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Contribution of phenolic compounds to the UV-B screening capacity of developing barley primary leaves in relation to DNA damage and repair under elevated UV-B levels

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Epidermally located UV-absorbing hydroxycinnamic acid conjugates and flavonoid glycosides are known to be efficient UV-B protectants in higher plants, although important biological molecules are not always fully protected. However, repair mechanisms also exist, such as repair of damaged DNA by photolyases. To distinguish between the relative importance of the phenolic compounds and of DNA repair, developing primary leaves of two barley lines, mutant ant 30-310, deficient in flavonoids, and its parent line Ca 33787, were grown under relatively high visible light (650–700 μmol m^{-2} s^{-1} max for 6 h in a 13 h photoperiod) and supplemented with (+ UV-B) or without (−UV-B) 12 kJ m^{-2} UV-B_{BE} for 6 h daily. UV-B screening capacity of the leaf phenolics was determined at 315 nm during leaf development and compared with thymine dimers (TD) accumulation, as an indicator of UV-B-induced DNA damage and potential subsequent repair. The degree of damage was related to the phenolic contents of the leaves. UV-B screening capacity was increased ca. 4-fold in the parent line (+ UV-B), mainly due to UV-induced flavonoid (saponarin, lutonarin) accumulation in epidermal and subepidermal mesophyll tissue, relative to the flavonoid-deficient mutant. Nevertheless, in the parent line an 8-fold increase in TD levels occurred over the growth period of 18 days, whereas the mutant accumulated additional DNA damage, with 6- to 9-fold higher TD amounts. Surprisingly, under the high UV-B irradiation, growth and development of the primary leaves in both lines were only slightly reduced.

Keywords: *Hordeum vulgare* L.; Poaceae; Flavonoid mutant; UV-B acclimatization; Hydroxycinnamic acid conjugates; Flavone glycosides; Saponarin; Lutonarin; DNA damage; Thymine dimers; DNA repair by photolyase

1. Introduction

Ozone reductions in the stratosphere are not confined to the Antarctic, but extend to mid-latitudes in both hemispheres (Tevini, 1994; Pyle, 1997; Madronich et al., 1998). This leads to selective increase in UV-B radiation (280–315 nm) at the earth's surface which damages DNA (Taylor et al., 1997), and may affect genome stability of plants (Ries et al., 2000), proteins and other essential molecules, for example, auxin (Rozema et al., 1997; Jansen et al., 1998). In the spring months, comparatively high UV-B:PAR ratios (photosynthetically active radiation, PAR, 400–700 nm) were measured in Erlangen, Germany, as a consequence of mini ozone holes over northern and mid Europe (Lebert et al., 2002). Such increasing UV-B irradiation could potentially damage growing seedlings and developing leaves.

Plants have evolved two major strategies for UV-B radiation tolerance, namely, repair and avoidance mechanisms. Repair mechanisms include, for example, DNA damage repair by excision and photoreactivation-mediated repair of photoproducts, e.g. repair by photolyase of cyclobutane pyrimidine dimers (CPD) (Taylor et al., 1997; Britt, 1999; Dany et al., 2001). Avoidance mechanisms include epidermal screening of UV-B radiation, by accumulation of phenolic compounds, which protects the mesophyll tissue (Bornman et al., 1997; Schnitzler et al., 1996; Burchard et al., 2000; Bilger et al., 2001; Kolb et al., 2001). Also important is the formation of antioxidants, such as glutathione, ascorbate

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and flavonoids, especially *ortho*-dihydroxylated compounds, which may scavenge peroxides and oxygen radicals (Jansen et al., 1998; Olsson et al., 1998; Ryan et al., 1998; 2001).

Mutants of *Arabidopsis thaliana* (Li et al., 1993; Landry et al., 1995; Sheahan, 1996; Booij-James et al., 2000), *Zea mays* (Stapleton and Walbot, 1994), *Oryza sativa* (Hidema et al., 1997) and *Hordeum vulgare* (Reuber et al., 1996, 1997) that are defective in hydroxycinnamate (HCA) and/or flavonoid biosynthesis, have become increasingly useful in elucidating potential functions of these compounds in plants grown under elevated UV-B. On the other hand, photolyase-deficient plants helped elucidate the importance of CPD photoreactivation in UV-B stressed plants (Landry et al., 1997; Hidema et al., 1997; Ries et al., 2000), which was shown to occur in *Arabidopsis thaliana* (Dany et al., 2001), *Pisum sativum* (Kalbin et al., 2001), *Sorghum bicolor* (Hada et al., 1996), *Triticum aestivum* (Taylor et al., 1996), *Oryza sativa* (Hidema and Kumagai, 1998) and *Zea mays* (Stapleton et al., 1997).

Little is known about the relative contribution of specific phenolic compounds to the UV-B screening capacity of developing leaves in relation to DNA damage and repair under elevated UV-B levels. Stapleton and Walbot (1994) compared two maize lines, one defective and the other high in its flavonoid (primarily anthocyanin) content, and showed major DNA protection against UV-B for the latter line, in short time exposures of the leaves. In a previous paper, we compared barley mutant ant 30-287, deficient in flavonoids, with its parent line Hege 550/75, accumulating saponarin **1** and lutonarin **2** (Reuber et al., 1996). An increased sensitivity of the primary leaf of the mutant to UV-B irradiation was observed, when various photosynthetic parameters were measured. This was accompanied by an increased UV-B penetration into the mutant leaf due to its flavonoid deficiency and low HCA content, and a significant growth reduction was measured as compared to the parent line Hege.

In the present study, two other barley lines, closely related to ant 30-287 and Hege 550/75 (Reuber et al., 1997), were investigated—the mutant ant 30-310 and its parent line Ca 33787. Primary leaves, either deficient in flavonoids or with different flavonoid and HCA contents, which represent various UV-B screening capacities, respectively, were grown in the presence or absence of elevated UV-B irradiation. Potential UV-B screening capacity was compared with the degree of DNA damage and DNA repair, as estimated by the levels of TD, the major UV-B-induced products of DNA damage in plants (Mitchell et al., 1992; Dany et al., 2001).

2. Results

2.1. Primary leaf development and accumulation of phenolic compounds under high UV-B irradiation and UV-B exclusion

As a first step, to gauge the general UV-B susceptibility and overall response to UV stress, growth parameters of the primary leaves grown under −UV-B and +UV-B irradiation (Fig. 1) were measured. These included total leaf length and fresh weight of the irradiated leaf segments above the coleoptile (Fig. 2). Under −UV-B, neither the parent line nor the mutant showed any difference in leaf length. Under +UV-B, length reduction of 12% ($P \leqslant 0.5\%$) in the parent line became apparent only after 11 days, whereas mutant leaves were reduced by 10% as compared to −UV-B ($P \leqslant 0.5\%$) after only 6 days. When fully expanded, 18-day-old primary leaves of the mutant were reduced by ca. 25% ($P \leqslant 0.5\%$) and leaves of the parent line by 10% ($P \leqslant 5\%$), as compared to those under −UV-B conditions. Fresh weight of the leaf part appearing above the coleoptile of 18-day-old leaves was ca. 15% lower under +UV-B for the mutant ($P \leqslant 0.5\%$) and 8% lower ($P \leqslant 5\%$) for the parent line.

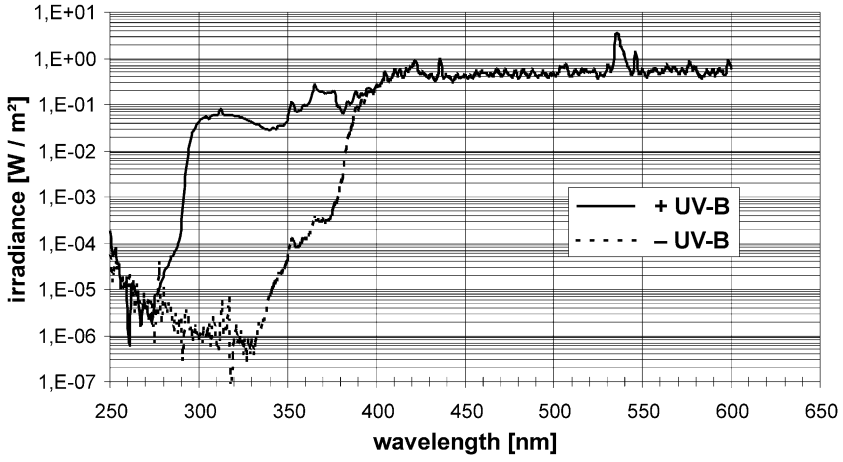

Fig. 1. Spectral distribution of irradiances for +UV-B and −UV-B radiation, at plant level. For details see Experimental.

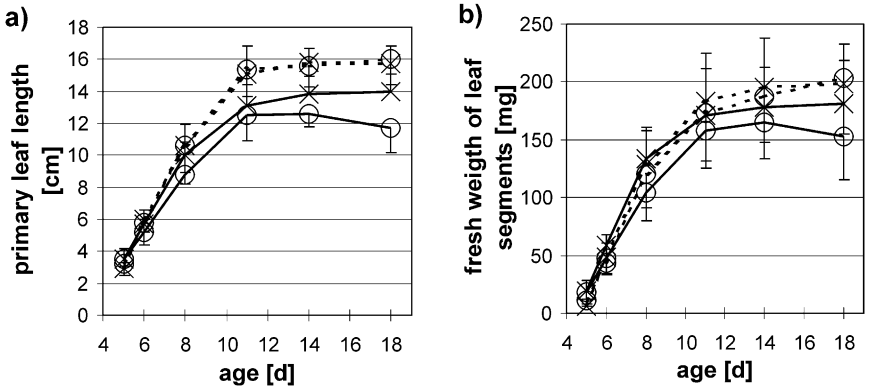

Fig. 2. Growth parameters of developing barley primary leaves, of the parent line Ca 33787 (X, *error bars upwards*) and mutant ant 30-310 (O, *error bars downwards*) both cultivated under the light regimes −UV-B (*dashed lines*) and +UV-B (*solid lines*): (a) total leaf length; (b) fresh weight of leaf segments above the coleoptile. Values are means±S.D. (n = 20–40) for each day and growth condition.

As shown in Fig. 3, saponarin **1** accumulated as the major phenolic compound during leaf development of the parent barley line. Under −UV-B approximately 2 nmol per mg fresh weight were measured with only little increase under +UV-B to about 3 nmol. Lutonarin **2**, although a minor compound, increased five-fold under +UV-B as compared to −UV-B and reached about 0.4 nmol during 18 days. The other flavones are not shown due to levels at or below the detection limit of the HPLC. Total flavone contents of the mutant were less than 1% of the parent line throughout the experimental period (mainly saponarin **1**; lutonarin **2** was below the detection limit; data not shown). Contents of soluble HCA derivatives showed lower levels, as compared to

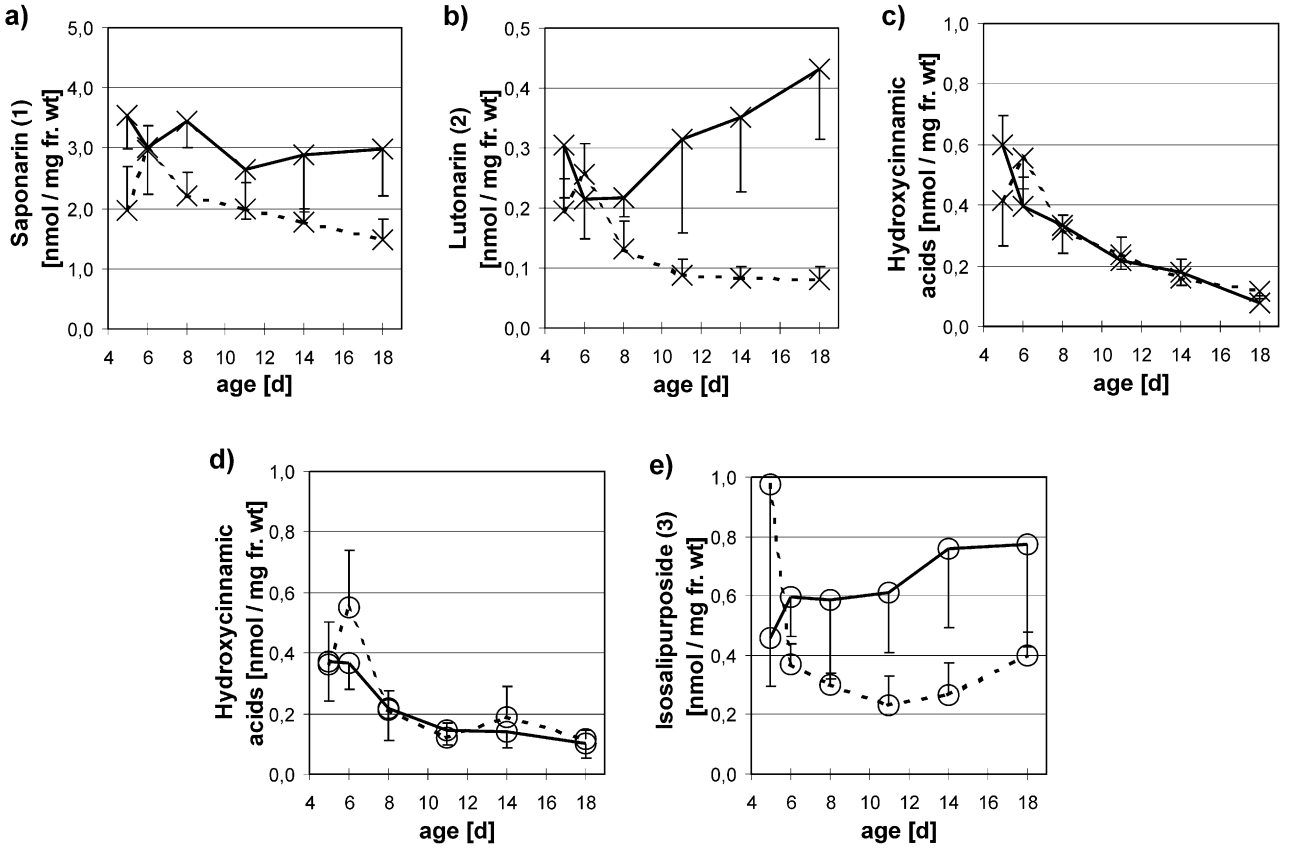

Fig. 3. Accumulation of phenolic compounds of developing primary leaves of barley lines Ca 33787 (X), and ant 30-310 (O), grown under −UV-B (*dashed lines*) and +UV-B (*solid lines*): (a) saponarin **1**, (b) lutonarin **2**, (c) and (d) soluble HCA derivatives, (e) isosalipurposide **3**, nmol per mg fresh weight of leaf segments. In the mutant leaves total flavone contents were less than 1% of the parent line (not shown). Values are means±S.D. (n = 8–15) for each day and growth condition.

the flavones, approximately 0.6 nmol per mg fresh weight in young stages. This amount decreased to 0.1 nmol within 18 days and was independent of the irradiation conditions (±UV-B). For both barley lines similar HCA levels were measured (Fig. 3c and d). Values for insoluble HCA were in the same low range (Table 1). However, there was an increasing tendency with age, especially under +UV-B, five-fold for Ca and 2.5-fold for ant, reaching 0.6 and 0.5 nmol per mg fresh weight, respectively. The amount of the mutant-specific isosalipurposide **3** (2′,4,4′,6′-tetrahydroxychalcone-2′-O-β-glucoside, see Reuber et al., 1997) was at 0.2–0.4 nmol per mg fresh weight (−UV-B). It increased about two-fold under +UV-B, reaching 0.8 nmol (Fig. 3e).

2.2. Potential UV-B screening capacity of phenolic compounds

In Fig. 4 the potential UV-B screening capacity of the various soluble phenolic compounds is shown as

Table 1
Amount (nmol) and potential UV-B screening capacity (*A* at 315 nm) of cell wall-bound HCA, mainly ferulic and minor *p*-coumaric acid, of both barley cultivars, Ca 33787 and ant 30-310, grown under +UV-B and −UV-B, respectively (c.f. Fig. 4)

Age [days]	Amounts of cell wall-bound HCA			
	Ca		ant	
	−UV-B	+UV-B	−UV-B	+UV-B
HCA [nmol/mg fresh weight]				
6	0.45±0.10	0.13±0.09	0.24±0.13	0.20±0.09
11	0.38±0.25	0.35±0.12	0.38±0.16	0.33±0.07
18	0.64±0.36	0.65±0.17	0.81±0.37	0.52±0.17
Potential UV-B screening capacity				
6	0.21±0.05	0.06±0,04	0.11±0.06	0.09±0.04
11	0.18±0.12	0.17±0.05	0.18±0.07	0.15±0.03
18	0.30±0.17	0.31±0.08	0.38±0.17	0.25±0.08

Values are means±S.D. (n=8–12) per mg fresh weight of leaf segments for each day and growth condition.

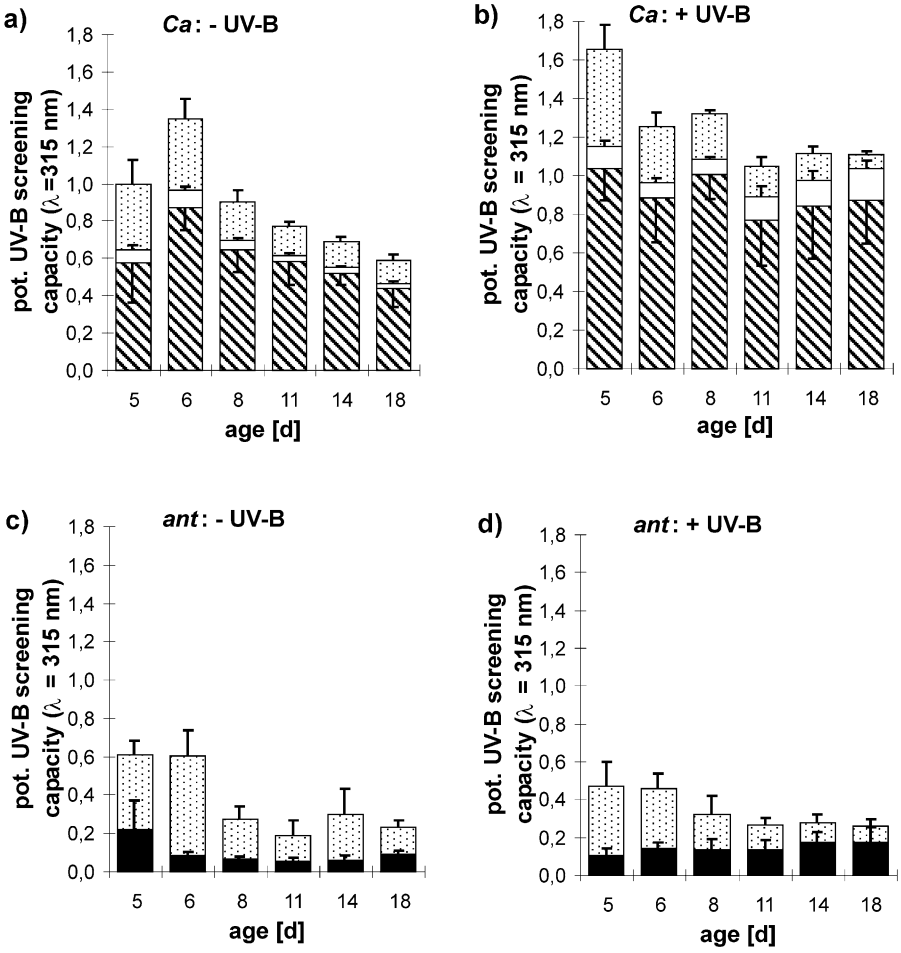

Fig. 4. Potential UV-B screening capacities of soluble phenolic compounds at 315 nm per mg fresh weight of leaf segments of both barley cultivars, Ca 33787 and ant 30-310: (a), (c) grown under −UV-B and under +UV-B (b), (d). The absorptivity was calculated from total phenolic peak integrations after HPLC at 315 nm, of saponarin (*hatched*), lutonarin (*white*), HCA derivatives (*dotted*) and isosalipurposide (*black*, for the mutant only). Values normalized to 1, for 5-day-old barley Ca 33787 (−UV-B). Values are means±S.D. (n=8–12) for each day and growth condition.

expressed by their absorptivity at 315 nm per mg fresh weight of leaf segments (for insoluble HCA see Table 1). The absorptivity was calculated from total peak integrations of the HPLC of 5- to 18-day-old primary leaves of the parent barley line Ca and the mutant ant grown under −UV-B and +UV-B irradiation, respectively. Values were set at 1 for 5-day-old leaf sections (−UV-B) from Ca 33787. After reaching a maximum UV-B screening capacity at 6 days, leaf segments of the parent barley irradiated with −UV-B showed a continuous decrease in this capacity with increasing age. However, this decrease was mainly due to an increase in fresh weight (see Fig. 2). Additional UV-B irradiation led to 40–80% higher screening capacity which was mainly due to the increased flavone biosynthesis, apparently as an acclimation response to UV-B irradiation. Again, the relative values decreased with increasing age. In all developmental stages for the +UV-B grown leaves, saponarin **1** was most effective in screening out the UV-B radiation, i.e. saponarin **1** accounted for 60–80% of the total screening capacity of all soluble phenolic compounds. However, in the youngest leaves, the HCA showed a rather high contribution of 30% to the overall screening capacity, which decreased strongly during further development. In comparison, UV-B absorptivity of the cell wall-bound HCA were in the same range as that of soluble HCA (0.4–0.05 units, Fig. 4b), but increased with age five-fold, from 0.06–0.31 (Table 1). Lutonarin **2**, as a minor flavone, contributed only with 7–15% of the total UV-B screening capacity. However, it was strongly induced (about five-fold) under +UV-B.

UV-B screening capacity of the mutant primary leaf was strongly reduced from 25 to 35% of the parent line, primarily due to its flavone deficiency (Fig. 4). Under +UV-B conditions, the mutant leaf accumulated HCA to similar concentrations as the parent leaf. Furthermore, when cell wall-bound HCA were analysed, their amounts were in the same range as the soluble HCA derivatives (Table 1). The UV-B screening capacity of isosalipurposide **3** was only 20–25% as compared to the soluble HCA of 5- to 6-day-old leaves of +UV-B irradiated plants, and increased only slightly until day 18. Similar ratios were found for the leaves under −UV-B conditions. The contribution of isosalipurposide **3** to UV-B absorption was low because of its spectral properties (absorption at 315 nm is much lower as compared to that at $\lambda_{max} = 368$ nm; see Reuber et al., 1997). In summary, flavonoid deficiency of the mutant could not be compensated for by other phenolic compounds.

2.3. Tissue and cellular localization of phenolic compounds under elevated UV-B levels

The data presented earlier represent rather rough or 'over all' measures of phenolic concentrations of whole leaf segments which consist of different tissue types. This raises the question of a possible tissue or even cell specific localization of phenolic compounds in epidermal or mesophyll tissues. A minor phenolic compound, as estimated per whole leaf, may occur in a particular tissue or even in single cells at high concentration.

In earlier work, which used epidermal peels of barley primary leaves (cv. Atlas; Liu et al., 1995) or leaf segments analysed for UV-B penetration by fibre-optic microprobes (cv. Hege; Reuber et al., 1996), the flavonoid compounds were suggested to be mainly localized in the epidermal layers and to some extent in the mesophyll. In the present study we analysed the barley lines Ca 33787 and ant 30-310 by direct microscopic observation of phenolic compounds of primary leaf sections using fluorescence microscopy. For specific detection of flavones the diagnostic reagent Naturstoffreagenz A (NA), diphenyl-boric acid 2-aminoethyl ester, was used (Hutzler et al., 1998). In Fig. 5 transverse, longitudinal and paradermal sections of 6-day-old primary leaves of the parent barley Ca, grown under +UV-B, are shown, prior to (A, C, E) and after incubation for 5 min in 0.1% NA (B, D, F). Without NA, besides the red-imaged chlorophyll fluorescence in the mesophyll, blue autofluorescence can be seen in the outer and anticlinal cell walls of epidermal cells, in cell walls of stomata and subsidiary cells. After incubation with 0.5% ammonia the blue fluorescence was strongly intensified and shifted to blue-green (not shown), indicative of ferulic acid derivatives (Hutzler et al., 1998, and literature cited therein). When freshly prepared leaf sections were incubated in NA, intense yellow fluorescence typical for flavones was obtained. This fluorescence was seen in both epidermal layers with higher intensity in the abaxial epidermis, in stomatal guard cells and subsidiary cells, and in the outermost cell layer of the mesophyll. The subcellular site of flavone accumulation appears to be primarily the vacuole (as was shown for rye leaves using confocal laser scanning microscopy; Hutzler et al., 1998). Cells in the right part of the epidermis in Fig. 5F were destroyed during sectioning and showed no yellow fluorescence, indicating that yellow flavone signals of intact cells came from protoplasts and not from cell walls. After NA staining and during viewing of the leaf sections the red fluorescence disappeared rapidly, whereas yellow fluorescence remained. Yellow fluorescence obtained in the various cell types seemed to be primarily caused by the presence of saponarin **1**, since authentic saponarin **1**, when analysed under UV radiation on a TLC plate, appeared non-fluorescent before NA treatment and became an intense fluorescent yellow after treatment, showing the same quality of colour. Lutonarin **2**, as a minor compound of the leaf, appearing distinctly different on TLC, fluoresced yellow-orange under UV. Lutonarin **2**-related fluorescence could not be detected in the cells, because of either a low

concentration or masking by the yellow fluorescing saponarin **1** complex. When ant 30-310 mutant leaves were sectioned and then analysed, the red chlorophyll fluorescence and blue auto-fluorescence were distributed identically as in parent barley Ca. Using 0.5% ammonia, the same observation was made as described earlier. After the addition of NA reagent no or only a very faint yellowish fluorescence signal was observed (Fig. 5G and H), which is in accordance with the analytical results, i.e. flavone deficiency.

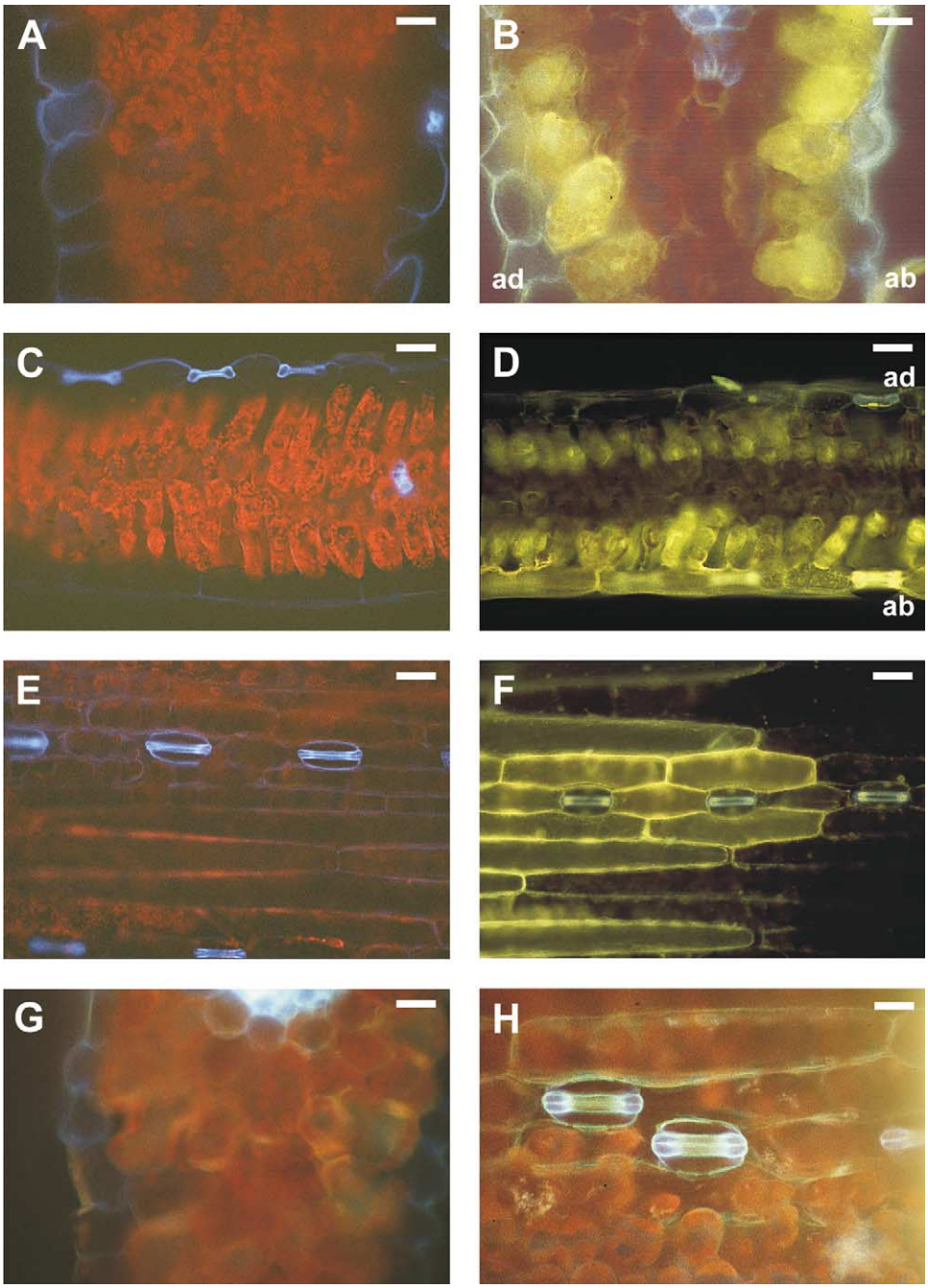

Fig. 5. Epifluorescence images (excitation 340–380 nm) of transverse sections (A, B, G), longitudinal sections (C, D) and paradermal sections (E, F, H) of 6-day-old primary leaves of barley Ca 33787 and mutant ant 30-310, respectively, grown under +UV-B, prior (A, C, E; autofluorescence) and after (B, D, F, G, H) incubation in Naturstoffreagenz A (NA) (0.1% (w/v) in sample buffer (100 mM $NaKP_i$ pH 5.8 with 1 mM $CaCl_2$). Note the yellow secondary fluorescence of flavones after NA staining in parent barley (B, D, F), mainly in epidermal cell layers including stomatal guard and subsidiary cells, and in subepidermal layers of the mesophyll. In the mutant a very faint (if any) yellowish fluorescence signal is observed after NA treatment (G, H). Blue fluorescence is due to HCA conjugates. Bar = 25 μm (in A, B, E–H) and = 50 μm (in C–D); ad = adaxial, ab = abaxial side.

2.4. Accumulation of thymine dimers and partial dimer repair

In primary leaves of parent barley Ca, the UV-B screening capacity was higher under supplementary UV-B irradiation (+UV-B), as compared to −UV-B (Fig. 4). Nevertheless, despite this acclimation response, TD increased and accumulated during leaf development. When measured at the beginning of the daily supplemental UV-B irradiation at 10:00 h, an eight-fold increase in TD was observed, from 5-day-old (value normalized to 1.0) to 18-day-old leaves (Fig. 6a). After 6 h of UV-B irradiation at 16:00 h, TD values were much higher as compared with those at 10:00 h, ranging from five- to two-fold during the experimental period. Thus, although DNA repair took place after 16:00 h (when UV-B was switched off) and during the subsequent night, TD accumulated further and DNA damage was continuously increased and apparently could not be prevented, neither by photorepair nor by repair in the dark (see later). DNA damage was much more pronounced in UV-B irradiated mutant leaves (Fig. 6b). When TD were estimated in young 5- to 6-day-old (or even 8-day-old) ant primary leaves at 10:00 h, when UV-B exposure started, their amounts were in the same order of magnitude as compared with those of the parent line (all values normalised to 1.0, for 5-day-old barley Ca at 10:00 h, see Fig. 6a). Apparently, repair mechanisms of young leaves were sufficient to remove lower TD amounts. Much higher damage occurred during 6 h of UV-B irradiation of 5- to 6-day-old mutant leaves, with TD values of 15–30 units, and only 4–6 units for Ca. In the older ant leaves, up to 18 days, accumulation of TD increased strongly, representing up to nine-fold higher damage at 10:00 h and six-fold higher damage at 16:00 h than in the parent line. In comparison, primary leaves of both mutant and parent barley grown under −UV-B conditions did not show any TD accumulation during their development (not shown).

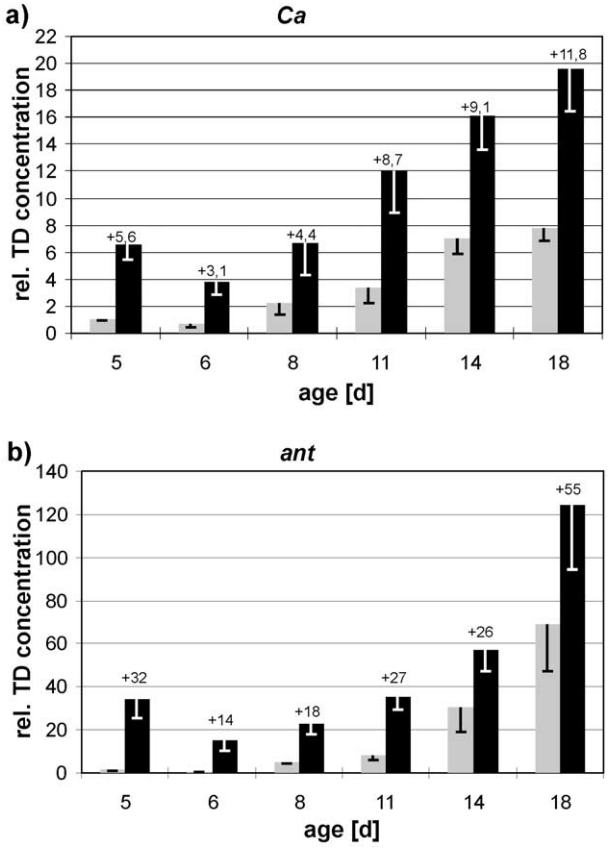

Fig. 6. Accumulation of TD in primary leaf segments of (a) barley Ca 33787 and (b) mutant ant 30-310, grown under +UV-B. Daily estimation of TD was at 10:00 (before UV-B irradiation, *grey columns*) and 16:00 (after UV-B irradiation, *black columns*). Relative TD concentrations, absorption units at 492 nm per 500 ng DNA, were normalized to 1, for 5-day-old barley Ca at 10:00. Values are means ± S.D. (n = 6–12) for each barley line, time value and day. +Numbers above columns represent the increase of TD concentrations during UV-B irradiation.

To characterize DNA damage and repair in more detail, mutant and parent barley seedlings were grown up to 6 days under the two irradiation regimes, −UV-B and +UV-B, respectively. Immediately afterwards both were further exposed to supplementary UV-B (+UV-B) for 6 h and the relative amounts of TD were estimated, normalized to 1.0 for barley Ca grown under −UV-B (see Fig. 7a, t = 0 h).

In comparison, for Ca leaves grown under +UV-B, the relative concentration was 0.6. The two values showed only slight differences ($P \leqslant 5\%$) and indicated similar, rather low DNA damage in both cases. For the mutant much higher values were obtained, 4.5 and 5.7 TD units, respectively, reflecting higher amounts of DNA damage (Fig. 7b, t = 0 h). During a subsequent acclimation period, irradiation for 3.5 h without UV-B, a strong decrease in TD within 1 h was observed in all cases, indicating an efficient photorepair (PR) of DNA. During the next 2.5 h only a slight further decrease occurred. Interestingly, the rates of TD repair were almost the same for both barley lines, although ant showed much higher damage than the parent line Ca. In complete darkness a rather slow decrease, by dark repair (DR), was observed for both lines.

3. Discussion

In the present work major aspects of UV-B tolerance in barley were investigated in two different lines, the parent line Ca 33787, showing increased flavonoid accumulation (flavones saponarin **1**, lutonarin **2**) under high UV-B irradiation in developing primary leaves, and the corresponding mutant line ant 30-310, characterized by its almost complete flavonoid deficiency in the leaves. Due to the accumulation of the chalcone

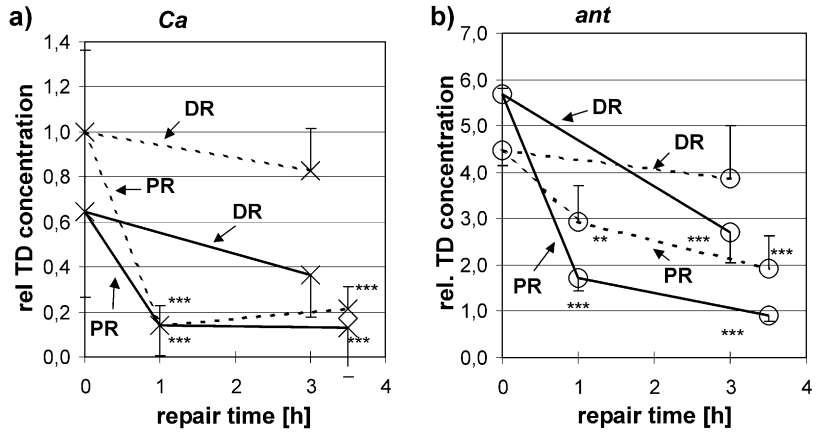

Fig. 7. Time-dependent dark repair (DR) and photo repair (PR) of TD within 3 and 3.5 h in leaf sections of (a) barley Ca 33787 (X) and (b) mutant ant 30-310 (O). Primary leaves of both barley lines were grown for 6 days under −UV-B (*dashed lines*) and under +UV-B (*solid lines*), respectively, followed by an additional 6 h irradiation with + UV-B in both cases. Relative TD concentrations were normalized to 1, for barley Ca grown under −UV-B. Values are means ± S.D. ($n = 7$–12) for each barley line and time value. Significance for $t = 1$ h and 3.5 h as compared to $t = 0$ h: ** $P \leqslant 1\%$; *** $P \leqslant 0.5\%$.

glucoside isosalipurposide **3**, the mutant was suggested to be defective in the chalcone isomerase (CHI) gene. The small flavone content of less than 1% of the parent line was attributed to a low autocyclization of the chalcone in vivo (Reuber et al., 1997). However, up to now there is no corresponding proof by molecular methods. On the other hand, genetically engineered maize cells that lack CHI still produced normal levels of flavonoids (Grotewold et al., 1998). Thus, further experiments are necessary to show whether or not CHI is expressed in barley ant 30 mutant line(s).

Our intention was to characterize relationships in both barley lines concerning (1) the efficiency of the UV-B screening capacity, as represented by flavones and/or HCA derivatives, with the amount of DNA damage sustained in developing leaves, (2) tissue localization, especially of the flavones as major UV protective substances in the parent line, (3) possible differences in DNA damage, measured as the relative accumulation of TD in the irradiated parts of the leaves of mutant and parent line, (4) subsequent photorepair or dark repair of DNA, and (5) growth and development of the primary leaves.

In several earlier investigations on the influence of increased UV-B on plants the concomitantly applied PAR was rather low and radiation-induced damage was overestimated (see Caldwell and Flint, 1994; and references cited therein). When a higher PAR:UV-B ratio was used, sensitivity towards UV-B was often strongly reduced, apparently due to increased photorepair (e.g. Taylor et al., 1997; Stapleton et al., 1997; Hidema et al., 1999). Therefore, in the present study, parent and mutant barley were grown with relatively high PAR in the presence or absence (controls) of elevated UV-B irradiation (Fig. 1).

As observed by fluorescence microscopy (Fig. 5), major flavone concentrations, especially of saponarin **1**, were specifically located in both epidermises and in the outermost cell layers of the mesophyll, primarily in the vacuoles. Thus, these cell layers represent a functional unit that absorbs potentially harmful UV-B radiation. HCA conjugates were localized in the outer and anticlinal cell walls and are probably also present in vacuoles, since both soluble and insoluble amounts of HCA were measured.

Lutonarin **2** did not contribute much to the over all screening capacity of the leaf and could not be attributed to a specific tissue or cell type. Since lutonarin **2** is an *ortho*-dihydroxylated flavonoid, which was markedly increased under elevated UV-B (ca. five-fold), it may have another protective function in scavenging oxygen radicals induced by UV-B irradiation (for discussion see Markham et al., 1998a,b; Olsson et al., 1998).

UV-B-induced increase in flavone accumulation in developing parent barley leaves was shown to be essential to protect the leaf, at least partially, against UV-B-induced DNA damage. Younger leaves appeared to be better protected than older ones, although protection was incomplete (Figs. 3, 4, 6). This may be an intrinsic property of barley line Ca 33787 in which the relatively high UV-B dose led to some damage in the biosynthetic pathway of flavones. It would be of interest to analyse whether lower doses allow sufficient or even better acclimatization in this line and also, whether differences exist in the UV-B sensitivity between Ca 33787 and other 'wild-type' barley lines.

Flavone-deficient mutant leaves were not able to acclimatize and suffered a much higher degree of damage (Fig. 6). HCA conjugates alone together with the low mutant-specific chalcone glucoside accumulation

could not prevent DNA damage. UV-B screening capacity was only ca. 25% of that of the parent leaves (Fig. 4). Soluble as well as insoluble (cell wall-bound) HCA conjugates of ferulic and *p*-coumaric acid accumulated as constitutive compounds in both lines with similar rather low amounts and were not increased under elevated UV-B irradiation (Figs. 3 and 4; Table 1). During early leaf development, both lines were able to reduce their daily UV-B damage to rather low levels by repair processes, apparently mainly by photorepair (c.f. Fig. 7). Growth and development of 5- to 8-day-old leaves were similar when compared with control leaves without UV-B irradiation (Fig. 2). However, the daily UV-B-induced DNA damage could not be completely repaired (Figs. 6 and 7) and TD accumulated continuously up to 18 days, reaching much higher values in the mutant than in the parent line. Up to now it is not known whether particularly the parent barley is unable to synthesize or accumulate sufficient amounts of active photolyase protein to keep TD levels low.

Even ambient solar UV-B in the field caused measurable DNA damage in young primary leaves of two other barley lines, in spite of a significant increase in UV-absorbing compounds (Mazza et al., 1999). In rice leaves a negative correlation was found between CPD levels induced by supplementary UV-B exposure and the content of UV-absorbing compounds estimated as total absorptivity at A_{330} nm (Kang et al., 1998). Thus, the leaves appeared to be well protected against harmful UV-B, although tissue localization of phenolics was not considered. In similar experiments, CPD levels of young rice leaves raised under additional UV-B were maintained within constant limits depending on the levels of visible radiation (Hidema et al., 1999). In these experiments, photo repair as well as dark repair was demonstrated but possible interacting effects of phenolic accumulation and DNA damage and repair had not been studied.

Increasing amounts of CPD were also found in pea leaves, mutant Argenteum, under acute high UV-B irradiation (10.3 kJ m^{-2} per 12 h) or after an acclimation to moderate UV-B (Kalbin et al., 2001). This pea line accumulates flavonol glucosides in both epidermal layers. However, although tissue localization of mRNAs for key enzymes of phenylpropanoid and flavonoid biosynthesis had been studied, the corresponding flavonoid amounts were not measured for an assessment of possible protective effects. One may speculate whether in parent barley leaves DNA damage occurred in both tissue types, epidermis and mesophyll. For pea, UV-B-induced DNA damage was found primarily in the epidermis but also in the mesophyll in varying amounts, which depended upon the actual UV-B exposure program (Kalbin et al., 2001). For maize leaves CPD were also detected in both tissue types (Stapleton et al., 1997).

In later developmental stages of the parent barley line, from 8 to 18 days, when TD concentration dramatically increased, leaf length and fresh weight were only slightly reduced. This was true even for the mutant, and no visible damage or morphological difference appeared in the phenotype of either line. Thus, the questions arises especially for the parent line: (1) are there negative effects for the primary leaf due to the observed DNA damage, and more important, (2) what are the consequences for further development of the adult plant and finally for its crop yield? A partial answer may be given as follows: in parallel experiments the barley line Hege 550/75 and its corresponding flavone-deficient mutant ant 30-287, closely related to the lines Ca 33787 and mutant ant 30-310 (see Section 1), were grown under the same controlled conditions in our growth chamber with additional high UV-B_{BE} irradiation of 12 kJ m^{-2} day^{-1}. Various photosynthetic parameters were measured during primary leaf development, the F_v/F_m-ratio, rate of net photosynthesis, O_2 evolution and D1 protein content, and showed only a slight, if any, reduction in both lines (Burchard, 2000). A similar behaviour may be expected for the two barley lines investigated in the present work. Apparently, cell differentiation, including functions of the photosynthetic apparatus, occurred mostly undisturbed in early developmental stages when DNA repair of TD was still highly efficient. One may speculate that in later stages when DNA damage had increased, this had little or no effect since differentiation had already been completed. It is also possible that UV-B-induced DNA damage took place mainly in active genes and was immediately repaired, whereas DNA damage accumulated mainly in the inactive genes (c.f. Suter et al., 1997; Schieferstein and Thoma, 1998).

4. Concluding remarks

Our comparative analysis of the leaves of parent and mutant barley lines showed the importance of the accumulation of UV-B screening compounds, HCA and flavonoids, as a major strategy for avoiding DNA damage under elevated UV-B irradiation. However, the acclimatization response, i.e. the rate and amount of phenolic accumulation, and therefore UV protection of the parent barley leaf, was incomplete. On the other hand, DNA repair capacity due to photolyases, a second strategy for UV-B tolerance, was also found to be essential, but turned out to be insufficient in either barley line, since DNA damage still accumulated during leaf development. It would be of interest to analyse whether this damage may have consequences for further growth and development of the plant, including the grain yield. Finally, it would be necessary to test economically important barley lines for their UV-B tolerance, with regard to the potential complementary nature of these protective strategies.

5. Experimental

5.1. Plant material

Seed material of barley (*Hordeum vulgare* L.) mutant ant 30-310 and corresponding parent line Ca 33787 was from the Carlsberg collection of proanthocyanidin-free mutants and parent lines (Carlsberg Research Laboratory, Copenhagen, Denmark; Jende-Strid, 1988, 1993; Reuber et al., 1997). The line ant 30-310 was propagated in a greenhouse and Ca 33787 in the field at the Max Planck Institut für Züchtungsforschung (Köln, Germany) to obtain sufficient amounts of caryopses for the experiments.

5.2. Growth and irradiation conditions

Caryopses of both lines were sown into a peat/soil mixture and covered with a glass plate for 72 h to ensure high humidity for an even germination and seedlings were grown under two different irradiation conditions, visible light without UV-B (−UV-B) or with additional UV-B radiation (+UV-B) up to 18 days in a growth chamber. The photoperiod was 13 h, with day and night temperatures of 20 and 10 °C, respectively. Relative humidity was 50% during days and 80% during nights.

Irradiation conditions for UV-B treatments were essentially the same as described previously (Burchard et al., 2000); in brief: PAR (400–700 nm, with eight halogen lamps, Powerstar HQI-T 400 W/D; Osram, Munich, Germany, and four incandescent bulbs, Krypton 100 W, Osram) was programmed to increase stepwise from 06:30 h to a maximum of 650–700 μmol m^{-2} s^{-1} at 10:00 h, lasting for 6 h. At 16:00 h, PAR was decreased in reversed sequence. UV radiation (supplied by four UV-B fluorescent lamps, TL 20 W/12; Philips, Hamburg, Germany) was added for 6 h during the maximum PAR intensity period to provide a constant UV-B irradiation of 12 kJ m^{-2} $UV\text{-}B_{BE}$ day^{-1} based on the generalized plant action spectrum (Caldwell, 1971) and calculated using a UV dosage model (see Burchard et al., 2000). This level of radiation was chosen for comparison with other laboratories. However, it corresponds to approximately ambient UV-B levels over much of Asia during the summer month (Bachelet et al., 1991) and is therefore a natural level. For UV-B treatment a 0.1 mm cellulose diacetate foil (URT Ultraphan 100; Digefra, Munich) was used, pre-exposed for 24 h for stable removal of irradiation $\leqslant$290 nm, and changed each week. Spectral irradiances at plant level were measured with a Bentham BC-150 spectroradiometer (Bentham Instruments, Reading, Berkshire, UK) at intervals of 1 nm between 250 and 600 nm (Fig. 1, +UV-B). Photon flux densities of PAR (400–700 nm) were measured with a LI-185 A quantum meter (LiCor, Lincoln, NE, USA). A Plexiglas filter (GS 231 Röhm, Darmstadt, Germany) was used to exclude the UV-B spectral region and part of the UV-A for the −UV-B conditions (Fig. 1, −UV-B)

5.3. Analysis of growth parameters

For the two barley lines grown under the various irradiation conditions, 10–15 primary leaves, representative for each developmental stage from 5 to 18 days, were harvested. Their total length was estimated in centimetres. The part of each leaf appearing above the coleoptile was cut with a razor blade and its fresh weight estimated individually.

5.4. Analysis of phenolic compounds

Each segment was extracted in 80% MeOH using standard protocols (Reuber et al., 1996; Burchard et al., 2000) and centrifuged. The crude extract was subjected to HPLC. The remaining pellet was hydrolysed with 1 M NaOH for 3 h at 80 °C, acidified and extracted using EtOAc. After evaporation, free HCA were dissolved in MeOH. Phenolics of crude extracts were separated using a water–acetonitrile gradient (flow rate: 1 ml/min) on a Nucleosil 100-5 C_{18} column (125×5 mm). Elution was started with 10% acetonitrile and increased to 14% within 16 min followed by an isocratic step of 10 min. Afterwards, acetonitrile concentration was linearly increased up to 22% within 15 min. After each run, the column was washed with 100% acetonitrile for 3 min and re-equilibrated to 10% acetonitrile within 5 min. The cell wall-bound HCA, hydrolysed from the pellet, were analysed by HPLC using 25% MeOH (flow rate: 1 ml/min) on the same column (see earlier). Elution was complete after 23 min. After each run the column was washed with 100% MeOH for 3 min and re-equilibrated to 25% MeOH within 5 min. Phenolic compounds were measured at 315 nm and identified by co-chromatography with reference substances and online spectral analysis during the HPLC runs, using a Shimadzu HPLC system (Tokyo, Japan) with UV/Vis absorbance detector (Shimadzu SD-10A) coupled to a Shimadzu C-R 5A integrator. Quantification was performed after calibration with authentic compounds from our laboratory collection. Values were expressed in nmol per mg fresh weight of leaf segments. HCA conjugates were characterized as ferulic and *p*-coumaric acid derivatives by their UV spectra (data not shown). The two aromatic acids were identified as the major chromophores, after alkaline hydrolysis of crude extracts and pellets, via HPLC as compared with reference compounds. These findings correspond with earlier results of Liu et al. (1995).

5.5. UV-B screening capacity of phenolic compounds

Potential UV-B screening capacity of phenolic compounds of both barley lines was measured at 315 nm

and calculated from the total phenolic peak area integrations of the HPLC, values per mg fresh weight of leaf segments.

5.6. DNA isolation and DNA damage analysis

Extraction and purification of DNA of the two barley lines was performed according to Rogers and Bendich (1994), except that RNase A (Roth GmbH, Karlsruhe, Germany) was added immediately to the extraction buffer. After final purification of barley DNA from both lines, RNA was absent as determined by agarose gel electrophoresis using ≈5 μg of purified DNA (data not shown). As a control, DNA extraction and purification without adding RNase A to the extraction buffer revealed an appreciable amount of RNA in the final preparation. Concentration of resuspended DNA was measured photometrically at 280 nm to test for protein impurities, at 260 nm for DNA, and at 230 nm for the presence of carbohydrates. According to the ratios obtained for the leaves of both barley varieties, $E_{260}/E_{280} \geqslant 1.9$ and $E_{260}/E_{230} \geqslant 2.1$, respectively, DNA preparations were sufficiently purified for further measurements. Accordingly, an optical density of $E_{260} = 1$ corresponded to 50 μg of DNA per ml.

Relative TD concentrations were estimated using an enzyme-linked immunosorbent assay (ELISA), originally described by Mori et al. (1991) with several modifications for the standard assay.

Standard assay: sterile cell culture plates with 96 cavities were used. Per cavity, 500 ng of purified DNA from irradiated or non-irradiated (control) barley in 50 μl phosphate buffered saline (PBS, 150 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4 in H_2O) were applied. The following sequence of incubations was carried out stepwise; in step (1) the plates remained uncovered; for steps (2)–(5) the plates were covered to avoid evaporation. After each step the cavities were washed five times using T-PBS (0.05% Tween 20 in PBS) and droplets were removed by inverting the plates. (1) Probes were heated for 1 h at 100 °C to adsorb DNA to the surface. (2) 200 μl of 1% (w/v) of low fat milk powder in PBS was added per cavity, and kept at 37 °C for 1 h. (3) 50 μl of the anti-TD mouse antibody (Kamiya Biomedical Co, Seattle, USA), diluted 1:1000, were incubated at 37 °C for 30 min. (4) 50 μl of the second antibody, biotin-conjugated goat anti-mouse antibody (Sigma, Saint Louis, USA), diluted 1:1000, at 37 °C for 30 min, and (5) 50 μl of Extravidin®-horse radish peroxidase conjugate (Sigma, Saint Louis, USA), diluted 1:500, at 37 °C for 30 min. After the last washing step with T-PBS two additional washings were performed using citrate phosphate buffer (50 mM Na_2HPO_4, 24 mM citric acid, pH 5). The colour reaction was started by the addition of 100 μl of a OPD-H_2O_2 solution (0.04% (w/v) *o*-phenylene diamine (OPD) and 0.007% (v/v) H_2O_2 in citrate phosphate buffer (H_2O_2 freshly added). Incubation was at 37 °C for 15 min, afterwards the reaction was terminated by the addition of 2 M H_2SO_4 (50 μl). Absorbance was read at 492 nm using the ELISA reader SpectraFluor Plus (Tecan, Salzburg, Austria).

The following control measurements for ELISA were performed on each plate: (1) PBS solution (50 μl) were added to six cavities, (2) PBS solution (50 μl) with of non-irradiated DNA (500 μg) were added into six different cavities, and (3) PBS-solution (50 μl) plus of irradiated, damaged DNA (100 μg), under standardized irradiation conditions. For standardization of the controls (2) and (3) DNA that had been isolated and purified from 5-day-old etiolated rye (*Secale cereale* L.) seedlings lacking TD, was used. Part of this 'dark-DNA' was used as described in (2), the other part was irradiated (3) for 30 min under + UV-B. The assay was linear between 50 ng up to 1000 ng of DNA, and from 0.1 up to 0.7 absorption units measured at A_{492}.

5.7. Epifluorescence microscopy

For the tissue and cellular localization of phenolic compounds a conventional microscope, Leica DMRB with epifluorescence equipment (Leitz, Wetzlar, Germany), was used. From the upper 2 cm of fresh and turgescent 6-day-old primary leaves of barley mutant and parent lines, both grown under + UV-B irradiation conditions, hand cuts were performed with a razor blade. Transverse, longitudinal and paradermal sections were immediately incubated in a droplet (100 μl) of sample buffer (100 mM K_2HPO_4/NaH_2PO_4, pH 5.8, 1 mM $CaCl_2$) on microscopic slides under a coverslip. After monitoring the autofluorescence of leaf sections, a droplet of freshly prepared 0.5% (w/v) ammonia in sample buffer was added and transferred under the cover glass using filter paper. Subsequent changes in fluorescence were also recorded. In a second set of cuttings, the autofluorescence of freshly prepared barley leaf sections was again monitored. Afterwards, a droplet of 0.1% (w/v) NA, diphenyl-boric acid-2-aminoethyl ester, in sample buffer, diagnostic for flavonoids (Hutzler et al., 1998), was transferred under the cover glass as described above and secondary fluorescence studied after 5 min of incubation. Epifluorescence images were obtained using a band pass filter, excitation 340–380 nm, beam splitter RKP 400 nm and long pass filter 425 nm. Photographs were taken with an Olympus OM-4 camera on Ektachrome EPL 135 (400 ASA), digitalized and prepared for printing using the image processing programs Adobe Photoshop (Adobe, USA) and iPhoto Plus (Ulead Systems GmbH, Braunschweig, Germany). Care was taken for highest possible colour reproduction.

5.8. Statistics

Student's *t*-tests were performed using SPSS 6.1.3 (SPSS Inc., USA).

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