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In situ localization of light-induced chalcone synthase mRNA, chalcone synthase, and flavonoid end products in epidermal cells of parsley leaves

(*in situ* RNA hybridization/immunohistochemistry/microspectrophotometry/UV-protective pigments/transcriptional regulation)

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ABSTRACT **Methods involving *in situ* RNA hybridization, immunohistochemistry, and microspectrophotometry of individual cells were used to localize the mRNA encoding chalcone synthase (the key enzyme of flavonoid biosynthesis), the enzyme protein, and the biosynthetic end products in cross sections of parsley leaves (*Petroselinum crispum*). The light-dependent, sequential occurrence of all three components was restricted to epidermal cells. The results are in agreement with the putative function of flavonoids (transcriptionally inducible, UV-protective pigments) and suggest that all biosynthetic steps occur in those cells in which the products accumulate.**

For plants, light is an essential source of both energy and external signals regulating developmental processes and adjustments to changes in the environment (1). In tissues exposed to potentially noxious UV irradiation, a major form of adjustment is the rapid accumulation of UV-protective compounds. Flavonoids accumulate in the vacuoles of epidermal cells (2) and absorb strongly, *in vivo* as well as *in vitro* (3, 4), in the critical range of 230–380 nm where damage caused by UV irradiation occurs. Flavonoids have therefore been implicated in the UV-protection mechanism of plants (5).

Extensive studies using cell-suspension cultures of parsley (*Petroselinum crispum*) and a few related species (6) have revealed that flavonoids are absent in dark-grown cells and accumulate rapidly upon UV irradiation. In cultured parsley cells, the exclusive site of accumulation is the vacuole (3). The concomitant accumulation of several structurally related flavone and flavonol glycosides is preceded by the transient, coordinated induction of the biosynthetic enzymes (6). For chalcone synthase, the key enzyme of the flavonoid glycoside pathway, the induction has been shown to occur at the transcriptional level (7). Although UV light is essential for induction, blue and red light have additional, modulating effects (8).

In view of the importance of a UV-protection mechanism in plants, we have extended our earlier investigations of intact plants (9) and have studied the site and mode of light-induced flavonoid accumulation in parsley leaves. We have used chalcone synthase cDNA for *in situ* RNA hybridization, a specific antiserum for the immunohistochemical detection of the enzyme, and spectrophotometric methods for the identification of flavonoids in individual cells. This combination of techniques enabled the tissue-specific localization of the four major steps in the induction process, from gene activation and enzyme synthesis to the formation and deposition of the biosynthetic end products.

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MATERIALS AND METHODS

Materials. ^{3}H-labeled nucleotides (dATP, dCTP, dTTP) and [^{32}P]dCTP were purchased from Amersham. Anti-rabbit immunoglobulin from sheep was obtained from the Institute Pasteur. DNA polymerase I from *Escherichia coli* Klenow fragment was from Pharmacia and Nuclear Truck emulsion NTB2 was from Kodak.

Growth of Plants. Parsley plants were grown for 1 yr in a greenhouse. The dark/light experiments were carried out in a phytochamber under controlled conditions at 70% relative humidity in a photoperiod of 10,000 lux for 16 hr (20°C) and 8 hr in the dark (17°C). White, UV-containing light was obtained from Philips TLD 36W/84 lamps.

RNA Blot Hybridization. Total cellular RNA was extracted from parsley leaves (10). Dot blot and RNA blot hybridizations of chalcone synthase mRNA were performed according to ref. 11.

Protein Blotting. Proteins of tissue homogenates were separated on a $NaDodSO_4$/polyacrylamide gradient gel (10–18%). The resulting bands were electrotransferred to nitrocellullose according to Burnette (12). The chalcone synthase protein was detected specifically by indirect immunoperoxidase staining using anti-chalcone synthase as the first antibody and peroxidase-linked anti-rabbit immunoglobulin from sheep as the second antibody.

Preparation of Tissue Sections for *in Situ* RNA Hybridization. Parsley leaves were cut into pieces of ≈ 2 mm^2 and immediately prefixed with 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 2 hr on ice. The fixed tissue was mounted in O.C.T. (Miles), quickly frozen in *n*-hexane (cooled with liquid N_2), and 12-μm sections were sliced in a cryostat (Reichert-Jung) at an object temperature of -15°C. Sections were picked up on gelatin (0.3%)-subbed slides, heated at 50°C for 2 min, postfixed for 20 min at room temperature in the same fixative as described above, and dehydrated through a series of ethanol concentrations (30%, 60%, 80%, 94%, and 96%) for 2 min each, except that in 80% ethanol the sections were kept for 5 min. The slides were air-dried and were stored for several weeks at 4°C.

Pretreatment of Sections. The following treatments were carried out prior to hybridization. The fixed sections were first extracted with 0.2 M HCl at room temperature for 20 min, rinsed with water, and incubated in 0.3 M NaCl/0.03 M sodium citrate, pH 7.0, at 70°C for 20 min. The slides were rinsed with water for 5 min at room temperature, incubated for 10 min in a freshly prepared solution of self-digested (13) Pronase (250 μg/ml in 50 mM Tris·HCl, pH 7.5/5 mM EDTA), and again fixed and dehydrated as described above.

Preparation of DNA Probe. DNA fragments were "oligolabeled" (14) in the presence of [^{3}H]dCTP, [^{3}H]dTTP, and [^{3}H]dATP as radioactive precursors. The labeled DNA was ethanol precipitated together with 250 μg of denatured

herring sperm DNA and redissolved in 50 μl of 10 mM Tris·HCl, pH 8.0/1 mM EDTA. The DNA probes were labeled to a specific radioactivity of 10^8 dpm/μg and had an average length of 50–200 base pairs.

***In Situ* RNA Hybridization.** The labeled DNA was denatured by boiling for 10 min and chilling on ice. The hybridization solution (2 μg of labeled DNA probe per ml/500 μg of denatured and sonicated herring sperm DNA per ml/50% deionized formamide/0.6 M NaCl/1 mM EDTA/10 mM Tris·HCl, pH 7.5/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.02% Ficoll/10% dextran sulfate) was applied to each slide (10 μl per 12–16 sections) and covered with siliconized coverslips (18 × 18 mm). The edges of the coverslips were sealed with rubber cement, diluted 1:1 with petroleum ether. The slides were incubated in a moist chamber for 15–24 hr at 42°C. For the washing procedure, the coverslips were carefully withdrawn and the slides were washed three times for 3 hr in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/50% formamide at 42°C and three times for 30 min in 1× SSC at room temperature. Finally, the slides were dehydrated by immersion in increasing concentrations of ethanol, as described above, and air-dried.

Autoradiography. NTB-2 emulsion was melted at 43°C and diluted 1:1 with 0.6 M ammonium acetate (pH 7.0). Dehydrated slides were coated with the photoemulsion by dipping in a narrow jar. The slides were set up end to end and air-dried at room temperature for 2 hr. The dry slides were exposed at 4°C in a dry light-tight box for appropriate times (7–14 days), developed in Kodak D-19 developer for 2 min, rinsed for 30 sec in H_2O, fixed in Kodak Unifixer for 4 min, rinsed again in three changes of H_2O for 15 min (all procedures at 15°C), and air-dried. The sections were mounted and examined under a light microscope (Zeiss). Photomicrographs were taken using Kodak Ektachrome X160 or Agfapan 25.

Acridine Orange Staining. The retention of cellular RNA in tissue sections during the various steps of the procedure was followed by staining with acridine orange (15). The sections were examined under the fluorescence microscope using blue-violet light. The bright orange staining of cellular RNA in freshly sliced sections was reduced to about one-half after all pretreatments and hybridization, including washing.

Immunohistochemistry. Chalcone synthase was localized in the tissue by indirect immunoperoxidase staining of cryosections as described elsewhere (16).

Localization of Flavonoid Glycosides. Flavonoid glycosides were analyzed spectrophotometrically in 80% methanolic leaf extracts (4 ml/g fresh weight). In cross sections of leaf tissue, absorbance spectra and fluorescence emission spectra (excitation at 365 nm) were obtained from vacuolar areas of single intact epidermal or mesophyll cells by *in vivo* microscopic spectral analysis carried out with the Universal Microscope Spectral Photometer UMSP 80 from Carl Zeiss (2). Fluorescence and bathochromic shift were induced by treatment with 0.05% NH_4OH. Diameters of the effective circular measuring fields were 6 μm or 10 μm, depending on the cell diameters.

RESULTS

Chalcone Synthase mRNA Induction. When the daily 16-hr light period of a 1-year-old parsley plant was discontinued for 2 days, drastic changes occurred in the amount of chalcone synthase mRNA in leaves. A reduction of the mRNA concentration by a factor of 2 during the dark period was followed by a large, transient increase. Several days later, the initial steady-state level was resumed (Fig. 1).

Illumination of 7-day-old etiolated leaves with the standard light program produced a similar pattern of changes in mRNA levels. A notable difference was the absence of detectable mRNA levels in the dark (Fig. 2, lane A). The increase was more rapid, and a peak was observed ≈10 hr after the onset of illumination (Fig. 2, lane B). These conditions were used for all subsequent experiments owing to the suitability of the young developing tissue for cross sectioning and the low background level of chalcone synthase mRNA. In contrast to the mRNA, the enzyme protein was detectable in extracts from etiolated leaves, but it was much more abundant at a maximum after 24 hr of standard light treatment (Fig. 2, lanes C and D). Fig. 2 also demonstrates that both the cDNA and the antiserum gave specific signals after blotting from separation gels.

***In Situ* RNA Hybridization.** Fig. 3 (*A–C*) shows a series of photomicrographs of cross-sectioned leaves hybridized *in*

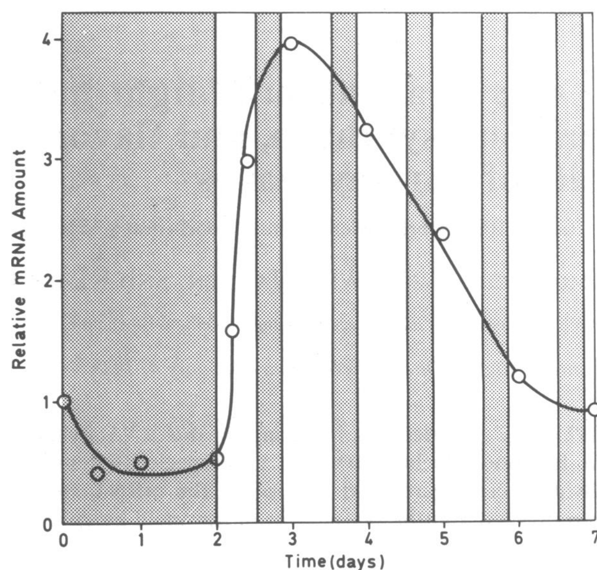

FIG. 1. Time course of changes in chalcone synthase mRNA levels in parsley leaves. RNA was isolated at the indicated times, blotted, and hybridized with ^{32}P-labeled chalcone synthase cDNA. Relative signal intensities (arbitrary units) were calculated by densitometric scanning of autoradiographs. Dark periods are indicated by shaded areas.

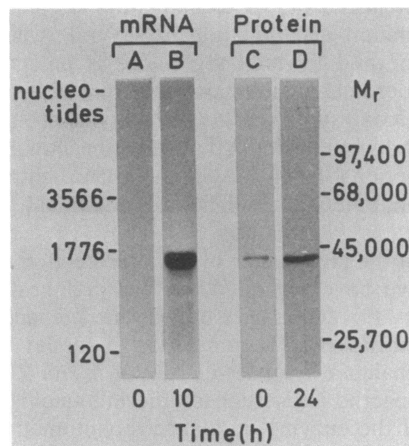

FIG. 2. Relative chalcone synthase mRNA and protein contents of etiolated and illuminated parsley leaves. Total RNA was isolated either from etiolated leaves (lane A) or from leaves illuminated for 10 hr (lane B) and analyzed by RNA blot hybridization with chalcone synthase cDNA. Protein extracts (4 μg) of etiolated leaves (lane C) or leaves treated with the standard light program for 24 hr (lane D) were separated and blotted electrophoretically. Chalcone synthase was visualized by indirect immunoperoxidase staining. Positions of rRNA and protein size markers are indicated.

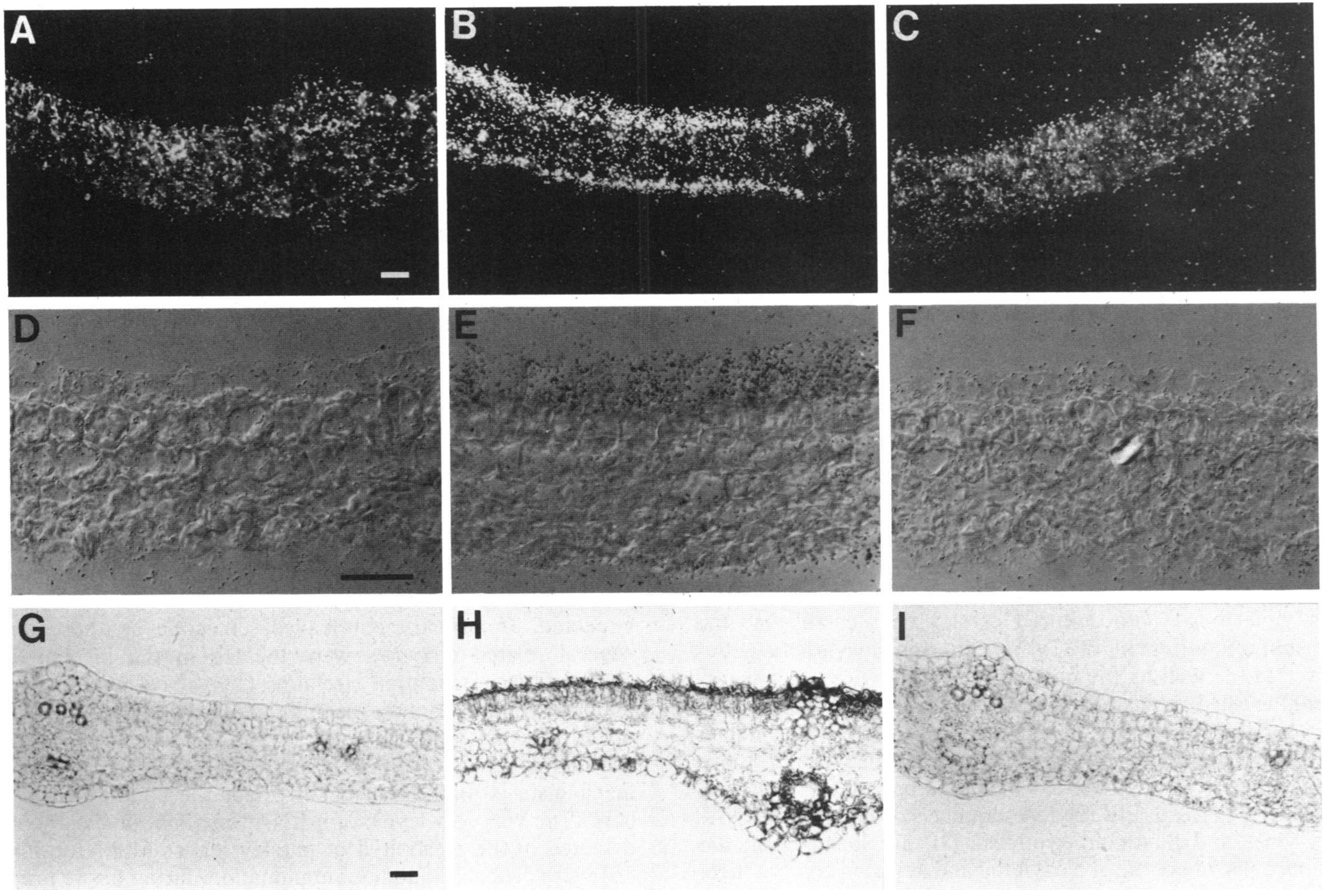

FIG. 3. *In situ* localization of chalcone synthase mRNA and protein. (*A–F*) Cross sections from etiolated leaves (*A* and *D*) and leaves illuminated for 10 hr (*B* and *E*) were hybridized *in situ* with chalcone synthase cDNA. As controls (*C* and *F*), cross sections from illuminated leaves (10 hr) were hybridized with pBR322. Photomicrographs *A–C* were taken under dark-field conditions, *D–F* were by differential interference contrast microscopy. (*G–I*) Localization of chalcone synthase protein by indirect immunoperoxidase staining. Cross sections from etiolated leaves (*G*) and leaves illuminated for 24 hr (*H*) were treated with enzyme-specific antibodies. As a control (*I*), a cross section from an illuminated leaf (24 hr) was treated with preimmune serum. (Bars = 30 μm.)

situ with ^{3}H-labeled DNA probes and analyzed by autoradiography. No tissue-specific hybridization with labeled chalcone synthase cDNA was observed in completely etiolated leaves (Fig. 3*A*), whereas chalcone synthase transcripts accumulated preferentially in the epidermis of previously etiolated leaves that had been illuminated for 10 hr (Fig. 3*B*). Treatment of illuminated tissue with ^{3}H-labeled pBR322 served as an additional control and was also negative (Fig. 3*C*). An analogous series (Fig. 3 *D–F*) is shown at a larger magnification under a differential interference contrast microscope. This technique demonstrates most clearly the high density of silver grains in the photographic emulsion over epidermal cells of illuminated tissue probed with chalcone synthase cDNA (Fig. 3*E*), as compared with mesophyll cells and the two controls (Fig. 3 *D* and *F*).

Immunohistochemistry. A third series of cross sections was used to localize the light-induced chalcone synthase protein *in situ* by indirect immunoperoxidase staining (Fig. 3 *G–I*). In this case, dark-grown tissue (Fig. 3*G*) was compared with tissue treated for 24 hr with the standard light program (Fig. 3*H*), and preimmune serum was used instead of chalcone synthase antiserum in an additional control experiment with illuminated tissue (Fig. 3*I*). Enzyme-specific staining occurred only in illuminated tissue and was largely confined to the narrow cytoplasmic layer of epidermal cells.

Flavonoid Accumulation. Under standard light/dark conditions, 7-day-old, etiolated leaves assumed the size and appearance of normal leaves ($\approx$250 mg) within another 7 days. During the first 2 days of this period, the increase in fresh weight was slow (from 25 to 35 mg), whereas the flavonoid glycoside content increased rapidly from a very low level to over half ($\approx$2.4 μmol per leaf) the value measured at day 7 ($\approx$4 μmol per leaf). In leaves etiolated for 14 days, fresh weight and flavonoid glycoside level did not change appreciably.

Microspectrophotometry. A comparison of cross sections from etiolated and green leaves by UV fluorescence microscopy under alkaline conditions (Fig. 4) showed the characteristic green fluorescence of flavonoids in the epidermal cells of green tissue. The identification of the fluorescing

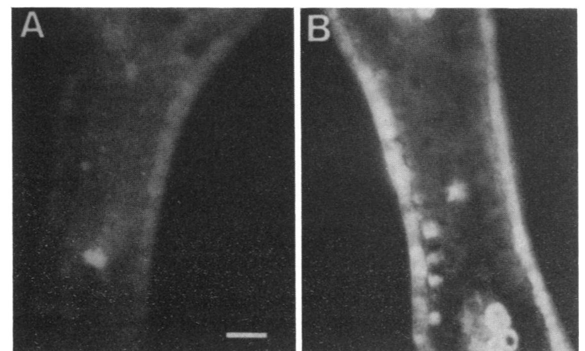

FIG. 4. Green fluorescence of flavonoids in alkalized hand sections of etiolated (*A*) and light-grown (7 day) parsley leaves (*B*). Alkali treatment was with 0.05% NH_4OH. (Bar = 30 μm.)

compounds as flavonoids is demonstrated in Fig. 5. Absorption spectra obtained by microspectrophotometry in the vacuolar region of single epidermal cells revealed two characteristic peaks at 270 and 330 nm. A bathochromic shift of the 330-nm peak to 390 nm occurred under alkaline conditions (Fig. 5*A*). These spectra were not observed in mesophyll cells. For further comparison of the two cell types, the emission spectra (excitation at 365 nm) of vacuolar areas were recorded in intact epidermal and mesophyll cells from a green leaf (Fig. 5*B*). While the epidermal cell clearly showed the fluorescence of flavonoids with an emission maximum at 510 nm, the mesophyll cell fluoresced mostly in the red range of the spectrum, due to the presence of chloroplasts. The yellow fluorescing compounds in vascular bundles (Fig. 4) are not flavonoids. Their chemical nature is unknown and was not investigated.

DISCUSSION

We have shown that epidermal cells are the site of both synthesis and accumulation of flavonoids in parsley leaves. Previous work with cultured parsley cells established the absolute requirement of UV light for this biosynthetic activity (17), as well as the complex coaction with other wavelengths (8). In this study, we used white light containing all potentially effective wavelengths, including UV, to simulate natural illumination as closely as possible. Under these conditions, we demonstrate that all steps of the induction process, the causally related sequence of *de novo* mRNA, enzyme, and flavonoid synthesis (7), are localized in the same cells where the glycosylated and acylated end products (18) accumulate. Chalcone synthase was used as a representative enzyme for two reasons. It catalyzes the first committed step and, hence, occupies the key position in the flavonoid pathway; and chalcone synthase mRNA and protein are far more abundant in light-stimulated parsley cells than those of any other flavonoid biosynthetic enzyme (19).

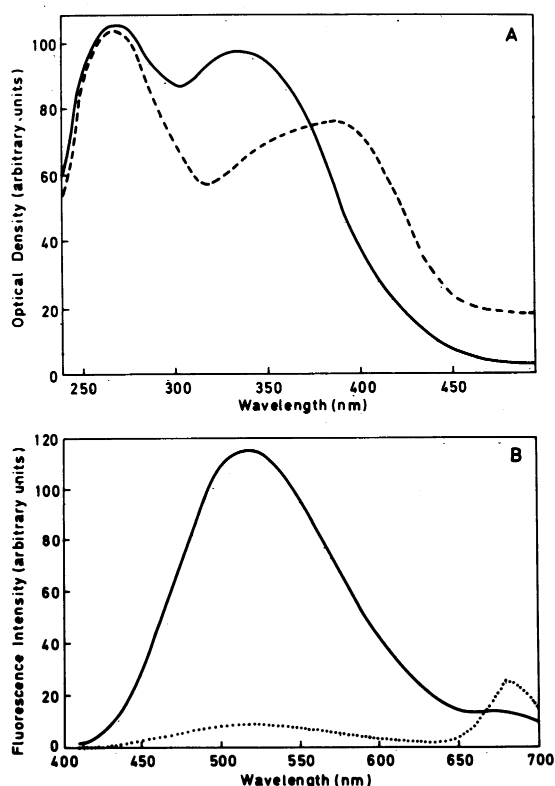

FIG. 5. Absorbance and fluorescence spectra of intact epidermal and mesophyll cells. (*A*) Absorbance spectra of the vacuolar area of an epidermal cell before (——) and after (------) alkali treatment. (*B*) Fluorescence spectra (excitation wavelength, 365 nm) of the vacuolar areas of alkalized epidermal (——) and mesophyll cells (········).

Etiolated leaves were a particularly suitable system. Their response to illumination was similar to green leaves temporarily kept in the dark (Fig. 1), except that relative changes started at lower levels and were therefore more readily detected. A detailed mathematical analysis demonstrating a direct causal relationship for the sequential, light-induced appearance of chalcone synthase mRNA, chalcone synthase, and flavonoid end products has been reported for cultured parsley cells (7). Similar timing of the same sequence of events has now been observed in etiolated leaves. Peaks occurred at approximately 10 and 24 hr for light-induced changes in the relative amounts of chalcone synthase mRNA and enzyme protein, respectively, and a plateau in the accumulation of flavonoids was reached between days 2 and 7.

These studies report the entire sequence of secondary product formation in intact plant tissue. The localization of all sequential steps in the same cell type was not necessarily expected. In primary oat leaves, chalcone synthase and several related enzymes were located in the mesophyll, whereas two structurally related C-glycosylflavones, the major flavonoid products, accumulated predominantly in the epidermis (20). The authors interpreted their results by postulating the involvement of intercellular transport. Such a mechanism is unlikely to occur under the conditions used here. The very low levels of mRNA, enzyme, or flavonoids detected in the mesophyll of parsley leaves (e.g., see Fig. 5*B*) were probably due to contamination during tissue preparation.

The rapid synthesis of flavonoids as a result of light-dependent gene activation in the outermost cell layer of parsley leaves, together with the previously demonstrated UV requirement for induction (8) and the strong UV absorbance of the products, strongly supports the putative role of these compounds as UV-protective pigments in plants.

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