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Arabidopsis Flavonoid Mutants Are Hypersensitive to UV-B Irradiation

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Increases in the terrestrial levels of ultraviolet-B (UV-B) radiation (280 to 320 nm) due to diminished stratospheric ozone have prompted an investigation of the protective mechanisms that contribute to UV-B tolerance in plants. In response to UV-B stress, flowering plants produce a variety of UV-absorptive secondary products derived from phenylalanine. Arabidopsis mutants with defects in the synthesis of these compounds were tested for UV-B sensitivity. The *transparent testa-4* (*tt4*) mutant, which has reduced flavonoids and normal levels of sinapate esters, is more sensitive to UV-B than the wild type when grown under high UV-B irradiance. The *tt5* and *tt6* mutants, which have reduced levels of UV-absorptive leaf flavonoids and the monocyclic sinapic acid ester phenolic compounds, are highly sensitive to the damaging effects of UV-B radiation. These results demonstrate that both flavonoids and other phenolic compounds play important roles in vivo in plant UV-B protection.

INTRODUCTION

Thinning of the stratospheric ozone layer is projected to increase ultraviolet-B radiation (UV-B; 280 to 320 nm) reaching the biosphere. These changes originate from human activities that release chlorofluorocarbons and other ozone antagonists, resulting in stratospheric ozone depletion (Anderson et al., 1991; Schoeberl and Hartmann, 1991). Plant growth and productivity are compromised by excessive UV-B because it damages DNA, RNA, and proteins (Teramura, 1983; Sisson, 1986; Tevini and Teramura, 1989; Quaite et al., 1992; Stapleton, 1992). Plants are thought to employ a variety of UV-B–protective mechanisms, including increases in UV-B–absorptive pigments, UV-B–reflective properties, and leaf thickness (Caldwell et al., 1983; Beggs et al., 1986).

Light-absorbing flavonoid compounds, a group of phenylalanine-derived aromatic secondary products, have been implicated in protecting plants from the damaging effects of UV-B radiation. This hypothesis seems plausible because UV-B–absorbing flavonoids accumulate in leaf epidermal cells, where they may protect the inner cell layers from UV-B damage (Caldwell et al., 1983; Beggs et al., 1986). Furthermore, the synthesis of these compounds is induced by UV-B in a variety of plant species, as might be expected for an adaptive response. Also, plants grown under conditions that induce flavonoid synthesis are more tolerant to UV irradiation (Tevini et al., 1983; Murali and Teramura, 1985). However, the environmental conditions used to induce flavonoid synthesis, such as nutrient limitation or high white light pretreatment, cause multiple physiological and developmental changes. Thus, results from these experiments do not conclusively establish a primary role for flavonoids in UV-B protection.

We are taking a genetic approach to evaluate the relative importance of the proposed UV-B–protective mechanisms in flowering plants. The availability of recessive *transparent testa* (*tt*) Arabidopsis mutants blocked in leaf flavonoid biosynthesis (Koornneef, 1981; Koornneef et al., 1982, 1983) allowed a direct evaluation of the contribution of phenolic compounds to UV-B protection in vivo. In this report, we show that two distinct classes of phenylalanine-derived, UV-absorptive secondary products, flavonols and sinapic acid esters, provide UV-B protection to Arabidopsis.

RESULTS

Growth of Arabidopsis wild type (ecotype Landsberg *erecta*) is relatively insensitive to daily UV-B dosages of up to 7.1 kJ m^{-2} UV-B, as shown in Table 1. This irradiance level is similar to that measured at temperate latitudes during the annual solar maximum. This insensitivity suggests that the wild-type plants have adaptive responses to potentially damaging amounts of UV-B. The protective response might include efficient repair and replacement of damaged nucleic acids (Pang and Hays, 1991) and proteins as well as the synthesis of screening pigments.

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Table 1. Effects of UV-B Radiation on Weight of Rosettes of Wild Type or *tt5*

	Rosette Dry Weight (mg)[a]								
	Experiment I[b]			Experiment II[c]			Experiment III[d]		
UV-B Dose kJ/m²/16 hr[e]	0	7.1	14.2	0	2.3	4.5	0	2.3	4.5
Wild type	5.4	4.1	2.9	3.8	3.6	3.5	5.9	4.6	4.7
tt5	4.9	1.7	0.95	2.9	1.7	0.95	4.6	1.9	1.1

[a] Data presented are dry weights calculated for the midpoint of the sampling interval (mean dry weights).
[b] A statistically significant effect of UV-B on both genotypes, but the effect was significantly greater for *tt5* (genotype by UV-B interaction).
[c] Effect of UV-B on wild type was not statistically significant. The *tt5* plants were significantly smaller than the wild type, and there was a statistically significant effect of UV-B on *tt5* (genotype and genotype by UV-B interaction).
[d] UV-B effect on *tt5* only (genotype by UV-B interaction).
[e] UV-B doses are expressed as general plant response biological equivalents normalized to 300 nm. UV-C doses at UV-B doses employed (UV-C values are kJ/m²/16 hr): 0.0 UV-B, 0.001 UV-C; 2.3 UV-B, 0.014 UV-C; 4.5 UV-B, 0.028 UV-C; 7.1 UV-B, 0.04 UV-C; 14.2 UV-B, 0.08 UV-C.

Similar results were observed by other investigators for UV-irradiated parsley suspension cells (Chappell and Hahlbrock, 1984) and etiolated Arabidopsis seedlings (Feinbaum et al., 1991).

Consistent with a possible role in Arabidopsis UV-B protection, induction of genes involved in phenylpropanoid synthesis is correlated with a twofold elevation of UV-absorptive leaf pigments within 2 days of UV-B treatment for wild-type Arabidopsis (Figure 2B). As shown in Figure 3, fractionation of the constituent pigments by HPLC revealed an increase in nine of 10 major peaks during UV-B treatment (compare Figures 3A and 3B). Two of the major UV-B–absorptive compounds are sinapate esters: peak 5 is *O*-sinapoyl-L-malate and peak 6 is 1-*O*-sinapoyl-β-D-glucose (Chapple et al., 1992). The biosynthetic pathway for these monocyclic phenolic acid esters diverges from the main phenylpropanoid pathway prior to the committing step in flavonoid biosynthesis (Figure 1). As with flavonoids (Schmelzer et al., 1988), these UV-absorptive compounds are sequestered in the vacuoles of plant epidermal cells (Sharma and Strack, 1985; Strack and Sharma, 1985; Strack et al., 1985). The observation that the concentration of flavonoid and sinapate compounds increases in response to UV-B treatment led us to hypothesize that both afford UV-B protection to Arabidopsis.

UV-B Induction of Secondary Product Biosynthesis

Although UV induction of phenylpropanoid gene expression was previously demonstrated for a variety of plant species, the tissue types, developmental states, and light conditions employed varied widely (for example, compare Bruns et al., 1986; Schmelzer et al., 1988; Feinbaum et al., 1991). This prompted an analysis of the effects of UV-B irradiation on the induction of gene expression for phenylpropanoid pathway enzymes and flavonoid synthesis in Arabidopsis under the experimental conditions employed in our laboratory. In these experiments, plants were grown under white light deficient in UV-B and then shifted to identical conditions with supplemental UV-B radiation.

The committing step in phenylalanine-derived secondary aromatic compound biosynthesis is catalyzed by phenylalanine ammonia-lyase (PAL), whereas chalcone synthase (CHS) is the initial enzyme in the flavonoid pathway, as shown in Figure 1 (steps 1 and 2, respectively). Figure 2A illustrates the rapid and transient increase in steady state concentration of both PAL and CHS mRNAs during exposure to UV-B radiation. Maximal accumulation of both transcripts was observed at 6 hr of UV-B treatment. The accumulation of the *PAL1* and *CHS1* mRNA in response to UV-B appears to be a transcriptional event, as indicated from analysis of transgenic plants containing *PAL1* and *CHS1* promoter–β-glucuronidase (GUS) fusions (data not shown). GUS activity increased fourfold from the uninduced levels within 24 hr of UV-B treatment. These results indicate that UV-B radiation rapidly induces PAL and CHS gene expression in white light–grown Arabidopsis plants.

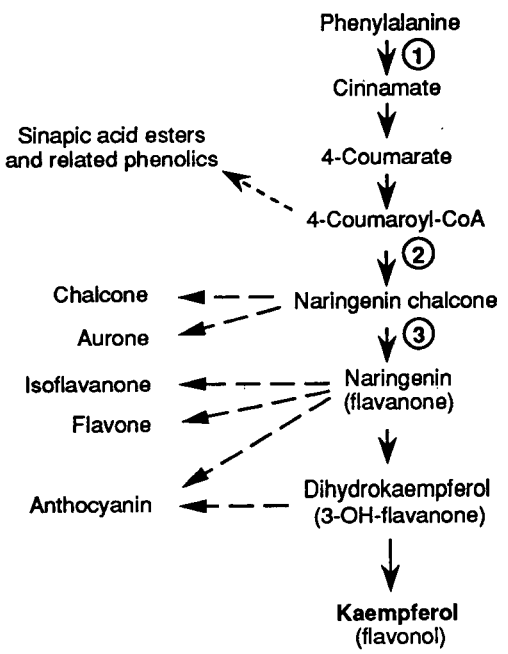

Figure 1. Pathway for Phenylalanine-Derived Secondary Product Biosynthesis in Plants.

A simplified pathway for the synthesis of sinapic esters and flavonoids derived from phenylalanine (Harborne, 1980) is presented with the relevant intermediates and products diagrammed. Circled numbers indicate enzymes discussed in the text: (1) PAL (EC 4.3.1.5), (2) CHS, encoded by the *TT4* gene, and (3) CHI (EC 5.5.1.6), encoded by the *TT5* gene.

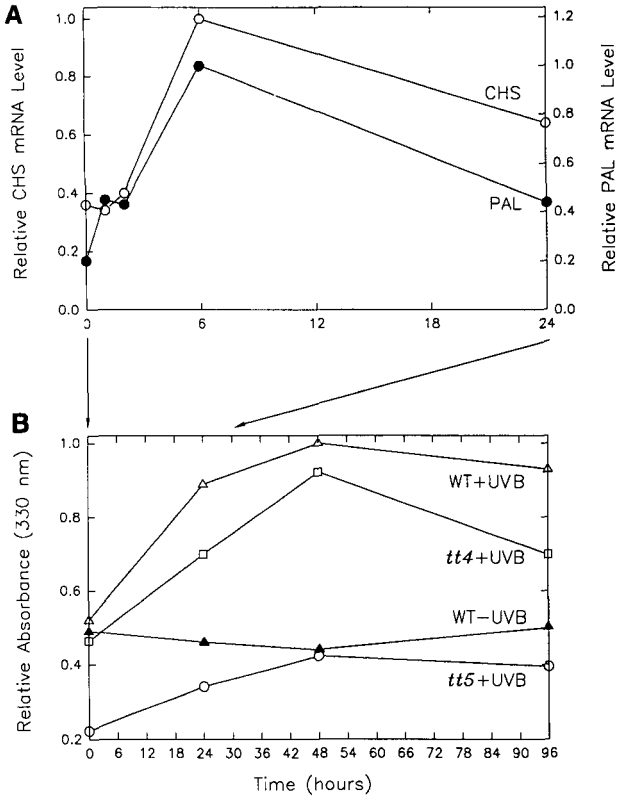

Figure 2. Induction of Leaf Phenolic Compound Biosynthesis in Response to UV-B.

Plants were grown in the absence of UV-B for 10 days following germination (0-hr samples) and then shifted to UV-B supplementation conditions.

(A) Relative levels of PAL (filled circles) and CHS (open circles) mRNA in wild-type plants are presented normalized such that 1.0 represents the level of either mRNA at 6 hr of UV-B irradiation (8.0 kJ m^{-2} day^{-1}).

(B) Relative absorbance at 330 nm of leaf extracts is reported for plants grown on a 16-hr photoperiod in the absence of UV-B or with 7.1 kJ m^{-2} day^{-1} UV-B supplementation. Wild type (WT) without UV-B treatment, filled triangles; wild type shifted to growth under UV-B, open triangles; *tt4* shifted to growth under UV-B, open squares; *tt5* shifted to growth under UV-B, open circles. The absorbance values were obtained by summation of the integrated values for HPLC peaks 1 to 10 (Figure 3) and are normalized such that 1.0 represents the value for wild type at 48 hr of irradiation.

UV-B Hypersensitivity of *tt* Mutants

As a test of this hypothesis, the effects of UV-B on the growth of representative *tt* mutants were compared to wild type. We chose the *tt4* and *tt5* mutants for study because they are devoid of spectrophotometrically detectable leaf anthocyanin and seedcoat pigmentation (T.-M. Ou-Lee and R. L. Last, unpublished results; Koornneef, 1981). *tt4* is a chalcone synthase (CHS) structural gene mutant (B. Shirley and H. Goodman, personal communication), and *tt5* is a chalcone isomerase (CHI) mutant (Shirley et al., 1992). The *tt4* mutant is more sensitive to high-irradiance UV-B treatment than wild-type Arabidopsis, as demonstrated in Figure 4. UV-B hypersensitivity of *tt4* is seen for plants propagated under 13 kJ m^{-2} day^{-1} UV-B, a dose that causes modest growth retardation of wild-type Landsberg *erecta* (Figure 4B). However, the growth rate and morphology of *tt4* plants resemble the wild type when plants are grown under 8.0 kJ m^{-2} day^{-1} UV-B (Figure 4A), a

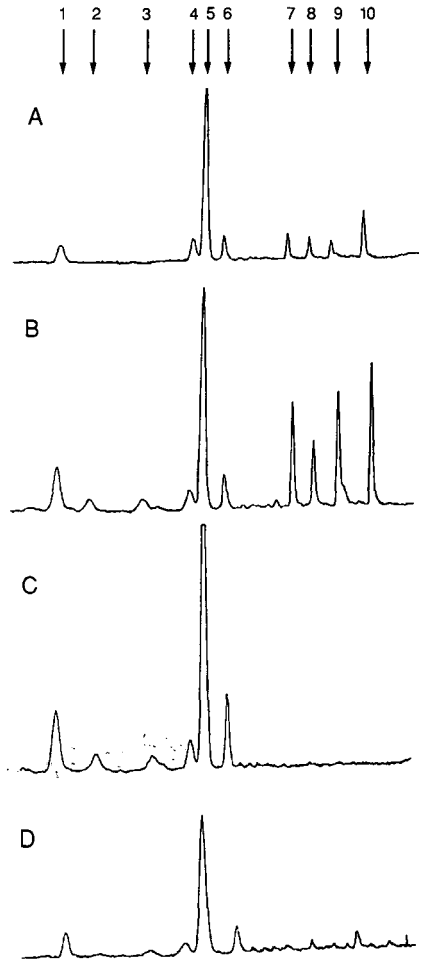

Figure 3. HPLC Separation of Leaf UV-Absorptive Compounds.

Extracts from rosette leaves were fractionated by reverse phase chromatography. The numbered arrows at the top indicate the 10 major UV-absorptive compounds identified by this chromatographic system.

(A) Extract from wild-type plants grown in the absence of UV-B supplementation.

(B) to **(D)** Extracts from plants shifted to growth under UV-B irradiation (7.1 kJ m^{-2} day^{-1}) for 2 days.

(B) Wild type.

(C) *tt4* mutant.

(D) *tt5* mutant.

The *tt6* mutant yielded profiles similar to those for *tt5*.

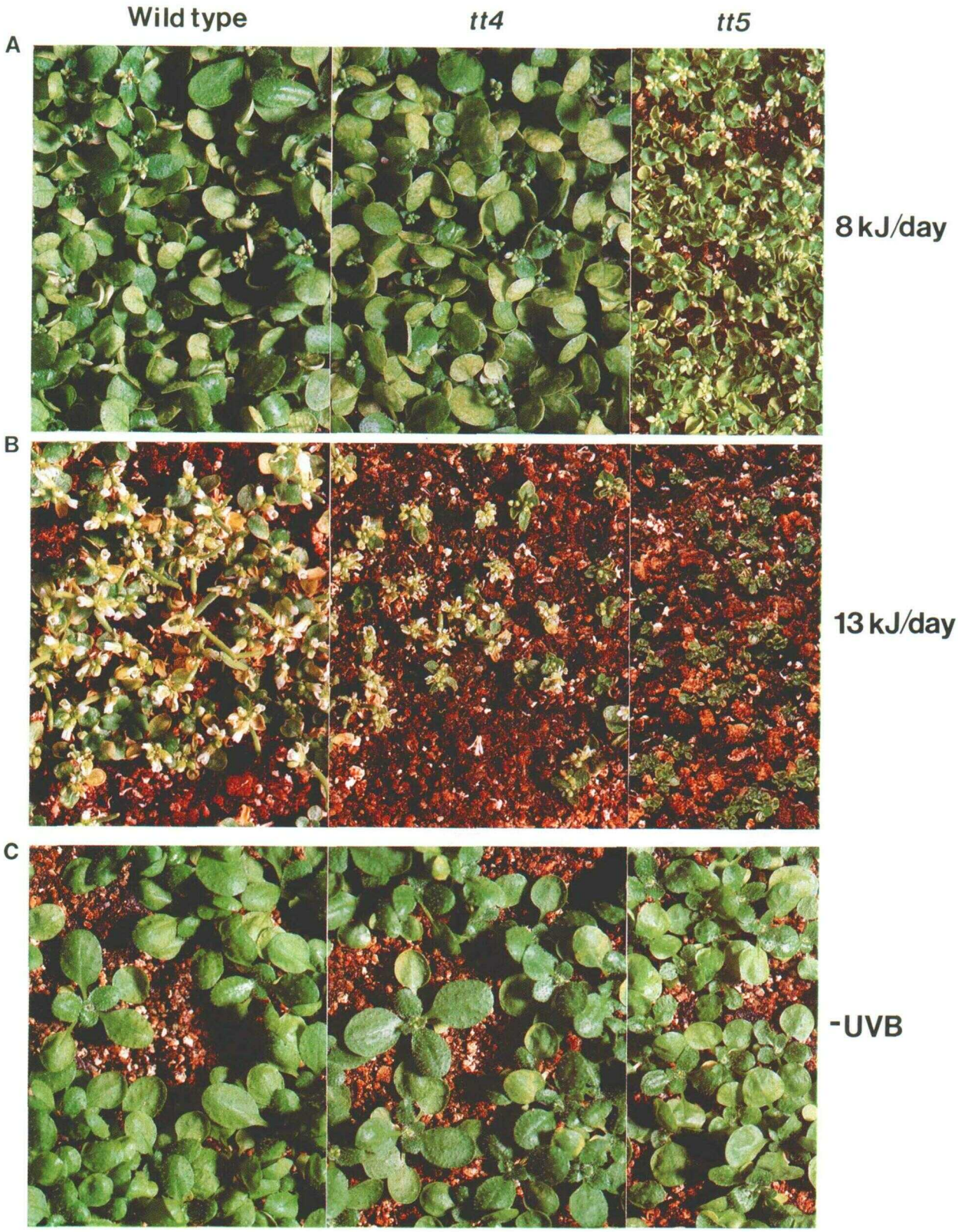

Figure 4. Growth of the *tt* Mutants Is Inhibited by UV-B.

dose similar to that of summer clear sky conditions in the United States. In contrast, growth of *tt5* mutants is inhibited at much lower irradiance levels (Figure 4A). As shown in Table 1, growth of the *tt5* mutants was decreased 41 to 59% compared with the control minus UV-B plants by a UV-B dose approximately one-quarter that of summer clear sky conditions in the United States (2.3 kJ m^{-2} day^{-1}). A higher UV-B dose (4.5 kJ m^{-2} day^{-1}) reduced *tt5* rosette tissue weights by 67 to 76%. In contrast, growth of the wild type and *tt4* was not significantly affected at either UV-B irradiance.

To test whether the extreme UV-B sensitivity of *tt5* plants is due to a single genetic locus, F_3 progeny were analyzed from a cross with the *TT5* wild-type Landsberg *erecta*. Plants from all eight *tt5*$^-$ lines tested were UV-B hypersensitive, whereas the 32 *TT5*$^+$ lines manifested wild-type UV-B resistance. The cosegregation of the flavonoid mutation with UV-B sensitivity is consistent with the hypothesis that the *tt5* mutation, rather than unlinked secondary mutations, is responsible for the poor growth of the mutant under UV-B. Further genetic and phenotypic studies are necessary to establish whether the poor growth of the *tt5* mutant in the absence of UV-B (Table 1 and Figure 4C) is caused by pleiotropic effects of the CHI mutation or uncharacterized secondary mutations in the mutant line employed.

Figure 2B shows that the total quantity of alcohol-extractable UV-absorptive compounds in leaves is correlated with the degree of UV-B tolerance of the Arabidopsis lines employed in this study. The *tt4* mutant accumulates slightly less UV-absorptive compounds than the wild type, whereas *tt5* is very deficient. In fact, extracts from UV-B–induced *tt5* are slightly less UV absorptive than wild type grown without UV-B. Our interpretation of these results is that the total level of UV-B–absorptive leaf compounds is a critical determinant of plant UV-B tolerance.

HPLC was employed to obtain a higher resolution view of the differences in UV-absorptive pigments in the UV-B–sensitive mutants. Figure 3C shows that the *tt4* mutant, which is sensitive to high-irradiance UV-B, is deficient for the major leaf flavonoid compounds (HPLC, peaks 7 to 10). These compounds were shown to be derivatives of the flavonol kaempferol by analysis of the flavonoid skeleton liberated by acid hydrolysis of the purified peaks. The UV spectrum of the peak 10 hydrolysis product was identical to that of authentic kaempferol in methanol and in the presence of the absorbance-shift reagents sodium methoxide, aluminum chloride, and acidic aluminum chloride (Mabry et al., 1970; Markham, 1982), as shown in Table 2. Electron impact–mass spectroscopy yielded the fragmentation pattern, molecular formula, and mass seen for pure kaempferol (Table 2) and reported in the literature (Hedin and Phillips, 1992). The hydrolysis products of peaks 7 to 9 yielded UV and mass spectra similar to those of peak 10. These results very strongly suggest that the unhydrolyzed compounds in peaks 7 to 10 represent kaempferol derivatives.

The HPLC profile in Figure 3D demonstrates that *tt5* is also deficient for the major extractable leaf flavonols (HPLC, peaks 7 to 10). Surprisingly, the *tt5* flavonoid biosynthetic mutant also has reproducibly reduced quantities of sinapate esters (HPLC, peaks 5 and 6). When grown without UV-B, *tt5* reproducibly accumulated 42% less sinapate esters than the wild type. Upon irradiation with 7.1 kJ m^{-2} day^{-1} UV-B, the difference between *tt5* and wild type decreased to 38% after 1 day, 25% after 2 days (see Figure 3D), and 17% after 4 days. These results demonstrate that the enhanced UV-B sensitivity of *tt5* compared with *tt4* is correlated with reduced sinapate esters.

DISCUSSION

These results demonstrate that phenylalanine-derived secondary products play an important in vivo role in plant UV-B protection. We find that growth of the Arabidopsis *tt4* CHS mutant, which fails to produce the flavonol kaempferol (Figure 3C), is affected under high UV-B irradiation (Figure 4). In contrast, the extreme UV-B sensitivity of the CHI mutant *tt5* (Table 1) is correlated with the absence of detectable kaempferol and reduced quantities of sinapate esters (Figure 3D). These results indicate that although flavonoids play a role in Arabidopsis UV-B protection, sinapic acid compounds also make a significant contribution.

Analysis of the *tt4* mutant probably underestimates the importance of flavonoids in Arabidopsis UV-B protection. In contrast to the loss of major flavonoid compounds (Figure 3, peaks 7 to 10), we observe 30 to 60% higher levels of sinapate esters (peaks 5 and 6) in leaves of *tt4* compared with the wild type. (The *tt4* extract in Figure 3C has 50% more sinapate esters than the wild-type extract in Figure 3B.) The difference in quantity of sinapate esters is seen in plants grown in the presence or absence of UV-B irradiation. These results suggest that if a flavonoid mutant with unaltered sinapate accumulation were available, it would be more sensitive to UV-B than is *tt4*. The increased accumulation of monocyclic phenolic

Figure 4. (continued).

Plants were grown from seed imbibition under constant 120 μmol m^{-2} sec^{-1} PAR cool-white fluorescent bulb illumination with different levels of UV-B supplementation. All three panels show (from left to right) wild-type, *tt4* mutant, and *tt5* mutant plants.

(A) Growth under 8.0 kJ m^{-1} day^{-1} UV-B. Plants were photographed 23 days after imbibition.

(B) The effects of growth under 13.0 kJ m^{-1} day^{-1} UV-B. Plants were photographed 34 days after imbibition.

(C) Plants grown without UV-B, photographed 23 days after imbibition.

Table 2. Characteristics of the Flavonoid Skeleton Liberated from Peak 10 by Acid Hydrolysis

	Ultraviolet-Visible Spectra (λ_{max}, nm)[a]				Mass Spectra		
	MeOH	NaOMe	$AlCl_3$	$AlCl_3$/HCl	Formula	Mass	Fragmentations[b]
P10H	365	423 (dec)	422	423	$C_{15}H_{10}O_6$	286.048	287 (18); 286 (100);
(Sample)	320sh	318sh	348	348			285 (22); 258 (19);
	265	281	305sh	305sh			229 (16); 153 (8);
			268	268			136 (9); 121 (28).
Kaempferol	365	423 (dec)	423	423	$C_{15}H_{10}O_6$	286.048	287 (19); 286 (100);
(Standard)	320sh	318sh	350	349			285 (26); 258 (12);
	266	281	305sh	305sh			229 (10); 153 (6);
			269	268			136 (5); 121 (18).

[a] NaOMe, sodium methoxide; $AlCl_3$/HCl, acidic aluminum chloride; P10H, flavonoid skeleton liberated from peak 10 by acid hydrolysis; dec, decomposition of sample evidenced by decreasing band intensity with time; sh, shoulder peak.
[b] Peak heights relative to the molecular ion (arbitratry value of 100) are presented parenthetically.

compounds in a CHS mutant might be caused by shunting of the CHS substrate 4-coumaryl-coenzyme A from the blocked flavonoid pathway into sinapate biosynthesis.

The importance of sinapate compounds for Arabidopsis UV-B protection is reinforced by two additional observations. First, growth of the sinapate ester–deficient mutant *sin1-2* is more sensitive to UV-B irradiation than the isogenic wild type (C. Chapple and R. L. Last, unpublished results). Second, the *tt6* mutant, which has a UV-absorptive pigment profile comparable to *tt5*, is similarly UV-B sensitive (J. Li and R. L. Last, unpublished results). Monocyclic phenolic compounds such as sinapate probably play an important role in UV-B protection in other plants because sinapate compounds and related phenolic molecules are widely distributed in crucifers (Bouchereau et al., 1991) and in many other plants (Harborne, 1980).

The result that the major UV-B–inducible peaks 7 to 10 are derivatives of the flavonol kaempferol is consistent with the fact that *tt4* and *tt5* mutants are blocked prior to dihydroflavonol and flavonol synthesis. In contrast, they are present in the dihydroflavonol 4-reductase mutant *tt3* (J. Li and R. L. Last, unpublished results), which is blocked later in the pathway (Shirley et al., 1992).

It is interesting that the CHI-deficient *tt5* mutant, which is blocked in an early step of the flavonoid pathway (Figure 1), has diminished leaf sinapate esters. This result is consistent with a novel regulatory interaction between flavonoid and sinapic acid ester biosynthesis. Perhaps Arabidopsis responds to accumulation of one or more flavonoid pathway intermediates by down-regulation of the general phenylpropanoid pathway or the specific branch leading to monocyclic phenolic compounds. Such a mechanism might serve to balance the synthesis of the products throughout this pathway in wild-type Arabidopsis. There is precedence for a phenylpropanoid biosynthetic pathway intermediate having a regulatory effect on key enzymes in the pathway. Exogenous *trans*-cinnamic acid, the product of the PAL reaction, blocks the induction of CHS and PAL expression in fungal elicitor–treated bean suspension cells (Bolwell et al., 1988; Mavandad et al., 1990).

An alternative explanation is that the difference in *tt* mutant sinapate levels, and therefore UV-B susceptibility, reflects the severity of their enzymatic defects and not the particular pathway intermediate that accumulates. For example, it seems likely that the highly UV-B sensitive *tt5* mutant has a complete absence of CHI enzyme activity because it is a deletion-inversion mutant that accumulates reduced amounts of mRNA for a truncated CHI protein (Shirley et al., 1992). In contrast, the *tt4* allele is a CHS point mutation (B. Shirley and H. Goodman, personal communication) with mRNA of normal size and abundance (T.-M. Ou-Lee and R. L. Last, unpublished results). This could be a leaky allele that produces a low level of enzyme activity that leads to flavonol accumulation below our level of detection. A definitive test of this hypothesis could be made if a known null *tt4* mutation, such as a deletion allele, becomes available.

The enhanced UV-B sensitivity of Arabidopsis flavonoid biosynthetic mutants has implications for applied plant biology. Previously reported differences in UV-B susceptibility of varieties of plants within a species (Teramura and Murali, 1986; Teramura et al., 1991) might be caused by variations in levels of these UV-B–absorptive phenolic compounds. In this scenario, breeding plants for agronomically useful traits, such as reduced colored flavonoid pigment synthesis or increased palatability due to loss of phenolic compounds, might inadvertently cause enhanced UV-B sensitivity. Transformation of a flavonoid-deficient cultivar with a recombinant gene that directs tissue-specific expression of the wild-type enzyme could restore UV-B tolerance without introducing undesirable characteristics.

These results demonstrate that Arabidopsis produces at least two classes of screening pigments that reduce UV-B damage: flavonoids and sinapate esters. Because mutants were used in this study, we were able to demonstrate the in vivo

significance of both classes of compounds. Further physiological and biochemical analysis of screening pigment deficient mutants should elucidate the biochemical processes most sensitive to UV-B. The isolation of new mutants with altered UV-B response should indicate other mechanisms that plants employ to attenuate or repair the damage caused by UV-B. Such mutants should also help to elucidate the UV-B signal transduction pathway.

METHODS

Plant Growth Conditions

Plants were grown on nonsterile Peat-Lite soil mixture (W. R. Grace, Inc., Cambridge, MA) by modification of previously described procedures (Last and Fink, 1988). The *Arabidopsis thaliana* Landsberg *erecta* wild type and *tt* mutants used in this study were obtained from M. Koornneef, Agricultural University, Wageningen, Netherlands. The *tt* mutants were originally identified by their abnormal seedcoat coloration: seeds from plants homozygous for these *tt* mutations range from lighter brown to yellow in color in contrast to the brown color of mature wild-type seeds (Koornneef et al., 1983). Unless otherwise indicated, UV-B and visible radiation were supplied by 400-W metal halide lamps (HQI-TS400; Osram Sales Corp., Newburgh, NY) and supplemental UV-B by a 400-W mercury halide lamp (HPA-400; Philips Lighting Company, Skaneateles, NY). Visible radiation was attenuated with Saran-type green shadecloth; UV-C radiation (below 280 to 290 nm) was filtered with layers of 0.127-mm cellulose acetate (McMaster-Carr, New Brunswick, NJ), whereas wavelengths below 320 nm (UV-B and UV-C) were removed by Mylar plastic (0.127 mm; AIN Plastics, Mt. Vernon, NY). Mylar and cellulose acetate filters were replaced every 7 to 14 days to maintain uniform optical properties.

UV-B and visible radiation were measured with a spectroradiometer (Model OL 752; Optronics Laboratory, Orlando, FL) that was calibrated with an OL 752-150 calibration module and OL 752-10 spectral irradiance standard. The biologically effective UV-B fluence was calculated for the generalized plant response, normalized to 280 nm (Caldwell, 1971), and converted to 300 nm normalization using the weighting factor 4.59 (M. Caldwell, personal communication). Photosynthetically active radiation (PAR) was ~150 μmol m^{-2} sec^{-1}. Plants were grown at 20 ± 0.5°C and 60 ± 5% relative humidity with a 16-hr photoperiod. UV-C was present at very low irradiance. At a daily UV-B dosage of 7.1 kJ m^{-2}, the fluences of UV-C radiation transmitted through cellulose acetate were (in watts per square meter): 250 nm, 2.07×10^{-6}; 252 nm, 7.53×10^{-7}; 254 nm, 1.01×10^{-6}; 256 nm, 7.19×10^{-7}; 258 nm, 5.62×10^{-7}; 260 nm, 6.39×10^{-7}; 262 nm, 9.41×10^{-7}; 264 nm, 9.45×10^{-7}; 266 nm, 5.77×10^{-7}; 268 nm, 8.14×10^{-7}; 270 nm, 3.28×10^{-6}; 272 nm, 1.12×10^{-6}; 274 nm, 3.91×10^{-5}; 276 nm, 1.68×10^{-4}; and 278 nm, 2.75×10^{-4}.

RNA Gel Blot Hybridization

Plants for RNA extraction were grown at 20 to 21°C under constant cool white fluorescent bulb illumination (120 μmol m^{-2} sec^{-1} PAR) with Mylar filtration of light from 40-W fluorescent UV lamps (Philips F40UVB) for 10 days after seed imbibition. The UV-B treatment (8.0 kJ m^{-2} day^{-1}) began by replacing the Mylar filter with cellulose acetate. Leaf tissue was harvested into liquid nitrogen at the indicated time following a shift to UV-B and then stored at −80°C. RNA isolation (Last et al., 1991), RNA gel blot preparation (Pruitt and Hanson, 1991), blot hybridizations (Church and Gilbert, 1984), and hybridization probe preparation (Feinberg and Vogelstein, 1983) were performed as described previously. The *CHS1* probe was the 3.9-kb HindIII insert of pCHS3.9 (Feinbaum and Ausubel, 1988), obtained from F. Ausubel, Harvard University (Cambridge, MA). The *PAL1* probe was the 0.52-kb HindIII PAL fragment (Davis et al., 1991), obtained from K. Davis, Ohio State University (Columbus, OH); this is not a gene-specific *PAL1* probe. Quantitation of filter-bound radioactivity was performed using a PhosphorImager (Model 400a; Molecular Dynamics, Inc., Sunnyvale, CA) and normalized to rRNA by hybridization to the 2.5-kb EcoRI fragment from pARR17 (Chory et al., 1989), obtained from E. Richards, Washington University (St. Louis, MO).

Analysis of UV-B-Absorptive Compounds

Two methods of quantitating total UV-absorptive materials yielded equivalent results. In the first, leaf tissue was extracted with 70% methanol/1% HCl (vol/vol), the extract was cleared by centrifugation at 27,000*g* for 10 min, followed by filtration through Whatman GF-B, and the absorbance of the supernatant was measured at 330 nm. In the second method, the sum of the integrated HPLC peak absorbance at 330 nm was calculated.

HPLC was performed by modification of the procedures of Graham (1991). The rosette stage leaf tissue (100 mg) was ground to a fine suspension in 400 μL of 80% (v/v) methanol in a small ground-glass homogenizer. The extracts were clarified at 14,000*g* for 3 min. Twenty microliters of the supernatant was injected into a C18 reverse phase column (4.00 × 250 mm Hibar RT cartridge containing 10-μm diameter Lichrosorb RP-18). Chromatography was performed at 25°C with a Beckman HPLC system starting with 0.01% (v/v) glacial acetic acid in water as the solvent. Samples were eluted at a flow rate of 1.5 mL/min with increasing concentrations of HPLC grade acetonitrile (a linear gradient of 0 to 7% for 5 min, constant 7% for 15 min, a linear gradient of 7 to 22% for 25 min, and finally a linear gradient of 22 to 100% for 10 min). Elution was monitored at 330 nm, although similar profiles were obtained at 236 or 254 nm. Peaks 5 and 6 co-elute during HPLC with, and have the same UV-absorption spectra as, purified *O*-sinapoyl-L-malate and 1-*O*-sinapoyl-β-D-glucose, respectively.

Purification and Identification of the Major Leaf UV-Inducible Compounds (Peaks 7 to 10).

UV-B–treated rosette stage leaf tissue (40 g) was ground to a fine suspension in 40 mL of 90% (v/v) methanol in a ground-glass homogenizer. The homogenate was subject to centrifugation at 10,000*g* for 10 min. The clear supernatant was transferred to a flask, the pellet was reextracted with 40 mL of 10% (v/v) methanol and recentrifuged, and the combined supernatants were concentrated to 1.0 mL under reduced pressure. The concentrated crude extract was clarified at 14,000*g* for 3 min before being loaded onto a C18 reverse phase column (Hibar RT cartridge containing 10-μm diameter Lichrosorb RP-18). Chromatography was performed at 25°C with a Beckman HPLC system equilibrated with 2% (v/v) of HPLC grade acetonitrile in 0.01% (v/v) glacial acetic acid and monitored at 330 nm. The peak 7 to 10 compounds were purified with a linear gradient of 9 to 16.5% (v/v) acetonitrile for 50 min. Each purified peak compound was brought to 1.0 M HCl

and 20% (v/v) methanol, and acid hydrolysis was conducted at 95°C for 1 hr. The hydrolysate was then concentrated to 0.4 mL under reduced pressure and separated on a C18 reverse phase HPLC column with a linear gradient of 2 to 55% (v/v) acetonitrile and monitored at 365 nm. The major peak was collected, dried under reduced pressure, and dissolved in 0.3 mL methanol. The UV-absorption characteristics of each hydrolysis product were determined by published methods (Mabry et al., 1970; Markham, 1982) with a spectrophotometer (Lambda 5; Perkin-Elmer), and electron impact–mass spectroscopy was performed using a VG70-VSE with the following parameters: acceleration voltage, 8 keV; electron energy, 70 eV; emission current, 100 μA; ion source temperature, 200°C. The UV and mass spectral results were identical to those obtained with authentic kaempferol (Sigma).

Quantitative Plant Growth Measurements

Rosettes were harvested every 2 or 3 days through the log growth phase prior to flower stalk elongation. Dry weights were determined after drying for 24 hr at 70°C. The analysis of variance method was used to test for statistical differences due to genotype and UV-B level and their interaction.

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