

# Flavonoids and UV Photoprotection in *Arabidopsis* Mutants

Ken G Ryan<sup>a\*</sup>, Ewald E Swinny<sup>a</sup>, Chris Winefield<sup>b</sup> and Kenneth R Markham<sup>a</sup>

<sup>a</sup> Industrial Research Ltd, PO Box 31 310, Lower Hutt, New Zealand. Fax: +64-4-5690132.  
E-mail: k.ryan@irl.cri.nz

<sup>b</sup> Crop and Food Research Ltd, Private Bag 11 600, Palmerston North, New Zealand

\*Author for correspondence and reprint requests

Z. Naturforsch. **56c**, 745–754 (2001); received April 2/May 7, 2001

*Arabidopsis*, Flavonoids, Kaempferol, Quercetin

Wild-type *Arabidopsis* L. leaves exposed to low ultraviolet-B (UVB) conditions contained predominantly kaempferol glycosides, with low levels of quercetin glycosides. The flavonoid level doubled on treatment with UVB and an increase in the ratio of quercetin: kaempferol was observed. These results suggest that flavonols protect *Arabidopsis* plants from UVB damage, and indicate that the flavonoid 3'-hydroxylase (F3'H) enzyme, which converts dihydrokaempferol to dihydroquercetin, may play a crucial role. The *tt7* mutant lacks this gene and, after treatment with sub-ambient UVB, contained kaempferol glycosides exclusively, to a level of total flavonols similar to that in wild-type *Arabidopsis*. Total flavonols after enhanced UVB treatment were higher in *tt7* than in similarly treated wild-type plants, and only kaempferol glycosides were detected. Despite this high level, *tt7* plants were less tolerant of UVB radiation than wild-type plants. These observations suggest that kaempferol is a less effective photoprotectant than quercetin. The chalcone isomerase (CHI) mutant (*tt5*) surprisingly did not accumulate naringenin chalcone, and this suggests that the mutation may not be restricted to the CHI gene alone. The concentration of hydroxycinnamic acid derivatives did not change with UVB treatment in most varieties indicating that their role in UV photoprotection may be subordinate to that of the flavonoids.

**Abbreviations:** CHI, Chalcone isomerase; CHS, Chalcone synthase; FLS, Flavonol synthase; F3H, Flavanone 3-hydroxylase; F3'H, Flavonoid 3'-hydroxylase; hc, hydroxycinnamic acids; HPLC, High pressure liquid chromatography; Ler, Landsberg *erecta* ecotype; PAL, Phenylalanine ammonia-lyase; RT, retention time; *tt*, transparent testa; Q, quercetin glycosides; K, kaempferol glycosides; Q:K, quercetin to kaempferol ratio; UVA, Ultraviolet-A radiation (320–400 nm); UVB, Ultraviolet-B radiation (280–320 nm); +UVB, enhanced UVB; amb, Ambient UVB; -UVB, low UVB.

## Introduction

The loss of polar ozone has become a global problem over the last decade due to movement of ozone-poor air to temperate latitudes where it may result in increases in biologically harmful solar UVB radiation (Madronich *et al.*, 1998). Unlike animals, plants cannot avoid exposure to incident radiation, and many studies have shown that plants grow poorly under enhanced levels of UVB flux (Caldwell *et al.*, 1998). In several of these studies, plants were grown under unnaturally excessive artificial UVB fluxes, and estimates of UVB induced damage may have been overstated. In many cases, plants appear to have adequate protection against moderate ozone-induced changes in UVB flux (Fiscus and Booker, 1995). However, even within a single species such as soybean, marked varietal differences in UVB sensitivity have been noted

(Teramura and Murali, 1986). Similarly, Hoffmann *et al.* (2000) have observed differences among clover varieties, where the more UV sensitive varieties were those bred for agronomically useful traits such as increased yield and palatability.

Plant breeding regimes might inadvertently enhance UVB sensitivity (Li *et al.*, 1993; Hoffmann *et al.*, 2000). With the advent of modern gene technology, plant breeders now have access to techniques to rapidly develop new phenotypes, increasing the risk of introducing undesirable UVB sensitivity. The latter may be particularly likely if the enhancements involve traits controlled by the general phenylpropanoid pathway, which is perhaps the best characterised of all metabolic pathways in plants. For example, increased palatability or altered flower colour can be achieved by modifying the genes controlling production of phenolic compounds including flavonoids, tannins

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and hydroxycinnamic acid derivatives. However, up-regulation of the general phenylpropanoid pathway is a common response to a number of environmental stresses in plants including UVB (Daugherty *et al.*, 1994). Some of these compounds absorb strongly in the UVB region of the solar spectrum, and many authors have suggested that they play a key role in the protection of plants from the effects of UVB radiation by acting as the plant's sunscreen (Jordan, 1996; Landrey *et al.*, 1995; Reuber *et al.*, 1996). It is therefore possible that a reduction in phenolic compounds in leaf tissue introduced by plant breeders may inadvertently lead to an increase in UVB sensitivity. A detailed understanding of the mechanisms of flavonoid biosynthesis in response to environmental stresses such as UVB is therefore required so that the potential for accidental introduction of UVB sensitivity is reduced.

Recent studies have demonstrated that *ortho*-dihydroxylated flavonoids may be synthesised in preference to their monohydroxylated counterparts in response to increasing UVB treatment (Ryan *et al.*, 1998; Markham *et al.*, 1997; Olssen *et al.*, 1998). The reasons for this preference are not clear because the UVB absorption profiles for both mono and dihydroxylated compounds are quite similar. In fact, as hydroxylation increases, the UVB absorption decreases slightly (Lavola *et al.*, 1997). *Ortho*-dihydroxylated flavonoids are more effective free radical scavengers/antioxidants than their monohydroxylated equivalents (Montesinos *et al.*, 1995), and we have suggested (Ryan *et al.*, 1998) that this may account for their production in plants under UV stress. Another possible benefit to the plant may be that *ortho*-dihydroxyflavonoids may be better able to dissipate absorbed UV energy in a harmless way (Smith and Markham 1998). It therefore appears that the biosynthetic conversion from mono- to *ortho*-dihydroxylation may be a crucial part of the plant's protection mechanism against UVB damage. The monohydroxylated and *ortho*-dihydroxylated flavonols that are found in *Arabidopsis thaliana* L. leaves are glycosides of kaempferol and quercetin respectively. The key step in the pathway therefore is the conversion of the kaempferol precursor to the quercetin precursor, which is catalysed by the cytochrome P<sub>450</sub> enzyme, flavonoid 3'-hydroxylase (F3'H) (Graham, 1998).

*Arabidopsis* has become increasingly valuable for studies on phenylpropanoid metabolism and many flavonoid biosynthesis mutant lines are available (Chapple *et al.*, 1994), some of which are susceptible to the damaging effects of UVB radiation (Li *et al.*, 1993). In particular, the *Arabidopsis* mutant *tt7* lacks the F3'H enzyme (Koornneef, 1982, 1990) and does not produce quercetin (Shirley *et al.*, 1995). To our knowledge, no detailed assessment has been made of the response of this mutant to ambient and enhanced UVB levels, both in terms of biomass production and in the quantities of total flavonoids produced. Given the importance we have assigned the F3'H enzyme affected in this mutant, it is clear that an analysis of the physiological and biochemical responses to enhanced UVB is required. Comparisons with wild-type and other *Arabidopsis* flavonoid biosynthetic mutants were also made, and the observations on the latter allow a direct assessment of the relative roles of sinapate esters and flavonoids in the protection of plants from UVB damage.

## Methods

### UVB treatments

Plants were grown in an outdoor plastic covered chamber which admitted natural solar radiation at slightly reduced intensity. The chamber was divided into three sections providing enhanced UVB, ambient UVB, and very low UVB. The cladding material was transparent to solar UVB in the +UVB and amb regions, and opaque to UVB in the -UVB section. The UVB illumination system was continuously adjusted to provide a constant enhancement of UVB over ambient levels throughout the day. For details of this system see Ryan *et al.* (1998) and Ryan and Ireland (1997). The experiment was conducted during autumn, when ambient levels of UVB were relatively low. The level of enhancement was set to provide UVB radiation similar to that on a clear day in mid summer, which amounted to approximately 50% over ambient at this time of the year. The -UVB region was clad in Mylar, which allows approximately 5% of ambient UVB to penetrate into the chamber. Similar levels of ambient UVA to those in the other two parts of the chamber were present in the -UVB region. The UV radiometer used was an International Light Inc. (Newburyport, MA),

type SED240/ACTS270W. This broad band radiometer has a response spectrum similar to that of many UV biological action spectra and the relative doses quoted here (5%, 100% and 150% of ambient UV) are equivalent to relative biologically effective doses.

### Plant material

*Arabidopsis thaliana* seeds of wild-type (Landsberg *erecta* ecotype, Ler) and mutant lines *tt4*, *tt5*, *tt6*, and *tt7* were obtained from the Nottingham *Arabidopsis* Stock Centre and germinated on sterile agar. Seedlings were obtained from all lines by germinating fresh seed on sterile agar in sealed plastic pots. Seedlings were transplanted into individual pots when the rosettes were about 1 cm diameter, and all were grown for 3 days in the -UVB region of the chamber to harden off. Plants of matched sizes were then allocated to the three regions of the chamber. An automatic system watered plants to excess. Measurements of rosette width were made at intervals during the following 3 week period. For each individual plant, a linear regression model of the following form was fitted:

$$W_i = c + rd_i \quad (1)$$

where  $W_i$  was the rosette width of the plant at day  $i$ ;  $c$ , was a constant term;  $r$ , was the rate of change in rosette width; and  $d$ , was the day of measure-

ment. During this period, the plants were in the linear phase of growth.

### Quantitative flavonoid analysis

At the end of the growth period, each plant was cut at soil level, weighed, rapidly frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Samples were later freeze-dried, ground to a fine powder and extracted under occasional vortex shaking for 24 h in MeOH-H<sub>2</sub>O-HOAc (70:27:3 v/v), followed by centrifugation and filtration. A 20  $\mu\text{l}$  sample was used for HPLC analysis.

Analytical HPLC was conducted using a Jasco PU-980 Intelligent HPLC solvent delivery system, Waters 994 programmable photodiode array detector, and a Gilson 234 autosampler. The column used was a Merck Supersphere LiChroCART 125-4 RP-18 endcapped (4  $\mu\text{m}$ , 4 mm  $\times$  119 mm) with a gradient solvent system comprising solvent A [1.5% H<sub>3</sub>PO<sub>4</sub>] and solvent B [HOAc-CH<sub>3</sub>CN-H<sub>3</sub>PO<sub>4</sub>-H<sub>2</sub>O (20:24:1.5:54.5 v/v)] mixed using a linear gradient starting with 80% A, decreasing to 33% A at 30 min, 10% A at 33 min and 0% at 39.3 min. Quercetin and kaempferol derivative peaks were identified on the basis of the on-line spectrum recorded for each identifiable peak. The total flavonol levels were calculated by adding the integrated areas of all flavonol peaks at 352 nm

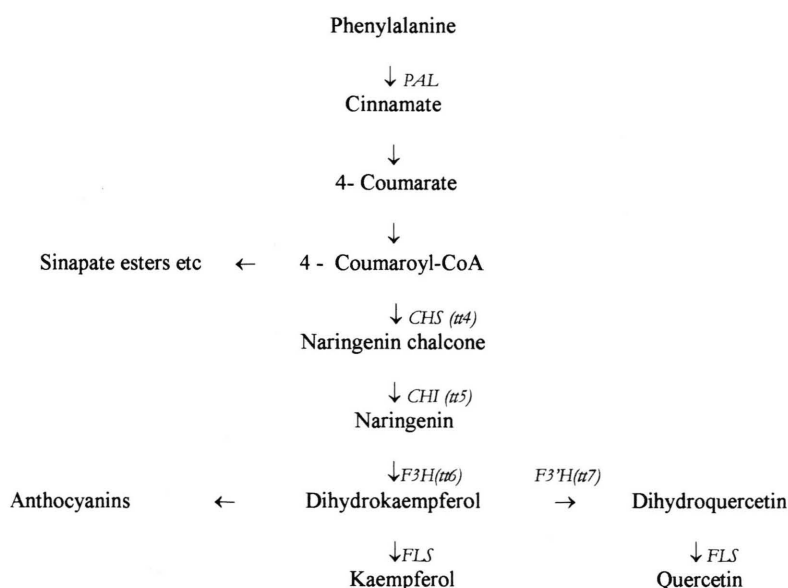


Fig. 1. Schematic diagram of part of the phenylpropanoid biosynthetic pathway. Relevant enzymes are indicated alongside the appropriate part of the pathway, and the mutant plants used in this study are indicated in brackets alongside the enzyme they lack. Abbreviations: PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3'H, flavanone 3 hydroxylase; F3'H, flavonoid 3' hydroxylase; FLS, flavonol synthase.

and the result compared to a standard curve prepared using rutin (quercetin-3-rutinoside) to calculate flavonoid levels in rutin equivalents. Naringenin was used as internal standard.

## Results

A simplified diagram of part of the phenylpropanoid pathway and the biosynthetic blocks characteristic of the various *Arabidopsis* mutants used in this study is given in Fig. 1. For references assigning mutations to genes, see Bharti and Khurana (1997) and Graham (1998). The pathway begins with the PAL enzyme. Plants mutant in each of the enzymes from CHS through to F3'H, were used in the current study.

Wild-type and mutant lines of *Arabidopsis* were grown under ambient solar radiation with reduced UVB radiation until they had established as small seedlings. Seedlings were then transferred to three different environments: ambient solar radiation, ambient solar radiation with reduced UVB, and ambient conditions with added UVB radiation. The ambient UV conditions during the study are illustrated in Fig. 2a, which are daily total UV data recorded at a site approximately 10km from our study site. UV dose varies from day to day due to differences in cloud cover.

### Effects of UVB on rate of increase in rosette width and biomass production

Plants grown in the presence of ambient or enhanced levels of UVB were generally smaller and less robust than were those grown in a low UVB environment. In support of this, our measurements indicate that the rate of increase in rosette diameter in wild-type plants was reduced under +UVB conditions. A typical plot of rosette width for wild type plants under -UVB treatment is given in Fig. 2b and a summary is given in Fig. 3. All four lines of *tt* mutants grown in this study had reduced (*tt4*, *tt6*, and *tt7*) or no (*tt5*) net change in rosette width under +UVB. Rosette widths even under -UVB were less than in the wild-type plants in most of the mutants, although *tt4* plants grew well in this environment. Despite the downward trend evident with increasing UVB in all the cultivars studied, none were statistically significant (Table I).

A comparison of the relative increase in dry weight on harvesting the samples is given in Fig. 4.

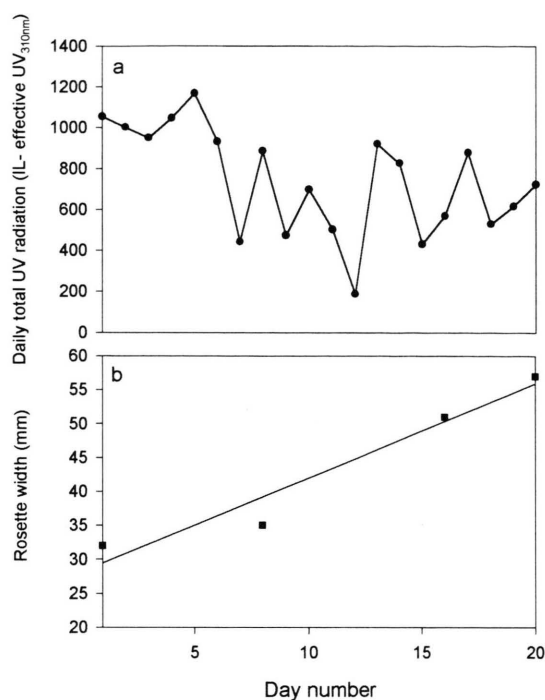


Fig. 2. a. Daily total UV irradiance (IL-effective UV<sub>310 nm</sub>) for the period of the study, recorded at a site 10km distant. Peaks represent days with little cloud cover, while low points are cloudy days. b. Linear regression fit to rosette widths recorded at intervals during the study on one individual Ler plant growing under -UVB conditions. Note that the plant is in the linear phase of growth. The slope of the regression was determined for each plant and a summary is given in Fig. 3.

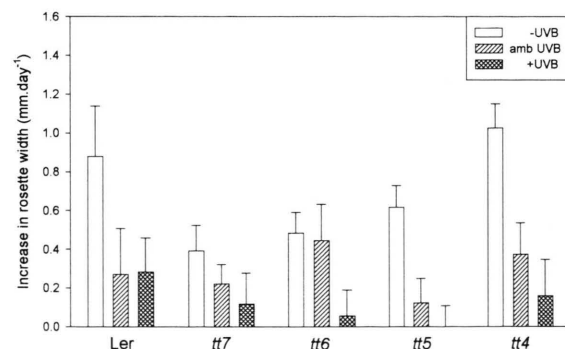


Fig. 3. Rate of increase in rosette width (estimated parameter *r* in equation 1) for each UVB treatment and plant variety. Values plotted are means and standard errors of the mean.



Table I: Values of *p* comparing low and ambient UVB (-UVB/amb), and low and enhanced UVB (-UVB/+UVB) for the same line.

Values of *p* higher than 0.1 are designated not significant (ns). Values of *p* less than or equal to 0.05 are statistically different and those less than 0.01 are highly significantly different. A comparison between ambient and enhanced UVB was also analysed and found to be not significantly different for any measurement, with the exception of fresh weight for *tt7* where *p* = 0.03.

	<i>Ler</i>		<i>tt7</i>		<i>tt6</i>		<i>tt5</i>		<i>tt4</i>	
	-UVB/ amb	-UVB/ +UVB	-UVB/ amb	-UVB/ +UVB	-UVB/ amb	-UVB/ +UVB	-UVB/ amb	-UVB/ +UVB	-UVB/ amb	-UVB/ +UVB
Biomass (dw)	ns	ns	ns	ns	ns	0.06	0.02	0.06	0.03	0.01
Biomass (fw)	ns	ns	ns	ns	ns	0.02	0.02	0.02	0.02	0.01
Rosette size	ns	ns	ns	ns	ns	0.03	0.02	0.07	0.01	0.003
Quercetin	0.03	0.04	—	—	0.0001	0.02	—	—	—	—
Kaempferol	ns	ns	0.02	0.0001	—	—	0.01	0.05	—	—
Q:K ratio	0.007	0.01	—	—	—	—	—	—	—	—
Total Flavonols	0.09	0.06	0.02	0.0001	0.0002	0.02	0.01	0.05	—	—

dw, fw = dry weight and fresh weight, respectively.

The initial dry weight could not be measured, as this would have involved harvesting the plants. An estimate was made by assuming that all plants with no increase in rosette width or seedling height during the study period represented plants that had no increase in dry weight during the study period (*n* = 14). The mean of these dry weights from all cultivars was used as the initial dry weight at the start of the study period. All mutants had lower yields than wild-type plants under +UVB conditions (Fig. 4), and this reflects the low to nil rosette growth-rates illustrated in Fig. 3. This trend was statistically significant at *p* < 0.05 (Table II). It is interesting to note that *tt4* and *tt5* mutants had higher yields than the wild-type plants under -UVB

treatments. Similar trends are evident in net fresh weights at harvest and all varieties showed reduction in fresh weight to dry weight ratio with increasing UVB treatment (data not shown).

#### Effects on flavonol levels

Dried samples were extracted and subjected to HPLC analysis. Two chromatograms are illustrated in Fig. 5, showing the changes in flavonol levels induced by UVB treatment. As noted by Graham (1998), the major flavonols produced in wild-type *Arabidopsis* are predominantly kaempferol glycosides along with a lower concentration of quercetin glycosides. Note that the two peaks of quercetin glycosides (*Q*<sub>1</sub> and *Q*<sub>2</sub>, Fig. 5) increased dramatically with UVB treatment. In addition there were two unidentified peaks (RT 14.4 and 18.5 min) with HPLC on-line absorption maxima at 330 nm. The shape of these absorption spectra indicates that these compounds are probably hydroxycinnamic acid derivatives (data not shown).

Wild-type *Arabidopsis* produced high levels of kaempferol glycosides per g dry weight of leaf material with lower amounts of quercetin glycosides (Fig. 6). The former are flavonols monohydroxylated on the B ring, and the latter are *ortho*-dihydroxylated. Approximately equal amounts of these two flavonols were additionally synthesised in the +UVB treated plants. Because the initial concentration of quercetin was low, the relative increase in quercetin concentration from 0.2 to

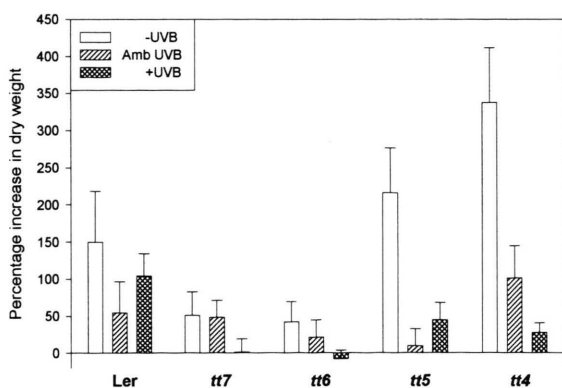


Fig. 4. Percentage increase in dry weight of whole plants (without roots) for each UVB treatment and plant variety. Values plotted are means and standard errors of the means.

Tabelle II: Values of *p* comparing different lines with Ler at the same UVB treatment. Values of *p* higher than 0.1 are designated not significant (ns). Values of *p* less than or equal to 0.05 are statistically different and those less than 0.01 are highly significantly different. Sample sizes for Ler were 5(-UVB), 4(amb), 6(+UVB).

	tt7			tt6			tt5			tt4		
	-UVB	amb	+UVB	-UVB	amb	+UVB	-UVB	amb	+UVB	-UVB	amb	+UVB
Biomass (dw)	ns	ns	0.02	ns	ns	0.01	ns	ns	ns	ns	ns	0.05
Biomass (fw)	ns	ns	0.04	ns	ns	0.05	ns	ns	ns	0.06	ns	ns
Rosette size	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Quercetin	—	—	—	0.05	0.03	0.06	—	—	—	—	—	—
Kaempferol	ns	0.06	0.002	0.003	0.02	0.003	0.004	0.02	0.003	—	—	—
Total flavonols	ns	ns	0.09	0.004	0.02	0.005	0.004	0.02	0.004	—	—	—
Sample size	5	5	8	5	6	6	6	4	4	5	8	7

dw, fw = dry weight and fresh weight, respectively.

1.7 mg; g<sup>-1</sup> dry weight (8.5×) (Fig. 6) was statistically significant at *p* < 0.05 (Table I). This relative increase was much greater than that of kaempferol, which increased from 3.1 to 4.9 mg; g<sup>-1</sup> dry weight (1.6×) in the same plants. Thus, although the absolute increase in each flavonol type was about the same, the ratio Q:K in wild-type plants increased from 0.07 under -UVB to 0.31 under +UVB. This fourfold increase was highly significant at *p* < 0.01 (Table I).

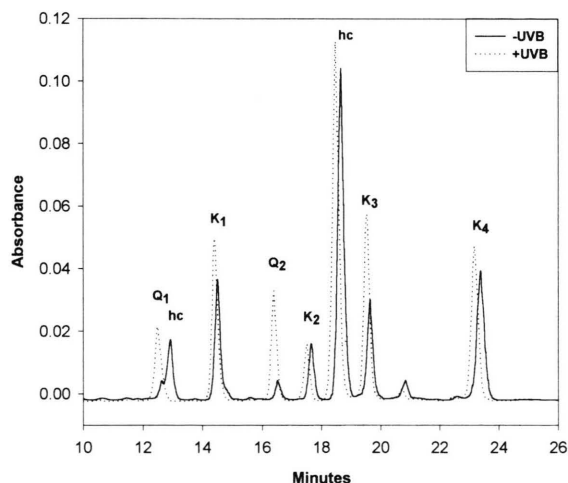


Fig. 5. Part of the HPLC spectra for wild-type (Ler) plants grown under -UVB and +UVB. Each spectrum was normalised to 25 mg dw of extracted material to enable direct comparisons. Peaks labelled Q<sub>1–2</sub> represent quercetin glycosides; K<sub>1–4</sub>, kaempferol glycosides; hc, hydroxycinnamic acid derivatives.

The total concentration of flavonols in the *tt7* mutant at the -UVB treatment was very similar to that in the wild-type plants, however, the leaves of these *tt7* plants exposed to +UVB accumulated almost 50% more kaempferol than similarly treated wild-type plants. This difference from the wild type was highly significant (Table II). Furthermore, no quercetin was detected following any treatment in this variety. Trace amounts of quercetin were detected in *tt6* mutants and trace amounts of kaempferol were detected in the *tt5* mutant, and there were increases in total flavonols in both mutants with increasing UVB, although

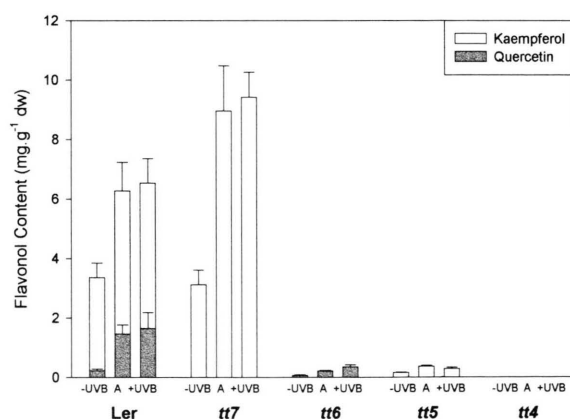


Fig. 6. Kaempferol and quercetin content in wild-type (Ler) and mutant plants grown under low, ambient and enhanced levels of UVB. The stacked bars show mean values with standard error of the mean. The upper limit of each error bar in Ler plants indicates the error associated with the kaempferol measurement.

the final levels under +UVB were still very low. No flavonols were detected in the *tt4* plants.

#### Effects on other compounds

The *tt6* mutant has been mapped to the F3H gene (Graham, 1998), and the substrate for this enzyme is naringenin. For this reason, the naringenin normally used as an internal standard was not included in a second HPLC run of the *tt6* samples. No naringenin peak was observed at the same retention time as the standard in *tt6* samples combined by treatment. However, a small peak was eluted at 17 min that has a similar absorption spectrum to naringenin and may be a naringenin glycoside. The amount of this compound increased with UVB treatment.

The *tt5* mutant has been mapped to the CHI gene (Shirley *et al.*, 1992), and the substrate for this enzyme is naringenin chalcone. Several samples were re run on the HPLC at the maximum absorbance wavelengths for chalcones (370 nm) rather than the 350 nm normally used to detect flavonoids, in order to detect chalcones. No peak representing naringenin chalcone or its derivatives was observed.

As noted above, several peaks with on-line spectra similar to those of hydroxycinnamic acid derivatives were observed in all HPLC profiles. In wild-type plants and in *tt7*, *tt6* and *tt5* mutants, the levels of hydroxycinnamic acid derivatives did not alter significantly with UVB treatment (Fig. 7). Insufficient HPLC data were recorded for *tt4* plants (CHS deficient mutants) to allow any conclusions to be drawn.

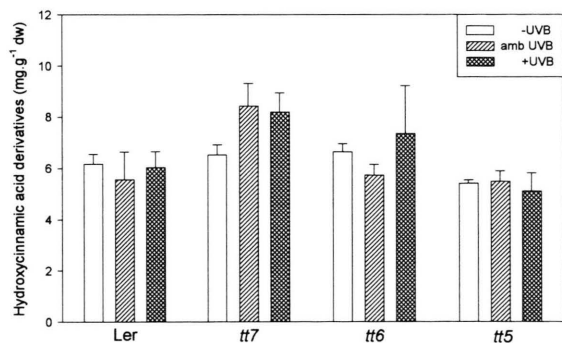


Fig. 7. Hydroxycinnamic acid derivatives content in wild-type (Ler) and mutant plants grown under low, ambient and enhanced levels of UVB. Values shown are mean with standard error of the mean.

## Discussion

### Physiological studies

Previous studies have shown that *Arabidopsis tt4*, and *tt5* mutants are smaller and less vigorous than wild type plants when grown under enhanced levels of UVB (Li *et al.*, 1993). Based on these visual assessments, Li *et al.* (1993) rank susceptibility to UVB in the order *tt5* > *tt4* > wild-type. These authors also quote unpublished results demonstrating that *tt6* is similar in UVB response to *tt5*. Our morphological observations and the data of Fig. 3 and 4, and Table I confirm these rankings, although the differences observed in the present study were not as severe as those illustrated in Li *et al.* (1993).

This lack of extremes is a common difference between experiments conducted under artificial radiation sources and those carried out under solar radiation as in our study (Fiscus and Booker, 1995). In external modulated systems such as ours, daily UVB doses fluctuate due to variations in cloud cover (see Fig. 2a), and periods of low dose may allow some degree of recovery or repair. External systems offer considerable advantage as far as ecological relevance is concerned, but artificial systems allow more controlled conditions. Once ecologically relevant doses are employed, the drastic reductions in growth rates observed in artificial irradiation systems tend not to occur (Fiscus and Booker, 1995). Thus although others have shown that wild-type *Arabidopsis* is susceptible to UVB (Li *et al.*, 1993; Lois, 1994), the reductions in rosette expansion and biomass accumulation evident in our wild-type plants were not statistically significant.

We are not aware of any previous analysis of UVB effects on *tt7* mutants. As with wild-type plants, the rates of rosette width expansion and biomass accumulation tended to decrease with UVB in these plants, but these decreases were not statistically significant. In *tt4*, *tt5* and *tt6*, either one or both of these downward trends were statistically significant (Table I). These results indicate at least that *tt7* and Ler are less susceptible to UVB than the other *tt* mutants. Further, as can be seen in Fig. 4, there was essentially no increase in dry weight in *tt7* plants under +UVB treatment, while the net dry weight of wild-type plants increased by about 100%. This difference was statistically sig-

nificant ( $p = 0.02$ , Table II), and this indicates that the *tt7* cultivar is more susceptible to UVB than the wild-type plants.

### Flavonoid analysis

As has been noted with previous species (Ryan *et al.*, 1998; Olssen *et al.*, 1998), wild-type *Arabidopsis* plants produced increased quantities of flavonoids per gram dry weight of leaf material with increasing UVB treatment. Two classes of flavonol glycosides were identified in wild-type plants by HPLC: the B-ring monohydroxylated kaempferol and the *ortho*-dihydroxylated quercetin. The absolute concentration of each flavonol increased by about the same amount with UVB treatment. However, since the initial levels of quercetin were low in plants under -UVB radiation, the increase in Q: K ratio with UVB was quite dramatic and highly significant (Table I). These results are consistent with previously published data (Ryan *et al.*, 1998; Markham *et al.*, 1997, 1998), and emphasise the important role that *ortho*-dihydroxylated flavonoids play in the protection of plants from UVB.

The *tt7* mutant is defective in the F3'H gene (Koornneef, 1982,1990) and should not be able to synthesise dihydroquercetin, the biosynthetic precursor of quercetin (see Fig. 1). Indeed, the only flavonol glycosides found in this variety were kaempferol (Fig. 6). While the total level of flavonols in *tt7* plants treated with -UVB was about the same as in wild-type plants, the total levels in plants exposed to ambient and enhanced levels of UVB were about 50% higher than in the wild-type. Despite this increased level, these mutant plants were still more susceptible to +UVB than similarly treated wild-type plants (Table II, biomass entries). These observations emphasise the beneficial effect of quercetin in the protection of plants from UVB. Smith and Markham (1998) have suggested that *ortho*-dihydroxylated flavonoids may be better able to harmlessly dissipate the energy of UVB photons than their monohydroxylated equivalents. It is likely that the increase in *ortho*-dihydroxylated flavonols in *Arabidopsis* (and other plants) is more related to their antioxidant, free radical quenching, or energy dissipating properties than to their UVB screening properties alone. This is also supported by the fact that the

absorption spectra of quercetin and kaempferol differ very little in the UVB region. Sheahan and Cheong (1998) discuss the different antioxidant properties of flavonols in *Arabidopsis* and note increasing efficacy with increasing hydroxylation of the flavonol B-ring, but were unable to confirm their role as antioxidants.

### *tt6* mutants

Graham (1998) has assigned *tt6* to the F3H enzyme and these plants should accumulate of naringenin and lack flavones (see Fig. 1). Our HPLC studies detected a small peak of an unknown compound in this mutant that increased with UVB dose. The absorption profile of this compound is consistent with it being a naringenin glycoside. In addition, small amounts of a mixture of quercetin glycosides were detected in this mutant, and these increased with UVB treatment.

### *tt5* mutants

The *tt5* mutant has been mapped to the CHI enzyme (Shirley *et al.*, 1992), and should therefore accumulate naringenin chalcone or its derivatives (see Fig. 1). However, our HPLC chromatograms did not contain any peaks representing chalcones, even when analysed using HPLC detection at their absorbance maximum of ca 370 nm. While Shirley *et al.* (1995) note that CHS activity is reduced from that in the wild-type, sufficient quantities of this enzyme should have been present for some chalcones to be detected in our studies. The reason for this lack is not known.

If sinapate esters are important in the protection of plants from UVB (Sheahan, 1996) then it is reasonable to expect them to increase in concentration with UVB treatment. However, there was no significant increase in the levels of hydroxycinnamic acid derivatives with UVB treatment in wild-type plants nor in the mutants (Fig. 7). Based on the observations of Li *et al.* (1993) on the same mutant lines, these derivatives are probably sinapate esters. These observations suggest that the role of sinapate esters in UVB protection may be subordinate to that of flavonoids.



*tt4* mutants

The *tt4* mutant had no detectable levels of flavonols under all three treatments (Fig. 6). These plants were sensitive to UVB and there was a highly significant reduction in the rate of increase in rosette width once plants were placed in the +UVB environment (Table I). Interestingly, under the -UVB treatment, the rosette and biomass measurements of *tt4* plants were higher than in all of the other varieties studied, including the wild-type. While neither parameter was statistically different

from the wild type at this treatment, they were highly significantly different from *tt7* and *tt6* ( $p \leq 0.01$  in all cases, analysis not shown). This may indicate that these plants will grow well under low levels of stress, and in this manner are similar to the clover varieties which were bred for high yield but were sensitive to UVB (Hoffmann *et al.*, 2000).

## Acknowledgments

We thank the New Zealand Foundation for Research Science and Technology for funding this research (Contract no CO 8804)

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