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DIFFERENTIAL FLAVONOID RESPONSE TO ENHANCED UV-B RADIATION IN *BRASSICA NAPUS*

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Key Word Index—*Brassica napus*; Brassicaceae; oilseed rape; flavonoids; kaempferol and quercetin 3-sophoroside-7-glucoside and 3-(2‴-*E*-sinapoylsophoroside)-7-glucoside; UV-B-radiation.

Abstract—We have examined the qualitative and quantitative differences in methanol-soluble flavonoids of leaves of two cultivars of *Brassica napus*, which were grown with or without (control) supplemental UV-B radiation. The flavonoids were identified using HPLC-diode array spectroscopy (-DAS), -electrospray ionization-mass spectroscopy (-ESI-MS) and ^{1}H and ^{13}C NMR, and quantitatively analysed by HPLC-DAS. After exposure to supplementary UV-B radiation, the overall amount of soluble flavonoids, kaempferol and quercetin glycosides, increased by ca 150% in cv. Paroll, compared to control plants. Cultivar Stallion showed a 70% increase, and also a lower overall content of soluble flavonoids compared to Paroll. The supplementary UV-B radiation resulted in a marked, specific increase in the amount of quercetin glycosides relative to the kaempferol glycosides with a 36- and 23-fold increase in cvs Paroll and Stallion, respectively. Four of the flavonol glycosides appearing after supplemental UV-B exposure were identified as quercetin- and kaempferol 3-sophoroside-7-glucoside and 3-(2‴-*E*-sinapoylsophoroside)-7-glucoside.

INTRODUCTION

A reduction in stratospheric ozone results in a selective increase in the amount of ultraviolet-B (UV-B, 280–320 nm) radiation reaching the earth's surface due to the absorbing properties of ozone. UV-B radiation is readily absorbed by many key biological molecules in living systems, making research in this field a continuing necessity in order to understand plant response.

The protective role of flavonoids in plants in the expression of tolerance to UV-B radiation has been strikingly demonstrated by comparison of flavonoid mutants and their corresponding mother variety or wild type [1]. Flavonoids afford a protective role not only through the absorption of UV-radiation, especially in the epidermal layers, but also through a selective increase after UV-B irradiation in those flavonoids which possess an additional hydroxyl group in the B-ring of the flavonoid skeleton, i.e. *ortho*-dihydroxyl substitution [1–3]. It is not known at present how extensive this selective induction is within the plant kingdom. In animal food technology and biomedical studies, extra hydroxyl groups have been shown to increase the antioxidant properties of the compounds [4].

Some of the flavonol glycosides in leaves of *Brassica* species have been characterized previously. For example, flavonol 3-sophoroside-7-glucosides have been reported in *Brassica napus* [5] kaempferol 3-sophoroside-7-glucoside and 3-(2‴-*E*-sinapoylsophoroside)-7-glucoside together with other acylated triglycosides, have been identified in *B. oleracea* convar. *capitata* L. Alef. var. *alba* DC [6]. Kaempferol 3-(2‴-*E*-sinapoylsophoroside)-7-glucoside has been found also in seeds of *Brassica napus* [7]. However, little detailed analysis of flavonoid compounds and their characterization has been done in comparative studies of plants grown under visible and UV-B radiation [8, soybean; 9, rye; 10, rye].

The aims of the present study were to (a) investigate further the selective increase in accumulation of soluble leaf flavonoid derivatives in response to UV-B irradiation in the two cultivars, Paroll and Stallion; (b) confirm the chemical structures of the compounds involved; and (c) consider to what extent the results point to further UV-B acclimation. A cultivar com-

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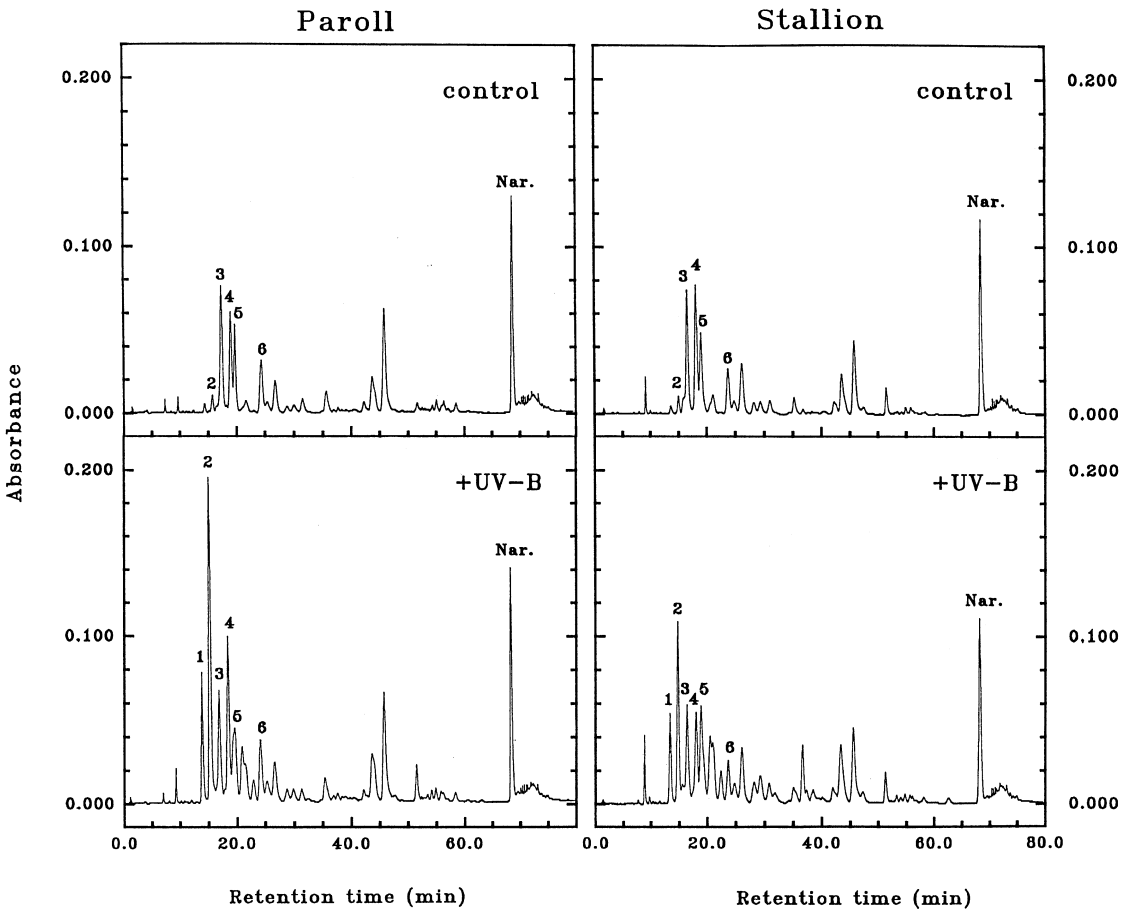

Fig. 1. Chromatograms (HPLC at 350 nm) of methanolic extracts of leaves of *Brassica napus*, cvs Paroll and Stallion, grown under 800 μmol m^{-2} s^{-1} with (+UV-B) or without (control) additional 12 kJ m^{-2} day^{-1} UV-B_{BE} radiation. The larger peaks with retention times around 45 min are hydroxycinnamic acid derivatives. Nar., naringenin, an internal standard used for quantification.

parison was made using specifically Paroll, an atrazine-sensitive cultivar, and Stallion, an atrazine-tolerant mutant, since in ongoing experiments we have found that these cultivars show differences in response when exposed to high visible radiation alone and in combination with enhanced UV-B radiation with respect to various photosynthetic parameters [11]. Thus in order to relate different plant response to potential phenolic accumulation, these cultivars were subjected to detailed analysis in the present study using the techniques of HPLC-electrospray ionization-mass spectroscopy (HPLC-ESI-MS), -diode array spectroscopy (-DAS), ^{1}H and ^{13}C NMR for the characterization of quantitative and qualitative differences in flavonoid response to UV-B radiation between the different cultivars under investigation.

RESULTS

Identification of compounds

Using column chromatography (CC) on polyamide and repeated CC on Sephadex LH 20, compounds **1**–**6** (Fig. 1) were isolated from a methanolic leaf extract of cv. Paroll plants, which were grown under either control conditions or supplemental UV-B radiation.

The structures of **1** and **3** from fraction III were analysed on HPLC-DAS, HPLC-ESI-MS and tandem mass spectroscopy (HPLC-ESI-MS/MS), but were not fully elucidated (Fig. 1). The HPLC-ESI-MS data (Table 1) showed that **1** had a protonated molecular mass of 1114 u, and the product ions in tandem mass spectroscopy revealed that **1** and **3** are derivatives of quercetin with m/z 303 and kaempferol with m/z 287, respectively. The structures of compounds **2**, **4**, **5** and **6** were established by HPLC-DAS, HPLC-ESI-MS and HPLC-ESI-MS/MS and extensive one dimensional (1D) and 2D ^{1}H and ^{13}C NMR experiments (Fig. 1). The protonated molecular masses of **2** and **4** 789 u and 773 u, respectively (Table 1), as determined by HPLC-ESI-MS from fraction I, together with the ^{13}C resonances (Table 2),, revealed that both compounds were flavonol glucosides containing three glucoses and that the aglycones differed by one hydroxyl group. The aglycone moieties were readily identified from the characteristic shifts and couplings in the ^{1}H (Table 3) and ^{13}C NMR spectra as quercetin and

Table 1. Characterization of ions detected by HPLC-ESI-MS/MS [*m*/*z*] (positive mode)

	Compound					
Precursor ions	**1**	**2**	**3**	**4**	**5**	**6**
$[M+H]^+$	1114	789	—	773	995	979
$[aglycone+H]^+$	303	303	287	287	303	287
$[sinapic\ acid-H_2O]^+$	—	—	—	—	206	206
$[sinapoylglucosyl]^+$	—	—	—	—	369	369
$[sinapoylsophorosyl]^+$	—	—	—	—	531	531
Product ions						
$[M-sinapoylsophorose+H]^+$	—	—	—	—	465	449
$[M-sophorose+H]^+$	—	465	—	449	—	—
$[M-glucose+H]^+$	—	627	—	611	—	817
$[M-sinapoylglucose+H]^+$	—	—	—	—	—	611

Table 2. ^{13}C NMR chemical shifts, δ [ppm] (J [Hz]) of compounds **2**, **4**–**6** (400 MHz, MeOH-d_6)

	Compound			
	2	**4**	**5**	**6**
C2	157.9	157.9	157.7	157.4
C3	135.1	135.3	134.9	134.8
C4	179.7	179.7	179.1	179.1
C5	161.5	162.5	162.1	162.1
C6	100.8	100.7	100.7	100.7
C7	164.5	164.5	164.2	164.2
C8	95.8	95.8	95.2	95.2
C9	157.9	157.9	157.5	157.3
C10	107.5	107.5	107.5	107.5
$C1^1$	122.8	122.6	122.9	122.9
$C2^1$	117.8	132.4	117.1	132.2
$C3^1$	145.8	116.23	145.9	116.1
$C4^1$	149.8	159.4	149.7	161.4
$C5^1$	116.2	116.23	116.0	116.1
$C6^1$	123.2	132.4	123.3	132.2
$C1^2$	101.4	101.4	97.0	97.0
$C2^2$	82.9	82.4	82.0	82.0
$C1^3$	104.6	104.9	98.5	98.5
$C2^3$	74.6	74.6	74.8	74.8
$C1^4$	101.6	101.6	101.4	101.4
$C1^5$	—	—	126.0	126.0
$C2^5$	—	—	105.6	105.6
$C3^5$	—	—	148.7	148.7
$C4^5$	—	—	138.7	138.7
$C5^5$	—	—	148.7	148.7
$C6^5$	—	—	105.6	105.6
$C7^5$	—	—	146.5	146.5
$C8^5$	—	—	116.0	116.0
$C9^5$	—	—	168.4	168.8
CH_3	—	—	56.3	56.3

kaempferol, respectively, with glycosylation at OH-3 and OH-7 [12]. This was in agreement with the typical absorption bands in HPLC on-line UV spectra of quercetin and kaempferol glycosides (Table 4) and the product ions in tandem mass spectroscopy at *m*/*z* 303 and 287, respectively (Table 1). The chemical shifts of the anomeric protons revealed that two glucose moieties are directly linked to the aglycone, whereas the third glucose is linked to a sugar. That was confirmed by correlation spectroscopy (^1H-^1H COSY), which showed also that the diglucoside (sophoroside) was bound at OH-3 on the aglycone and the interglucosidic linkage was 2 → 1 [12]. Thus, the structures of **2** and **4** were confirmed as quercetin and kaempferol 3-*O*-*β*-D-[*β*-D-glucopyranosyl(1 → 2)glucopyranoside]-7-*O*-*β*-glucopyranoside, respectively.

The structures of **5** and **6** from fraction II were deduced analogously as quercetin and kaempferol 3-*O*-*β*-D-[2-*E*-sinapoyl-*β*-D-glucopyranosyl(1 → 2) glucopyranoside]-7-*O*-*β*-glucopyranoside, respectively, as derivatives of **2** and **4** acylated with sinapic acid. This was in agreement with the protonated molecular masses, 995 u and 979 u, found in HPLC-ESI-MS experiments (Table 1). The site and nature of acylation was identified from the ^1H and ^{13}C NMR spectra (Tables 3 and 2, respectively), ^1H-^1H COSY and in HPLC-ESI-MS/MS experiments (Table 1). Upon collision activation of their respective precursor ions, sequential loss of substituents attached to the flavonoid moieties resulted in product ions *m*/*z* 817, 611 and 449 (**6**) or 465 (**5**). In addition, the acylated compounds **5** and **6** revealed product ions characteristic for sinapic acid at *m*/*z* 206, acylated glucose at *m*/*z* 369 and acylated sophorose at *m*/*z* 531, respectively.

The quite different shift at δ 6.19 and 6.18 for the H-1^2 anomeric protons of **5** and **6** measured in MeOH-d_6 in comparison to the ^1H NMR data published for **6** [6] in DMSO-d_6 at δ 5.76 was confirmed by comparison with an authentic sample (Prof. H. Geiger, Stuttgart University, Germany) measured in both solvents. The data from ^1H and ^{13}C NMR of the authentic sample measured in MeOH-d_6 were identical to those of **6** and when the authentic sample was measured in DMSO-d_6 the data were identical to those reported by Nielsen *et al*. [6] for the same compound.

Cultivar comparison and quantification

By using HPLC-DAS for the on-line UV spectra we obtained main absorption band maxima for **2** at 255 and 351 nm and for **4** at 265 and 345 nm (Table 4, cv. Paroll, +UV-B). When the latter was acylated (**6**) it had absorption maxima at 267 and 333 nm. These results show a difference in the lower absorption band maxima of around 10 nm between kaempferol and quercetin and a shift to lower wavelengths of the higher absorption band maxima of acylated compared to non-acylated compounds.

The cultivars showed the same flavonoid pattern,

Table 3. ^{1}H NMR chemical shifts, δ [ppm] (J [Hz]) of compounds **2**, **4**–**6** (300 MHz, MeOH-d_6)

	Compound			
	2	**4**	**5**	**6**
H6	6.52 *d*(1.9)	6.52 *d*(1.9)	6.28 *d*(2.0)	6.30 *d*(2.0)
H8	6.78 *d*(2.0)	6.80 *d*(2.0)	6.45 *d*(2.0)	6.41 *d*(2.0)
H2^1	7.74 *d*(2.1)	8.11 *d*(8.9)	7.55 *d*(2.1)	7.95 *d*(8.9)
H3^1	—	6.95 *d*(8.9)	—	6.94 *d*(8.9)
H5^1	6.93 *d*(8.4)	6.95 *d*(8.9)	6.92 *d*(8.5)	6.94 *d*(8.9)
H6^1	7.61 *dd*(8.4/2.1)	8.11 *d*(8.9)	7.50 *dd*(8.5/2.1)	7.95 *d*(8.9)
H1^2	5.43 *d*(7.5)	5.52 *d*(7.5)	6.18 *d*(8.2)	6.19 *d*(8.2)
H1^3	4.81 *d*(7.2)	4.81 *d*(7.2)	5.26 *d*(7.8)	5.26 *d*(7.8)
H1^4	5.10 *d*(6.9)	5.10 *d*(6.9)	5.14 *d*(7.5)	5.14 *d*(7.5)
H2^3	—	—	4.96 *t*	4.96 *t*
H2^5	—	—	6.29 *s*	6.31 *s*
H6^5	—	—	6.29 *s*	6.31 *s*
H7^5	—	—	6.13 *d*(15.9)	6.14 *d*(15.9)
H8^5	—	—	7.35 *d*(15.9)	7.35 *d*(15.9)
CH_3	—	—	3.64 *s*	3.64 *s*

Table 4. Retention times (R_t in min) and absorption maxima (λ_{max} in nm) measured by on-line HPLC-DAS of methanol-soluble compounds in *Brassica napus*, cvs Paroll and Stallion (see Fig. 1), grown under 800 μmol m^{-2} s^{-1} with (+UV-B) or without (control) additional 13 kJ m^{-2} day^{-1} UV-B$_{BE}$ radiation

	Paroll				Stallion			
	Control		+UV-B		Control		+UV-B	
Comp.	R_t	λ_{max}	R_t	λ_{max}	R_t	λ_{max}	R_t	λ_{max}
	9.7	324	9.6	325	9.1	324	9.0	325
1	—	—	14.3	244, 339	—	—	13.6	244,338
2	15.7	251, 349	15.7	255, 351	14.9	252, 349	15.0	254, 345
3	17.3	268, 331	17.3	268, 331	16.5	268, 331	16.6	268, 331
4	18.9	265, 345	18.9	265, 345	18.0	265, 345	18.2	265, 344
x+**5***	19.7	267, 333	19.3	266, 338	18.9	267, 332	19.0	242, 267, 332
5	—	—	20.1	243, 340	—	—	—	—
	—	—	21.3	251, 338	20.7	251, 340	20.7	251, 339
	—	265, 337	21.8	264, 342	21.0	264, 345	21.2	264, 344
	—	—	23.3	257, 324	—	—	22.6	257, 322
6	24.4	267, 332	24.6	268, 333	23.8	267, 333	23.9	268, 333
	25.4	267, 331	25.6	267, 331	24.8	268, 331	25.0	267, 331
	26.8	267, 331	27.0	268, 331	26.2	268, 331	26.3	268, 331
	—	—	—	—	—	—	37.0	254, 352
	44.0	327	44.3	328	43.8	327	43.8	328
	46.2	329	46.4	329	46.0	329	46.1	329
	52.0	264, 337	52.1	264, 342	51.8	265, 341	51.9	265, 341

* Compound **5** co-eluted with an unidentified kaempferol glycoside, x, in cv. Stallion, +UV-B.

although they differed in the amount of single compounds (Fig. 1). The on-line spectra of the detected flavonoids indicated that in the control leaves, grown without supplemental UV-B radiation, almost all the methanol-soluble flavonoids of both cultivars were kaempferol glycosides, of which around half were acylated (Tables 4 and 5, control). The flavonoid content was 20% higher in Stallion than in Paroll, mainly due to a higher amount of kaempferol glycosides (Table 5, control).

With supplemental UV-B radiation, the total amount of flavonoids was instead higher in Paroll

Table 5. Flavonoid content, mg (g dry weight)$^{-1}$ in *Brassica napus*, cvs Paroll and Stallion (see Fig. 1), grown under 800 μmol m^{-2} s^{-1} with (+UV-B) or without (control) additional 13 kJ m^{-2} day^{-1} UV-B_{BE} radiation

	Paroll		Stallion	
Compound	Control	+UV-B	Control	+UV-B
Total flavonoids	0.38	0.96	0.47	0.80
Total kaempferol glycosides	0.37	0.50	0.45	0.36–0.49
Total quercetin glycosides	0.0095	0.46	0.017	0.32–0.43
Unidentified kaempferol glycosides	0.048	0.18	0.10	0.16
Unidentified quercetin glycosides	—	0.066	0.006	0.13
1 Qu glycoside	—	0.091	—	0.067
2 Qu-3-sophoroside-7-glucoside	0.0095	0.24	0.011	0.12
3 Km glycoside	0.11	0.11	0.096	0.076
4 Km 3-sophoroside-7-glucoside	0.091	0.16	0.12	0.084
x Unidentified Km glycoside	0.068	0.054	0.087	?
5 Qu 3-2″-sinapoylsophoroside-7-glucoside	—	0.063	—	?
x+**5***	0.068	0.12	0.087	0.12
6 Km 3-2″-sinapoylsophoroside-7-glucoside	0.053	0.065	0.044	0.045

* Compound **5** co-eluted with an unidentified kaempferol glycoside, x, in cv. Stallion, +UV-B.

than in Stallion, but still around half of the flavonoids in both cultivars were acylated (Tables 4 and 5, +UV-B). In Paroll, the total amount of soluble flavonoids increased by ca 150% compared to conditions without supplemental UV-B radiation (Table 5), and in Stallion by only 70%. The quercetin glycosides increased more than the kaempferol glycosides in both cultivars such that after growth under supplemental UV-B radiation there was a 36-fold increase in the amount of quercetin as compared to kaempferol glycosides in Paroll, and in Stallion this was 23-fold. In contrast to Paroll, the amount of kaempferol glycosides did not change with supplemental UV-B radiation in Stallion. Both cultivars showed a slight increase in the amount of hydroxycinnamic acids (HCAs) with absorption maxima around 324–327 nm after supplemental UV-B radiation (Fig. 1, amounts not shown).

The most striking effect of supplemental UV-B radiation was the pronounced higher increase in quercetin glycosides compared to the increase in kaempferol. When comparing cultivars, the major difference was in the relative increase in total amount of flavonoids with additional UV-B radiation (Table 5), which was higher in Paroll (+150%) than in Stallion (+70%). Another difference was expressed in the lack of increase in kaempferol after exposure to UV-B radiation in Stallion. Some kaempferol glycosides appeared to even decrease after additional UV-B treatment in Stallion (Table 5, compounds **3** and **4**).

DISCUSSION

This study has examined the influence of supplemental UV-B radiation on quantitative and qualitative flavonoid content in two cultivars of *Brassica napus*. Apart from the efficient UV-filtering function of phenolic compounds, the interesting phenomenon of an actual switch in the relative amounts of the two major groups of flavonoid compounds, kaempferol and quercetin glycosides (Table 5), prompted a more detailed study of the components involved. *In vitro* studies have shown that flavonoids are good quenchers of hydroxyl radicals (OH*), superoxide anion radicals (O_2^-) and other active oxygen species, e.g. H_2O_2 [13–16]. Flavonoids with an *ortho*-dihydroxyl group in ring B of the flavonoid skeleton have a potentially increased antioxidant activity compared to those without [17, 18]. The shift from kaempferol to quercetin is thus of interest, since the quercetin chromophore has an additional hydroxyl group on position 3′ in the B-ring compared to kaempferol. Quercetin has been shown to be a better scavenger of superoxide than kaempferol *in vitro* [19, 20]. The same shift in a major flavonoid compound with supplemental UV-B radiation was demonstrated in a pilot study in leaves of *Brassica napus*, cv. Ceres [3], and also in *Hordeum vulgare* [1, 2], in the latter from an apigenin to a luteolin derivative.

The consequences of such a change after exposure to UV-B radiation may indicate an additional protective role, giving flavonoids both UV-B screening and antioxidant properties. An increase in flavonoid antioxidant activity would be highly beneficial, since UV-B radiation generates free radicals [21, 22]. UV photolysis of hydrogen peroxide radicals induces hydroxyl radicals, which are better scavenged by quercetin than by kaempferol [23]. The large increase in quercetin (or luteolin) derivatives after an enhanced UV-B exposure in species capable of the shift in compound, possibly may be taken as an indicator of stress, especially if this translates into a greater capacity for antioxidative functions.

Soluble phenolic compounds finally accumulate in the vacuoles [24–26], and predominately in those of the upper epidermal cells in *Brassica napus* [3, 27]. The location of the compounds in the vacuoles may complicate the explanation of the antioxidative activities of the flavonoids concerning processes occurring outside the vacuoles in the cells. However, under high stress conditions H_2O_2 is able to diffuse across membranes and enter the vacuoles [28]. The oxidation of flavonols by H_2O_2 requires peroxidases, which are localized in the vacuoles of leaves. Therefore it might be an advantage for the plant to have a co-localization of flavonoids and peroxidase in the vacuole [29]. There might also be unknown pools of flavonoids in the cytosol, where they are synthesized, before storage in the vacuoles [25]. Studies by Takahama [30] showed that in mesophyll cells of *Vicia faba*, both kaempferol and quercetin glycosides increased in the presence of H_2O_2, with a tendency for the quercetin derivatives to increase relative to controls. Takahama [31] has also shown that flavonols *in vivo* are oxidized by H_2O_2 in epidermal strips. The quercetin glycosides were also more sensitive to H_2O_2 than the kaempferol glycosides. It should, however, be kept in mind that (a) the antioxidant function of flavonoids is dependent on their redox potentials as pointed out by Bors *et al.* [32]; (b) the glycosylation of the flavonoids *in vivo* in plants probably decreases their antioxidant activity [33, 34] compared to the aglycones; and (c) their localization-functional relationship as potential antioxidants is not yet fully elucidated. Nevertheless, one may speculate that, because of the large increase in quercetin as opposed to kaempferol glycosides after UV-B exposure in our study, these compounds may increase antioxidant activity in plants grown under enhanced levels of UV-B radiation. The ability of quercetin derivatives to dissipate excitation energy more efficiently than kaempferol derivatives (personal communication, G. J. Smith and K. R. Markham) may serve as an additional explanation for their increase in plants irradiated with supplemental UV-B radiation during which they are exposed to high energy levels. Light is also absorbed in the vacuoles, which makes the latter aspect important with respect to the relationship between function and localization of the major part of soluble flavonoids.

Previous studies have shown that the photosynthetic apparatus is more susceptible to photoinhibitory radiation in cv. Stallion, the atrazine-resistant mutant, than in cv. Paroll [35] and also to UV-B radiation [11]. Although the increase in flavonoids was lower in cv. Stallion compared to that in Paroll after exposure to supplemental UV-B radiation, Stallion seemed to put more energy into producing quercetin glycosides rather than kaempferol glycosides (Table 5). The higher increase in quercetin compared to that in kaempferol glycosides may be an advantage, with regard to antioxidative and energy dissipating properties. This may be particularly important for cv. Stallion, since it is even more susceptible to high energy levels than Paroll.

EXPERIMENTAL

Plant material

Oilseed rape seed (*Brassica napus* L., cvs Paroll and Stallion) was purchased from Weibull AB, Landskrona, Sweden and grown in a greenhouse chamber under 800 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR, 400–700 nm) supplied by a bank of Osram Power Star dysprosium lamps (Awake 400 W/D, Germany). Half of the plants were exposed to additional 13 kJ m^{-2} day^{-1} biologically effective UV-B ($UV\text{-}B_{BE}$) radiation. Supplemental UV-B radiation was from Q-PANEL UV313 lamps (Largo, Göteborg, Sweden) and the radiation was filtered through 3.0 mm Plexiglas (FBL2458, Röhm GMBH, Chemische Fabrik, Germany) and 0.13 mm cellulose diacetate, which effectively removed radiation below 290 nm. The Plexiglas was included since it extends the life of the cellulose diacetate, which was changed every 5th day. The PAR and UV spectra were measured with a Model 752 spectroradiometer (Optronic Laboratories, Orlando, FL, U.S.A.). Weighted irradiance was calculated using a UV dosage model [36] and the generalized plant action spectrum of Caldwell [37], and the values normalized to 300 nm. The photoperiod was 12 h with a light-dark temperature of 20/16°. Supplemental UV-B radiation was given around solar noon for 3 h. After 16 days of light treatment the second and third leaf pairs were harvested and freeze-dried.

Extraction and isolation

The combined and freeze-dried plant material (104 g) of cv. Paroll was suspended in MeOH (4 l) at 50° and applied to a glass column. After 18 h the extract was collected and further extraction was achieved by a continuous flow of MeOH (2 l) at 20° through the glass column. Then 50% MeOH (600 ml) was added and after 12 h the extract was collected. This last step was then repeated, followed by a further extraction for 12 h in 1 l of 100% MeOH + 1% ammonia before collection. The combined extracts were evaporated to 1 l and further separation was achieved by CC on polyamide SC 6 (Macherey and Nagel, Dueren, Germany). The phenolic compounds were eluted with H_2O containing increasing concentrations of aq. MeOH and finally with 100% MeOH + 1% NH_3 as solvent. Fractions of 200 ml were collected and run on TLC silica plates, in EtOAc–H_2O–HOAc–HCO_2H (100:26:12:12). The TLC plates were sprayed with 1% Naturstoff Reagenz in MeOH and 5% polyethylene 400 in MeOH. Fractions containing similar phenolic TLC patterns were combined and evaporated to dryness or freeze-dried when containing only H_2O as solvent. The combined fractions were run on

TLC and HPLC-DAS (see below). Further purification using LPLC was carried out on Sephadex LH 20 (Pharmacia, Freiburg, Germany) or RP-18 Silica material (Kronlab, Berlin, Germany) with H_2O–MeOH gradients. The final purification step was CC on Sephadex LH 20 with Me_2CO–H_2O–MeOH (2:1:1) as solvent. Two fractions I and II, both containing a pair of the corresponding kaempferol and quercetin glycosides, were collected for HPLC-DAS, -ESI-MS and NMR analysis. After drying, fraction I and II gave 121 and 123 mg, respectively. Another fraction (III) was analysed on HPLC-DAS and -ESI-MS only.

Cultivar comparison

For each cultivar and treatment, 1.0 g of the freeze-dried plant material was extracted with 20.0 ml MeOH, containing naringenin (Sigma, U.S.A.) as internal standard (1 g l^{-1}) and stirred for 30 min. The extracts were then centrifuged at 2000 *g* for 10 min. The supernatant was collected and the pellet resuspended in 10 ml of 50% methanol and stirred for 30 min followed by centrifugation at 2000 *g* for 10 min. The supernatant was combined with the first one and analysed on HPLC-DAS as described above. The comparison between cultivars was carried out from the leaf extracts, each taken from 16 different plants with a total of 32 leaves.

Instrumentation

HPLC-DAS: Aliquots were used for analysis on an HPLC system with pumps (type 126), detector (PDA, type 168), autosampler (type 502) and an integration software (GOLD) from Beckman (Munich, Germany). The samples were run on a gradient of MeOH (A) and 0.15% aq. H_3PO_4, pH = 2 (B), in a stepwise linear gradient: 0 to 20% (A) for 2 min, 20–31% (A) for 28 min, 31–32% (A) for 5 min, 32–40% (A) for 10 min, 40–42% (A) for 5 min, 41–100% (A) for 15 min and finally isocratically for 15 min (100% A) with a flow rate of 1 ml min^{-1}. Injection volume was 20 μl. The column (Merck Supersphere RP 18 endcap, 125 × 4 mm) and guard column (Merck Lichrochart RP 18) were thermostated to 15°. Detection was at 330 to 350 nm and peak identities determined by on-line UV-spectra; HPLC-ESI-MS: Chromatographic separation was performed on a Nucleosil 120 C-18 column (5 μm, 2 × 125 mm, Knauer, Berlin, Germany) with a binary gradient delivered by an Applied Biosystems (Bai, Bensheim, Germany) 140B pump. The solvents were MeOH (A) and H_2O (B), both containing 0.05% TFA. The samples were run in a linear gradient: initial conditions 10% A at 0.2 ml min^{-1}, 10–60% (A) for 10 min, isocratically for 5 min (60% A) and finally 5 min equilibration (10% A). Injection volume was 5 μl; HPLC-ESI-MS/MS: Analysis was performed on a Finnigan (Finnigan, Bremen, Germany) TSQ 7000 triple-stage quadruple tandem mass spectrometer. For electrospray ionization, the capillary was set to 4.0 kV. N_2 served both as sheath (50 psi) and auxilliary gas. Positive ions were detected scanning from 200 to 1000 u with a total scan duration of 1.0 s. The MS/MS experiments were performed at a collision energy of 18 eV with argon (0.24 Pa) serving as collision gas, scanning a mass range from 200–1000 u; NMR: The spectra for one dimensional (1D) and 2D, ^1H and ^{13}C NMR were recorded at ambient temp. on Bruker AM 300 and ARX 4000 spectrometers locked to the deuterium resonance of the solvent, MeOH-d_6, using the standard Bruker software package. The standard was TMS.

Quantification

Calibration data for HPLC quantification was obtained from rutin for all quercetin derivatives, and from kaempferol 3-rutinoside (gift from H. Geiger, Saarbruecken, Germany) for all kaempferol derivatives, and from naringenin for the int. standard added to the crude extracts. The standards were run in the same HPLC gradient as the samples. Since no acylated reference compounds were available for calibration, the amount of acylated flavonol glycosides was calculated with the calibration data of the non-acylated compounds.

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