et al., 2006; Štroch et al., 2008). It is widely accepted that the protective role of zeaxanthin is, besides the stimulation of non-radiative dissipation of absorbed light, also based on the antioxidative activity preventing oxidative damage of thylakoid membrane (Baroli et al., 2004; Havaux et al., 2007). Taking into account the mentioned role of zeaxanthin in mitigation of oxidative stress, the enhanced VAZ size can also contribute to protection against UV stress (Láposi et al., 2009). Higher VAZ accumulation and a slight increase in Chl *a/b* indicate that UV radiation
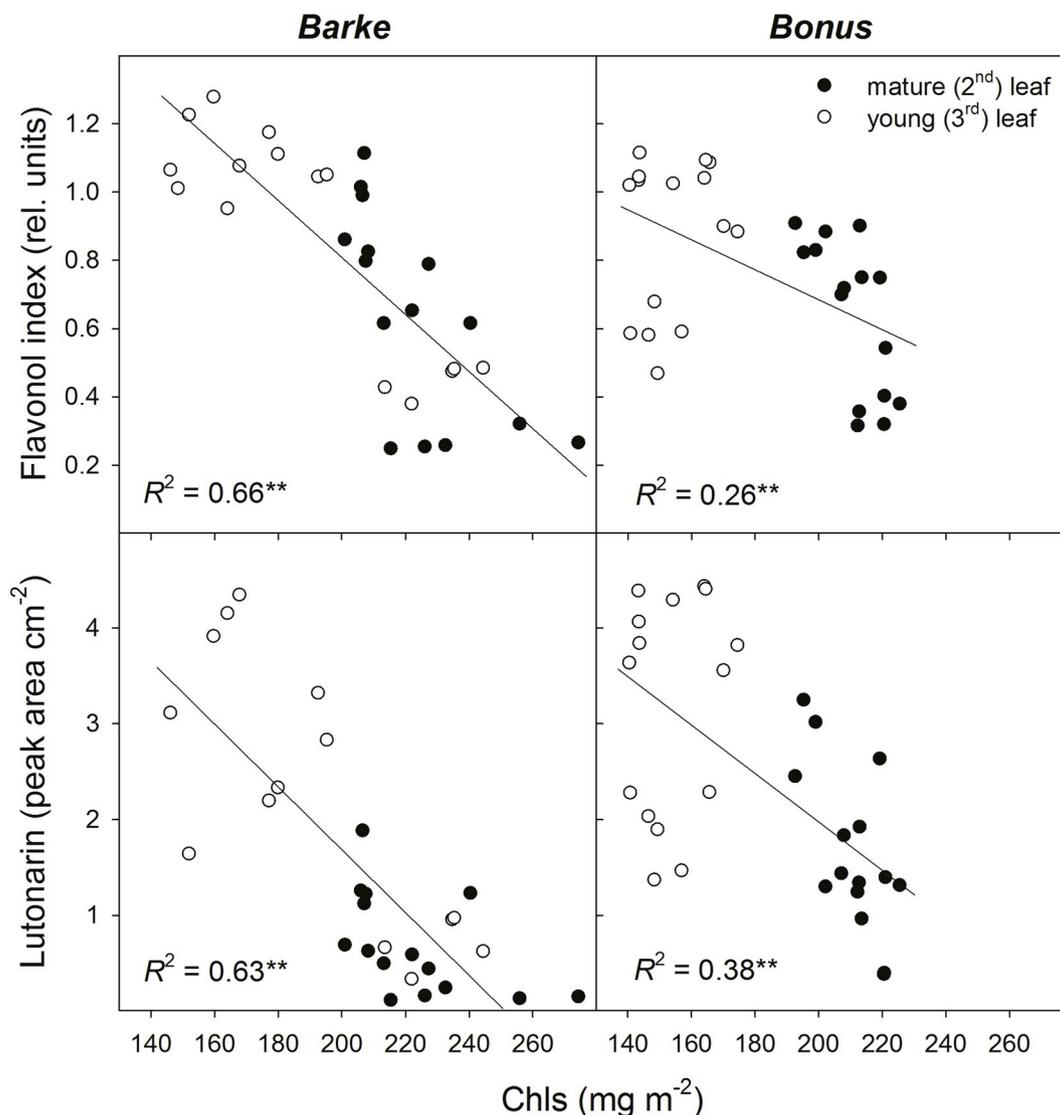
Fig. 8. The relationship between flavonol index measured *in vivo* using the instrument Dualex 4 Flav, lutonarin and the total content of chlorophylls (Chl $a + b$; Chls) at the end of UV and PAR treatments. A linear function was fitted to the data of both mature and young leaves together in two barley genotypes Bonus and Barke. Points represent individual replicates. Coefficients of determination (R^2) and significance levels (**$p \leq 0.01$) are shown.

induced in our study mainly accumulation of the xanthophyll cycle pigments that were not bound to pigment-protein complexes. Although this evidence is indirect, it seems reasonable that UV radiation stimulates induction of several sources of antioxidative protection in addition to PhCs.

In addition, RDA revealed a negative association between VAZ and total chlorophylls in both genotypes (Fig. 6). On the other hand, we found a strong positive association of VAZ and lutonarin accumulation, irrespective of leaf age (Fig. 7). Particularly for the Barke genotype, this relationship reveals a hyperbolic character with an asymptote at high VAZ contents. This means that a slight increase of lutonarin content is accompanied by a considerable increase of VAZ pool size, but later on, VAZ becomes almost saturated whereas the accumulation of lutonarin continues. Although a similar trend in the relation between lutonarin content and VAZ pool size was observed for the Bonus genotype, the hyperbolic shape was less pronounced, revealing a more proportional association between lutonarin content and VAZ pool size. Similar results were found for the relationship between contents of some other PhCs (e.g., HCA_1, LD_2) and the VAZ pool. The difference between Barke and Bonus genotypes may result from the fact that both constitutive and UV or PAR induced accumulation of lutonarin is strongly limited in

mature Barke leaves as compared to mature leaves of Bonus genotype, whereas in young leaves similar lutonarin content can be induced by UV and PAR treatment in both genotypes. It is thus argued that insufficient accumulation of lutonarin in mature Barke leaves can be thus compensated by an increased VAZ pool (Fig. 4). In support, we have recently found substantially reduced accumulation of PhCs with antioxidative activity, including lutonarin, in a barley mutant deficient in chlorophyll b (Clo-f2) grown under high PAR. However, the VAZ pool was considerably enhanced in this mutant (Nezval et al., 2017). This finding also supports the hypothesis that VAZ accumulation can compensate for the insufficient efficiency of antioxidative protection mediated by PhCs.

Our results also revealed significant relationships between lutonarin content and DEPS, but this was strictly separated by leaf age. Higher DEPS levels relatively to lutonarin content in mature leaves indicate that in mature leaves there was a higher demand for the protective role of xanthophyll cycle pigments due to lower PhCs accumulation. The steeper slope of the relationship between lutonarin and DEPS in mature leaves of genotype Barke may indicate that higher sensitivity of this genotype to oxidative stress mainly concerns the mature leaves.

From our results, the inverse relationship between total chlorophylls

and lutonarin or flavonol index (Fig. 8) is evident. Inverse relationships between total chlorophylls and epidermal UV-screening as a response to irradiance and nitrogen availability was also demonstrated by Meyer et al. (2006) in different species. Decreasing chlorophyll content with increasing PAR is a well-known process. Due to acclimation to high irradiance, leaves are expected to allocate less nitrogen to chlorophylls and more to photosynthetic electron transport components and to soluble proteins like Rubisco (e.g., Niinemets and Tenhunen, 1997). At the same time PAR and UV radiation induce expression of PAL and chalcone synthase (Krizek, 2004). Such acclimation to higher PAR and UV radiation resulting in higher flavonol index and a decrease in chlorophyll content, limit the absorption of PAR and penetration of UV radiation through the leaf and therefore protect mesophyll from photodamaging processes.

4.4. The stability of the flavonol index under low PAR and UV intensities

We hypothesized that epidermal flavonols under low radiation conditions remain relatively stable until they are degraded by oxidative stress (Takahama, 1988). We also assumed that the stability of epidermal flavonols varies depending on the induction by UV and PAR. Our results (Fig. 7) indicated, however, high stability of flavonol index irrespective of UV or PAR treatment, barley genotype, and leaf age. Although young leaves had higher flavonol index than mature ones, the differences between these age groups slightly decreased with the time of relaxation.

Only limited attention has been paid to the stability of flavonoids in the plant leaves. In barley, the stability of epidermal flavonols following the transition to low-light condition was documented by Nezval et al. (2017). More results about the stability of flavonoids are available for harvested vegetables. Bergquist et al. (2005) showed that total flavonoids content in spinach was relatively stable during normal retail storage conditions, although some individual flavonoid compounds showed considerable variation in terms of stability. The rather stable total content of flavonoids in spinach has also been reported by Gil et al. (1999). Conversely, a decrease in flavonoid glycoside content during storage was found by DuPont et al. (2000) in lettuce and endive genotypes. Based on our results, we assume that the oxidation rate of flavonoids under non-stressful conditions is low. However, the oxidative degradation of flavonoids can be increased under the influence of various stress conditions (Blokhina et al., 2003) since flavonoids act as scavengers of free radicals such as reactive oxygen species. Flavonoid oxidation in plants is mainly catalysed by polyphenol oxidases (catechol oxidases and laccases), and peroxidases and these activities are induced by environmental stressors (Pourcel et al., 2007).

We conclude, that leaf age strongly modulates accumulation of PhCs. Levels of hydroxycinnamic acids, lutonarin, and one luteolin derivative were higher in young leaves. On the contrary, an apigenin derivative was more accumulated in mature as compared to young leaves. It was also shown that constitutive accumulation of PhCs is significantly lower in variety sensitive to oxidative stress (Barke). We also documented the stability of flavonol index after exposure of plants to excluded UV and low PAR intensities. Accumulation of some PhCs (particularly lutonarin) was positively correlated to DEPS and VAZ and negatively to chlorophyll content, however, the non-linear relationships between lutonarin and VAZ indicated some compensation between these two protective mechanisms.

Acknowledgment

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