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Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves

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Abstract

The effects of UVB radiation on plant growth rate, gene expression and flavonoid content in wild-type, and in transgenic and mutant F3′H deficient *Petunia* lines have been studied for the first time. In wild-type *Petunia*, UVB induced an increase in total levels of flavonols and this was due to an up-regulation of several genes in the phenylpropanoid pathway. Furthermore, UVB induced a higher rate of production of dihydroxylated flavonols than mono-hydroxylated equivalents. Thus, the ratio of quercetin (*ortho*-dihydroxylated) to kaempferol (monohydroxylated) increased. In the F3H deficient mutant line, increasing UVB resulted in up-regulation of all of the basic flavonoid biosynthetic genes. Total flavonoids increased to levels significantly higher than in control plants, and the predominant flavonoid was kaempferol. The leaves of these plants grew at a significantly slower rate than comparably treated wild-type plants under ambient or enhanced UVB radiation. This suggests that the predominance of quercetin in the wild-type confers a protective advantage that is not matched in the mutant, even with higher overall flavonoid levels. In contrast, the antisense F3H construct produced an unexpected down-regulation of C4H, CHS and CHI transcription. This resulted in lower total flavonoid production in these plants. The growth rate of these plants was not impaired in UVB to a statistically significant extent, and the Q:K ratio did not change with increasing UVB radiation. This investigation has established a likely correlation between the effect of UVB on plant growth rate, the level of activity of the F3′H gene, and the proposed photoprotection afforded by an increased Q:K ratio.

Keywords: *Petunia axillaris* × (*P. axillaris* × *P. hybrida)*; Solanaceae; Mitchell Petunia; UVB; Ultraviolet; Transgenic; Mutant; Flavonoids; Kaempferol; Quercetin; Photoprotection

1. Introduction

Depletion of stratospheric ozone in polar latitudes may produce increases in biologically harmful solar UVB radiation (280–320 nm) over temperate and tropical regions (Madronich et al., 1998). Many plants grow poorly under enhanced levels of UVB flux (Caldwell et al., 1998) although different species and even different varieties (Hofmann et al., 2000) vary in their sensitivity. The up-regulation of the genes of the general phenylpropanoid pathway is a common response to a number of environmental stresses in plants including UVB (Daugherty et al., 1994). This pathway produces phenolic compounds including flavonoids, tannins and hydroxycinnamic acid derivatives. UVB treatment therefore often results in a dramatic increase in the concentration of flavonoid compounds within leaves and flowers.

Flavonoids absorb strongly in the UVB region of the solar spectrum, and there is good evidence that they protect plants from the effects of UVB radiation by acting as a simple sunscreen (Jordan, 1996; Reuber, et al., 1996). For example, in *Brassica napus,* flavonoids reduce UVB induced degradation of the D1 photosystem II reaction centre (Wilson and Greenberg, 1993), and in *Arabidopsis*, plants lacking flavonoids have enhanced UVB injury and oxidative damage (Landrey et al., 1995). However, while this protective screening function is important, recent studies have shown that flavonoids with a higher level of B-ring hydroxylation are preferentially synthesised following UVB treatment (Markham et al., 1997; Olsson et al., 1998; Ryan et al., 1998). Yet both monohydroxylated and *ortho*-dihydroxylated compounds have similar UVB absorption

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profiles, and in fact, the UVB absorption decreases slightly as hydroxylation increases (Lavola et al., 1997). These observations suggest a more complicated role for flavonoids in plant UVB protection than simply a sunscreen function.

Ortho-dihydroxylated flavonoids are more effective free radical scavengers/antioxidants than their mono-hydroxylated equivalents (Montesinos et al., 1995), and we have suggested (Ryan et al., 1998) that this may account for their enhanced biosynthesis in plants under UV stress. They may also be better able to dissipate absorbed UV energy (Smith and Markham, 1998). A crucial component of the plant's protection mechanism against UVB damage therefore may be the biosynthetic conversion of B-ring mono-hydroxylated flavonoids to their o*rtho*-dihydroxylated equivalents. This conversion is catalysed by the cytochrome P_{450} enzyme, flavonoid 3′hydroxylase (F3′H) (Graham, 1998). In *Petunia* leaves, the relevant compounds are glycosides of the flavonols, kaempferol and quercetin, respectively.

We have chosen two *Petunia* plant lines mutant or modified at the F3′H locus. The antisense F3H construct (Lewis et al., 2001) was expected to produce sufficient exogenous transcript to block or reduce the production of the endogenous F3′H mRNA, and thus result in leaves with reduced levels of quercetin glycosides. The mutant line *ht1,* is an almost complete null mutation of the F3′H gene and should not produce quercetin (Brugliera et al., 1999). Depending on the amount of quercetin loss, these lines should provide a gradient of F3H removal. An increase in susceptibility to UVB would confirm a specific protectant role for *ortho*-dihydroxylated flavonols.

2. Results

A simplified diagram of part of the phenylpropanoid pathway is given in Fig. 1. The pathway begins with the phenylalanine ammonia lyase (PAL) enzyme. The plants used in this study were modified in the F3′H enzyme, which converts dihydrokaempferol to dihydroquercetin.

2.1. Effects of UVB treatment on flavonol levels

Petunia plants were grown in an outdoor plastic-covered chamber which admitted natural solar radiation at slightly reduced intensity (see Ryan and Ireland, 1997). Ambient solar UVB radiation is recorded at a site approximately 10km away as part of a national network of UVB monitoring (Ryan et al., 1996). This data is displayed for the relevant periods of plant growth in

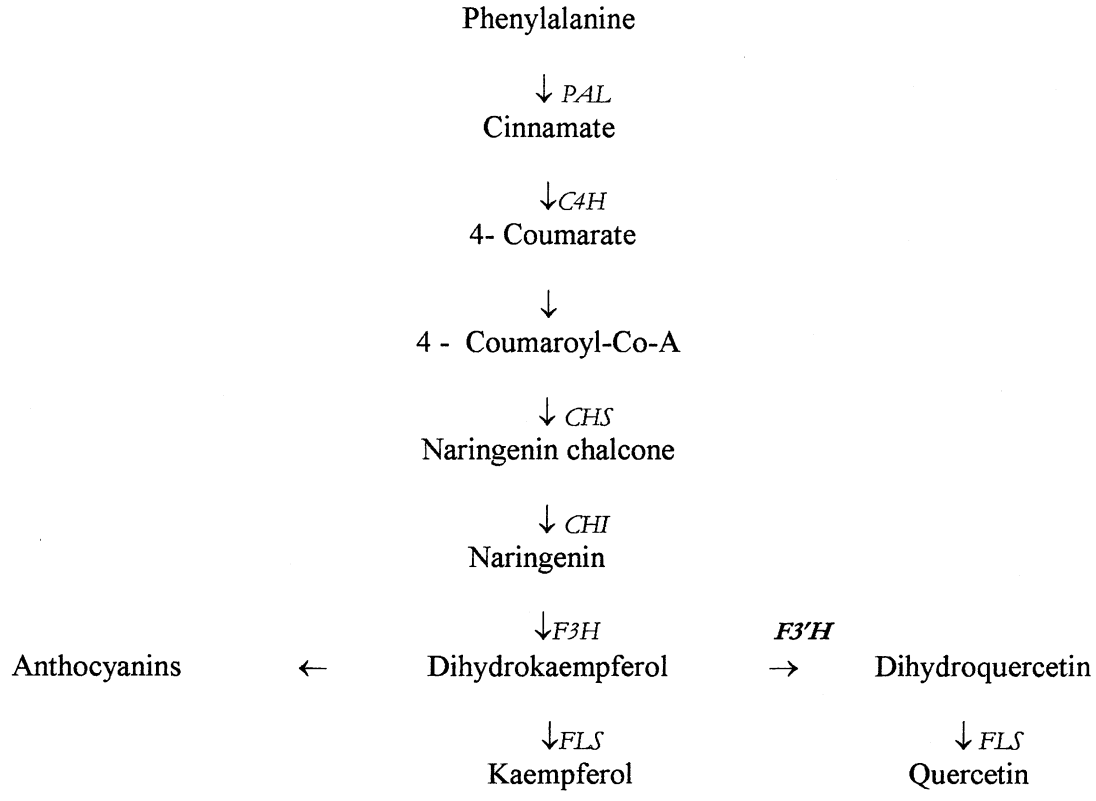

Fig. 1. Schematic diagram of part of the phenylpropanoid biosynthetic pathway. Relevant enzymes are indicated alongside the appropriate part of the pathway. Abbreviations: PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3 hydroxylase; F3′H, flavonoid 3′ hydroxylase; FLS, flavonol synthase.

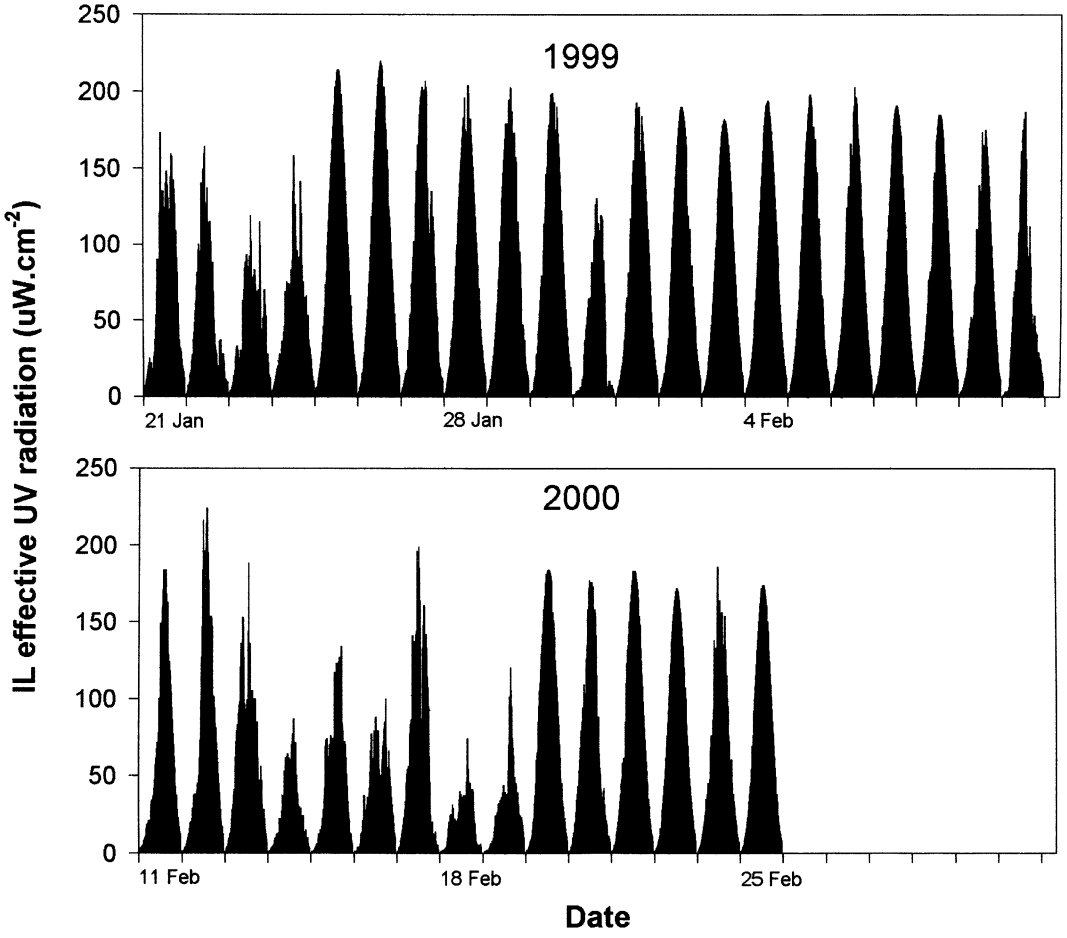

Fig. 2. IL-effective solar UV radiation recorded in Wellington during the experimental periods. Data are recorded as 10 min averages throughout the day. A smooth bell shaped profile of ambient UVB indicates a clear cloudless day, with a maximum at solar noon. Lower levels of UVB radiation occur in the early morning and late afternoon. Low, irregular profiles reflect days of varying cloud cover.

Fig. 2. As can be seen in these profiles UVB reaches a maximum at mid-day and is very low in the early mornings and late afternoons. The influence of cloud cover on solar UVB flux is considerable (e.g. 18 Feb 2000, Fig. 2). Plants were exposed to low UVB (−UVB), ambient UVB and enhanced UVB (+UVB). HPLC analysis showed that wild-type *Petunia* produced increased levels of flavonols per g dry weight of leaf material with increasing UVB treatment in both experiments (Fig. 3a, 1999; and Fig. 3b, 2000). These increases were

Table 1
Results from a statistical analysis comparing −UVB and ambient UVB, and −UVB and +UVB for the same line[a]

	Wild type		Transgenic as-F3′H		Wild Type		Mutant F3′H	
	−UVB/amb	−UVB/+UVB	−UVB/amb	−UVB/+UVB	−UVB/amb	−UVB/+UVB	−UVB/amb	−UVB/+UVB
Total flavonoids	**	***	**	**	**	***	**	***
Quercetin	**	***	**	**	**	***	***	**
Kaempferol	ns	*	**	**	*	*	*	**
Q:K Ratio	*	**	ns	ns	*	*	ns	*
Leaf size	ns	**	ns	ns	ns	ns	ns	ns

[a] Values of P less than 0.05 are significantly different (*) and those less than 0.01 (**) are highly significantly different. ns, not significantly different. A comparison between ambient and +UVB was also analysed and found to be not significantly different for any measurement, with the exception of total flavonoids and quercetin for the wildtype controls in the transgenic experiment. The level of significance was $P<0.05$ in both cases.

* $0.05>P>0.01$.

** $0.01>P>0.001$.

*** $0.001>P$.

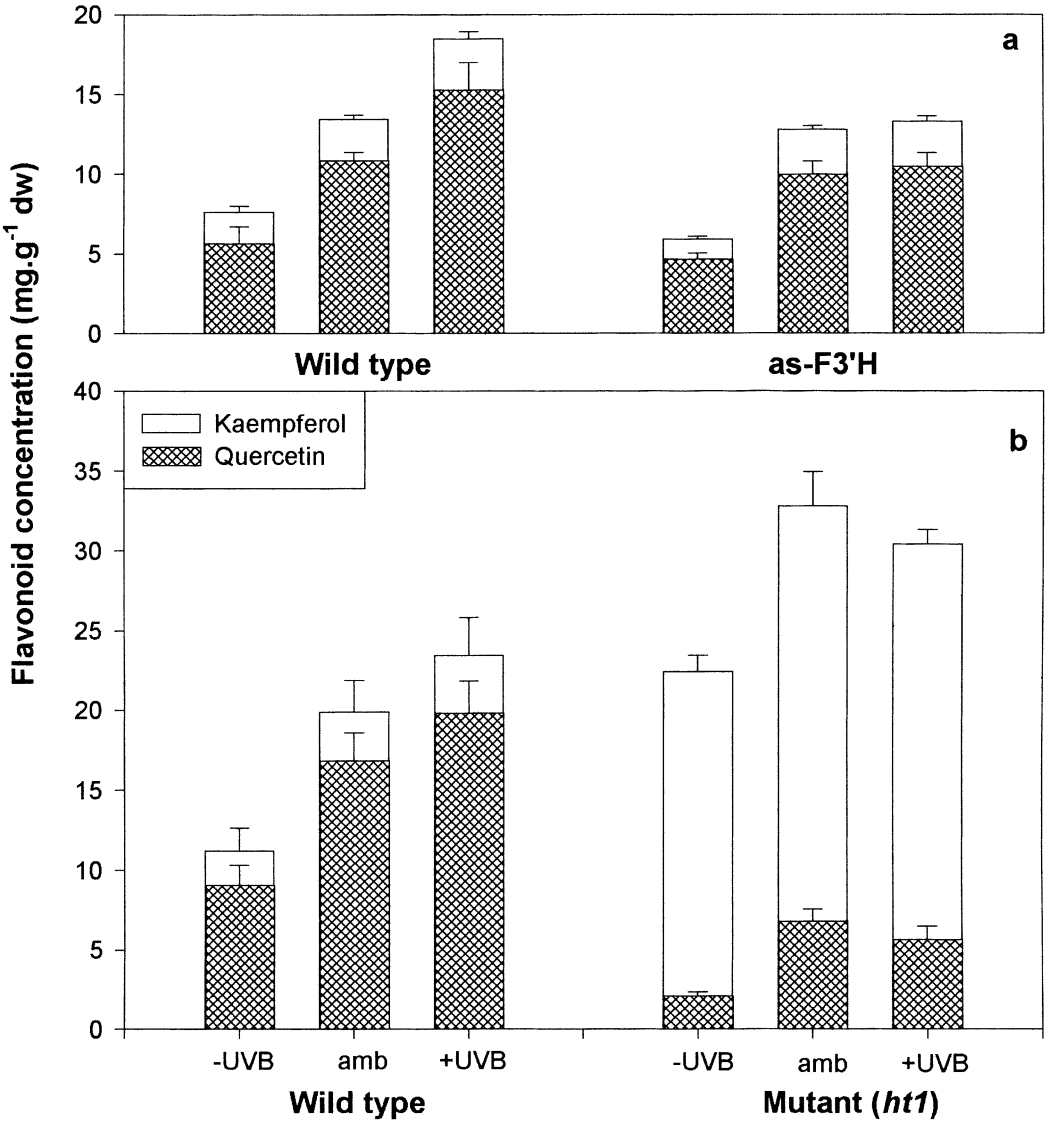

Fig. 3. Kaempferol and quercetin content in wild-type and transgenic as-F3′H plants (a), and in wild-type and mutant plants lacking the F3′H enzyme (b), grown under −UVB, ambient, and +UVB. The stacked bars show mean values with standard error of the mean.

highly significant ($P<0.01$, Table 1). The flavonoids detected were glycosides of the two flavonols, quercetin and kaempferol. In both experiments, most of the additional flavonols produced in wild-type plants after UVB treatment were quercetin glycosides ($P<0.01$, Table 1). There was an increase in kaempferol as well ($P<0.05$), although this was at a lower rate. The resulting ratio of quercetin to kaempferol (Q:K) increased with UVB treatment ($P<0.05$).

Total flavonoids also increased with UVB treatment in as-F3′H plants (Fig. 3a) and these increases were highly significant ($P<0.01$, Table 1). In contrast to wild-type plants, the two flavonoids were synthesised in the same proportion in all three treatments, and so the Q:K ratio did not change with UVB treatment in antisense plants. The as-F3′H plants had lower levels of total flavonoids than wild-type plants in all three treatments, and this trend was significant at +UVB ($P=0.05$, Table 2).

Mutant *ht1* plants contained very high levels of flavonoids, even under-UVB treatment (Fig. 3b). The increase from −UVB to +UVB was highly significant ($P<0.01$, Table 1). After +UVB treatment these plants exhibited the highest levels of flavonoid we have ever recorded in *Petunia* plants, and these were dramatically different from wild-type plants in all parameters measured ($P<0.02$ in all cases, Table 2). The predominant flavonoid in these plants was kaempferol, not quercetin, and the Q:K ratios were therefore very low.

2.2. Effects of UVB treatment on transcripts in the transgenic as-F3′H line

The relative transcript levels of the flavonoid biosynthetic genes PAL, C4H, CHS, CHI and FLS were compared between wild-type and the transgenic plant line

Table 2
Results from a statistical analysis comparing different lines at the same UVB treatment[a]

	Wild type—transgenic as-F3′H			Wild type—mutant F3′H		
	−UVB	Ambient	+UVB	−UVB	Ambient	+UVB
Total flavonoids	ns	ns	*	***	***	*
Quercetin	ns	ns	*	***	***	***
Kaempferol	.1	ns	ns	***	***	***
Q:K ratio	ns	.1	**	***	***	***
Leaf size	ns	ns	ns	ns	**	**

[a] Values of P less than 0.05 are statistically different (*) and those less than 0.01 (**) are highly significantly different. ns: not significantly different.
* $0.05 > P > 0.01$.
** $0.01 > P > 0.001$.
*** $0.001 > P$.

containing antisense copies of the F3′H gene (see Fig. 1 for abbreviations). In the wild-type plants, all genes analysed were up-regulated after +UVB treatment. There was no discernable difference in the expression levels of PAL between transgenic lines and wild-type *Petunia* (Fig. 4). Similar observations were made for FLS (data not shown). However, in the transgenic plants, expression of C4H, CHS and to some extent CHI was noticeably down-regulated relative to wild-type under all three UVB treatments. At the levels of total RNA loading in Fig. 4 (10 μg) the F3′H probe did not detect any transcript in either the wild type or the antisense mutant and so this lane is not shown.

2.3. Effects of UVB treatment on transcripts in mutant F3′H line (ht1)

The same genes were also probed in mutant F3′H plants (Fig. 5). In this series the wildtype +UVB lane was slightly overloaded (see control blots), although this has had little impact on the results. These results indicate some up regulation in a +UVB environment of the PAL, CHS (Fig. 5) and CHI (data not shown) transcripts in both the mutant and wildtype lines. No trend was evident for the C4H enzyme (data not shown). These observations are consistent with the observed increases in flavonoid levels in both lines as determined by HPLC analysis.

There was no production of the F3′H transcript in the mutant plants, even though these blots have 3× more RNA (30 μg) than the other Northerns illustrated. This extra loading was required because of the very low levels of expression of this gene in leaves. The lack of expression of the F3′H gene in the mutant plants is consistent

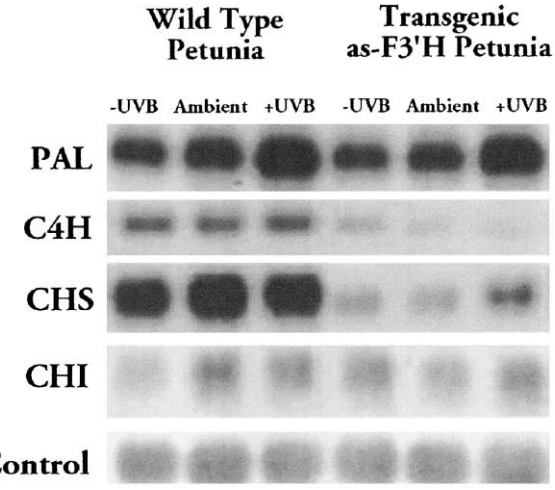

Fig. 4. Gel electrophoresis (Northern Analysis) of total RNA of wild-type and transgenic as-F3′H plants after exposure to −UVB, ambient and +UVB radiation. The control blots were probed with a ribosomal RNA probe to indicate loading levels.

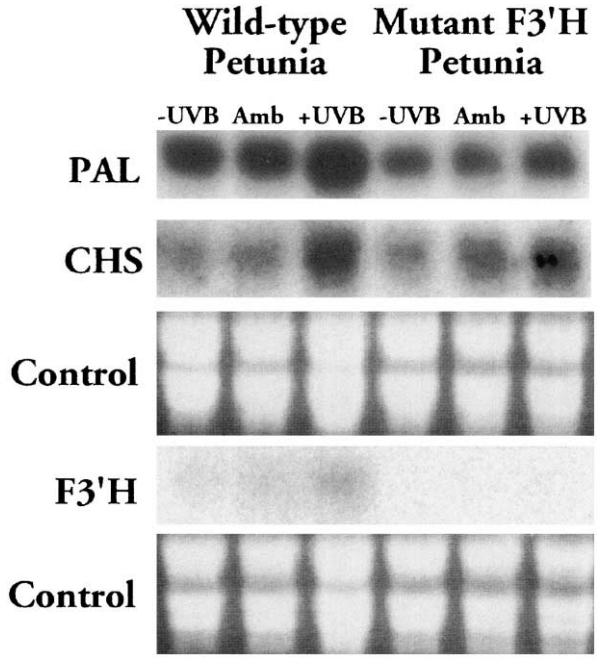

Fig. 5. Gel electrophoresis (Northern Analysis) of total RNA of wild-type and mutant plants lacking the F3′H enzyme after exposure to −UVB, ambient and +UVB radiation. The control blots were stained with ethidium bromide to indicate loading of RNA for each experiment.

with the low levels of quercetin found in these samples by HPLC.

2.4. Effects of UVB treatment on growth rates

Wild-type, transgenic and mutant lines of *Petunia* were grown in the −UVB part of the growth chamber, until they had established as small seedlings. They were then transferred to the three different environments. The growth rate of both the *as*-F3′H and the wild-type plants declined with increasing UVB treatment (Fig. 6a). This downward trend was statistically significant at the $P<0.05$ level between −UVB and +UVB for wild-type plants but not for *as*-F3′H (Table 1). There was no statistically significant effect of UVB on growth rate in wild-type or mutant plants in the 2000 experiment (Fig. 6b, Table 1). However, mutant plants grew significantly slower than wild-type plants when the two varieties were compared at the same treatment (Table 2). At ambient UVB levels, the level of significance was $P<0.01$ while at +UVB, $P<0.05$.

3. Discussion

In outdoor modulated irradiance systems such as ours, daily UVB doses vary due to differences in cloudiness. The applied UVB dose in our chamber is set to provide a constant enhancement over ambient conditions. It is likely that periods of low dose such as are illustrated in Fig. 2 for 2000 may allow for some degree of recovery or repair. In order to minimise this, samples were collected after a period of clear days in both experiments. In most indoor growth chambers using artificial illumination, the ratio of UVB:UVA:visible radiation bears little resemblance to that in natural solar radiation. Furthermore, the UVB dose is often applied at a constant rate for several hours about noon, and doses are often excessive

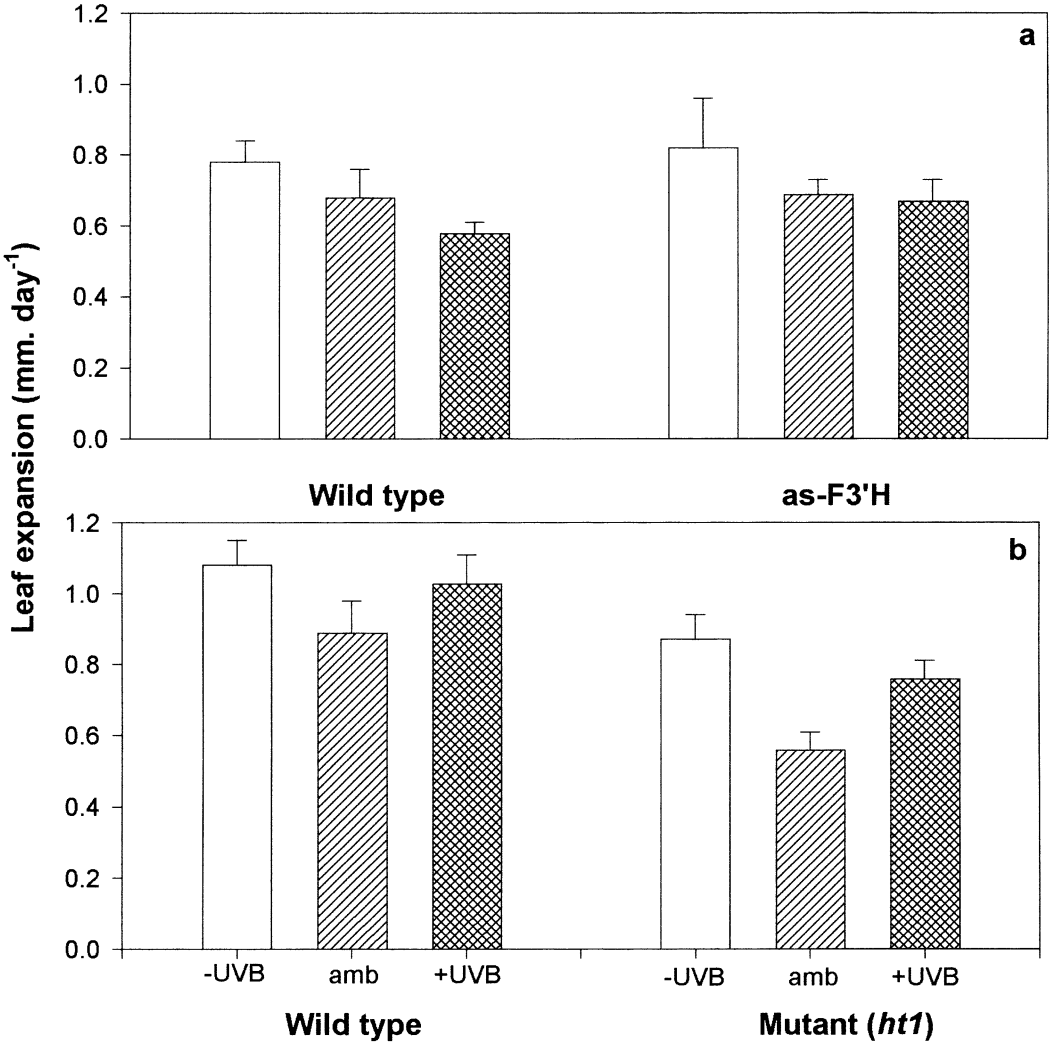

Fig. 6. Increase in leaf width [estimated parameter r in Eq. (1)] for wild-type and transgenic as-F3′H plants (a) and for wild-type and mutant plants lacking F3′H (b) after exposure to −UVB, ambient and +UVB radiation. Values are mean and standard error of the mean.

(Fiscus and Booker, 1995). Plants are adapted to and respond to natural solar UVB radiation that varies constantly during the day (see profiles in Fig. 2). Estimates of UVB sensitivity based on indoor growth chambers using unnatural and/or excessive artificial UV and visible irradiances are therefore often misleading (Fiscus and Booker, 1995).

The downward trend in growth rate of wild-type plants with increasing UVB treatment (Fig. 6) is consistent with previous observations (Markham et al., 1997, 1998; Ryan et al., 1998, 2001). The rate of leaf expansion in wild-type plants in the 2000 experiment was slightly higher than in 1999 (Fig. 6), and there was no difference between treatments in the former. Both of these different rates may be accounted for by examining the UVB readings shown in Fig. 2. These show a sustained period of low UVB during the first half of the 2000 experiment. In the 1999 experiment, UVB levels were higher throughout the growing period. Lower levels of UVB in the early stages of the 2000 experiment would account both for higher growth rates than in 1999, and for little difference between treatments. Flavonoid content responds relatively quickly to ambient UVB conditions, even over a period of hours (Veit, 1996). Consequently, our measurements of flavonoid content, reflect the UVB conditions just prior to harvest, where in both experiments, UVB levels had been at normal mid-summer levels.

In wild-type *Petunia*, UVB induced an up-regulation of genes coding for key enzymes in the phenylpropanoid pathway including PAL, C4H, CHS and CHI. (Figs. 4 and 5). This pattern of expression was observed in both experiments (Figs. 4 and 5), and is consistent with the increase in flavonoid concentration with UVB treatment. In addition, there was up-regulation of the F3′H gene with UVB treatment (Fig. 5). This resulted in a higher rate of production of quercetin glycosides than of kaempferol glycosides, and a consequent increase in the ratio of quercetin to kaempferol.

Transgenic plants transformed with the antisense F3′H constructs grew at a rate similar to wild-type plants. However, northern blot analysis revealed that there was down-regulation of CH4 and CHS upon UVB treatment compared to wild-type plants. As a result, total flavonoid production was lower than in wild-type plants, and statistical analysis revealed that this was significant in the +UVB treatment (Table 2). Although we could not demonstrate it in our Northern blots due to a low level of total RNA loading, the transgenic line used here does express the antisense transgene (Lewis et al., 2001). In the latter study, increasing levels of transcript expression correlated well with loss of F3′H activity among several as-F3′H lines examined. In each of these lines there were multiple copies (greater than three independent insertion events per transgenic line) of the transgene transferred to the plants (D. Lewis, pers. commun.).

The Q:K ratio in the *as*-F3′H plants remained the same regardless of UVB treatment. This was in contrast to the situation in the wild-type where Q:K increased. Thus after +UVB treatment, the *as*-F3′H Q:K ratio was significantly lower than the wild-type control ($P<0.01$, Table 2). These results were unexpected, because the exogenous mRNA should have blocked the expression of the endogenous gene and the levels of quercetin should have been markedly reduced. It is clear that some influence has been exerted on quercetin production because the Q:K ratio did not increase with UVB. The reason for this result may be that there was insufficient expression of the exogenous construct in these plants to effectively block the expression of the endogenous gene, and its influence was incomplete. Other plant lines with different antisense F3′H constructs were also grown in this study (data not shown). All had lower levels of total flavonoids than the wild-type, and in all cases there was down-regulation of C4H and CHS, while the expression of CHI varied. (see Lewis et al., 2001). Each antisense line had a unique Q:K ratio, and in most lines studied, this ratio did not change significantly with UVB treatment (data not shown). These observations indicate that each antisense line has a unique number of copies of the exogenous construct, and each responds to UV to a greater or lesser extent.

The reasons behind the unexpected alterations of transcript abundance for C4H, CHS and CHI are unclear. As the antisense F3′H construct was made using the entire cDNA it is possible that conserved regions between the cytP450s F3′H and C4H has resulted in an inappropriate downregulation of C4H transcript through an antisense mediated mechanism. However, this does not account for the alterations in transcript abundance observed for either CHS or CHI. It is possible that through alteration of F3′H enzyme activity in the transgenic plants a feedback mechanism has been activated that results in the observable changes in transcript abundance for C4H, CHS, and CHI. Blount et al. (2000) have proposed that C4H is a critical step regulating flavonoid metabolism through its action on PAL, suggesting that feedback loops between post-translational and translational control mechanisms may exist for the flavonoid pathway. Further, the levels of a single member of the pathway may have an observable effect on levels of other biosynthetic enzymes, as in *Arabidopsis* (Pelletier et al., 1999). There may be a biosynthetic feedback mechanism (product inhibition, substrate inhibition) in the antisense mutants which affects these enzymes, perhaps by signalling back to the regulatory side of the pathway but, if this were so, then we would expect similar effects in the mutant, and this was not seen (Fig. 5).

Mutant plants lacking this F3′H gene were grown under similar UV conditions. Under +UVB radiation, mutant plants grew at a rate significantly less than

comparably treated wild-type plants. As in wild-type plants, all transcripts of the flavonoid pathway were up regulated, and total flavonoids therefore increased to high levels. Flavonoid levels were significantly higher than in control plants, and since little or no F3′H transcript was produced in the leaves of this mutant (Brugliera et al., 1999), kaempferol was predominant. The low levels of quercetin even in the −UVB treatment indicate that this variety may produce some quercetin via an alternative pathway, or that there may be some low level non-enzymatic conversion. Despite the fact that the mutant plants had high total concentrations of flavonol glycosides, they still grew at a significantly slower rate than wild-type plants exposed to the same UVB treatments (Fig. 6b, Table 2). These observations suggest that the predominance of quercetin in the wild-type, confers a protective advantage that is not matched in the mutant, even with higher overall flavonol levels. This advantage is thought to be related to the higher antioxidant or energy dissipating capacity of quercetin (Montesinos et al., 1995; Smith and Markham, 1998; Ryan et al., 2001).

A similar observation has also been made by Olsson et al. (1998), in commenting on the enhanced level of B-ring *ortho*-dihydroxylated flavonoids in *Brassica napus* following UVB irradiation. They noted that the vacuolar location of flavonoids was far from ideal for their purported protective antioxidant function. They suggested that there might be undetected pools of flavonoids in the cytoplasm. This has in fact recently been demonstrated, at least in flower petals. In lisianthus (*Eustoma grandiflorum*) petals, around 14% of the petal flavonoids were shown to reside within the cytoplasm (Markham et al., 2001). Flavonoids are synthesised in the cytoplasm (Winkel-Shirley, 1999) and when retained there, are well sited to interact with reactive oxygen species generated therein. Furthermore, their function as antioxidants would be enhanced markedly in the near neutral pH of the cytoplasm compared with the acidic vacuole (Davies, 1997). Of possible importance also is that immediately following UVB irradiation, the newly synthesised flavonoids would be predominantly of the more effective *ortho*-dihydroxylated type (i.e. quercetin in *Petunia*). A vacuolar location of flavonoids however, would present no difficulties for the alternative (or additional) protective role, that of effective dissipation of absorbed UV energy. Indeed, it can be argued that an epidermal vacuolar location is ideal.

Through an investigation for the first time of both mutant and transgenic *Petunia* lines deficient in the F3′H enzyme, the present study has highlighted the relationship between UVB, plant growth rates, gene expression and flavonoid content. These findings provide valuable insights into the biochemical response of plants to UV stress, which results in the production of a more protective flavonoid profile.

4. Experimental

4.1. General experimental procedures

The outdoor plastic covered chamber was divided into three sections providing +UVB, ambient UVB, and very low UVB. The cladding material was transparent to solar UVB in the +UVB and ambient regions (cladding material teflon FEP), and opaque to UVB in the −UVB section. The −UVB region was clad in Mylar, which does allow a trace of UVB to penetrate into the chamber. The levels of UVB were approximately 5% of ambient in this part of the chamber. 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 level of enhancement was set to provide UVB radiation approximately 25% over ambient at this time of the year.

Solar UVB radiation was recorded continuously using Actinic UV radiometers consisting of a diffuser, an interference filter and a solar blind phototube (type SED240/ACTS270/W International Lingt Inc., Newburyport, MA, USA). Calibration procedures etc are described in Ryan et al. (1996).

4.2. Plant material

Wild-type Mitchell *Petunia* [*Petunia axillaris* × (*P. axillaris* × *P. hybrida*] seed was obtained from the University of Auckland, New Zealand. The antisense line, 114e/17096 was produced and analysed as described in Lewis et al. (2001). The mutant line *ht1* originated from INRA (France) and was kindly provided by F Brugliera of Florigene. In the first experiment (1999), seedlings of wild-type and as-F3′H lines were germinated from fresh seed on trays of commercial potting mix, covered with plastic to maintain a high level of humidity. In the second experiment (2000), seedlings of *ht1* were germinated on agar in sterile plastic tubs, as described in Deroles et al. (1996). In both experiments, seedlings were transplanted into individual pots when the seedlings were about 1cm high, 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 leaf width were made at intervals during the following 3-week period. For each individual plant, the rate of change in leaf width was determined by fitting a linear regression of the following form:

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

where W_i was the leaf width of the plant at day i; c was a constant term; r was the rate of change in leaf width; and d was the day of measurement. During this period,

the plants were in the linear phase of growth, and the rate of change in leaf width is referred herein as growth rates. The mean and standard error of the mean of these values were plotted in the figures.

4.3. 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 °C. Samples were later freeze-dried, ground to a fine powder and extracted under occasional vortex shaking for 24 h in MeOH–H_2O–HOAc (70:27:3), followed by centrifugation and filtration. A 15 μl 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 μm, 4×119 mm) with a gradient solvent system comprising solvent A (1.5% H_3PO_4) and solvent B [HOAc–CH_3CN–H_3PO_4–H_2O (20:24:1.5:54.5)] 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 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.

4.4. Northern analyses

Total RNA was isolated from 1 g of young plant material using TriZol reagent (Life Technologies, Auckland, New Zealand) according to manufacturers instructions. Total RNA (10 or 30 μg per sample) was loaded onto formaldehyde denaturing gels, subjected to electrophoresis according to Sambrook et al. (1989). The fractionated RNA was blotted onto Hybond N+ nylon membrane (Amersham Pharmacia Biotech, Auckland, New Zealand) according to the manufacturers instructions. The membranes were prehybridised and hybridised in modified Church and Gilbert solution (Church and Gilbert, 1984).

Plasmids containing petunia PAL, C4H, CHS, CHI, FLS, F3′H cDNAs were kindly gifted by Florigene, Australia. Inserts were prepared from these plasmids and subsequently radiolabelled using a T7 Quickprime kit (Amersham Pharmacia Biotech, Auckland, New Zealand) according to manufacturers instructions. RNA blots were hybridised with each probe overnight at 65 °C, washed to a final stringency of 0.1×SSC + 0.1% SDS at 65 °C, and subsequently autoradiographed. Loading levels for each sample were checked by either hybridising the blots with a ribosomal RNA probe or by comparison of ethidium bromide stained gels prior to transfer of the RNA to the membranes.

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