

Effects of Epidermal Cell Shape and Pigmentation on Optical Properties of *Antirrhinum* Petals at Visible and Ultraviolet Wavelengths¹

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We used the *Mixta*⁺ and *mixta*[−] lines of *Antirrhinum majus* as a model system to investigate the effects of epidermal cell shape and pigmentation on tissue optical properties in the visible and ultraviolet (UV) spectral regions. Adaxial epidermal cells of *Mixta*⁺ flowers have a conical-papillate shape; in the *mixta*[−] line the cells are slightly domed. *Mixta*⁺ cells contained significantly more anthocyanin and other flavonoids than *mixta*[−] cells when plants were grown under either high- or low-UV conditions. *Mixta*⁺ cells focused light (3.5–4.7 times incident) within their pigmented interiors, whereas *mixta*[−] cells focused light (2.1–2.7 times incident) in the unpigmented mesophyll. UV light penetrated the epidermis (commonly 20–50% transmittance at 312 nm) mainly through the unpigmented peripheral regions of the cells that were similar for the two lines, so that overall penetration through *Mixta*⁺ and *mixta*[−] epidermises was equal. However, maximum UV absorption in the central region of epidermal cells was slightly greater in *Mixta*⁺ than *mixta*[−], and intact *Mixta*⁺ flowers reflected less light in the spectral regions with intermediate flavonoid absorbance. In both cases, about 50 to 75% of the difference could be attributed to cell shape and resulting changes in the optical pathlength or focusing.

Plants are bathed daily in UV-B, UV-A, visible, and IR radiation from the sun. Some wavelengths are harmful, some provide photomorphogenic signals, some drive photosynthesis, some provide visual cues for insects, and some supply warmth. The optical properties of plant organs and tissues control the penetration and internal distribution of all of these wavelengths. Here we will consider how epidermal cell shape and pigmentation affect the penetration of UV-B, UV-A, and visible radiation into the underlying tissues of *Antirrhinum* petals. This study has implications for the penetration of light into leaves as well as other plant organs.

Because the stratospheric ozone layer has become depleted, attention has been focused on the harmful effects of UV-B radiation on living organisms. The atmospheric ozone layer absorbs radiation of wavelengths less than 300 nm and absorption increases strongly with decreasing wavelength. As the stratospheric ozone layer becomes thin-

ner, there will be an increase in radiation at the earth's surface within the UV-B band, mainly between 290 and 315 nm. Shorter wavelengths are so strongly absorbed by ozone that they will not reach the earth even with a thinner ozone layer, and longer wavelengths are not absorbed appreciably even with an intact ozone layer. The effects of UV-B radiation on higher plants (Tevini and Teramura, 1989; Teramura et al., 1991; Bornman and Teramura, 1993; Caldwell and Flint, 1994; Caldwell et al., 1995), as well as the evidence that links an increase in UV-B radiation to a decreased stratospheric ozone layer, have been reviewed (Blumthaler and Ambach, 1990; Madronich et al., 1991, 1995; Madronich, 1993).

Although UV-B light has important regulatory and photomorphogenic roles (Ballaré et al., 1995; Björn, 1996), excess UV light is clearly harmful and most research has concentrated on its damaging effects. Optimally, a leaf's structure would allow the penetration of photosynthetically active light to the interior while limiting the penetration of harmful UV rays. In fact, considerable evidence exists that penetration of UV-B radiation is strongly reduced by UV-absorbing compounds, chiefly flavonoids, in epidermal tissues (Robberecht and Caldwell, 1978, 1983; Tevini et al., 1991; Braun and Tevini, 1993; Day et al., 1993; Stapleton and Walbot, 1994). However, in herbaceous annuals UV-screening pigments are restricted mainly to the vacuoles of the epidermal cells, and UV-B can leak into the interior of the leaf along the anticlinal walls (Day et al., 1993).

Although UV absorption by epidermal pigments plays an important protective role in leaves, it serves an additional role in the corollas of many bee-pollinated flowers. UV-absorption patterns, although invisible to humans, are perceived by bees, which are most sensitive to wavelengths in the UV-A through yellow spectral regions (Kevan, 1983). These patterns were documented photographically, and a role for UV-absorption patterns, as cues for pollinator orientation, was proposed more than 70 years ago (Richtmyer, 1923; Lutz, 1924).

UV-absorption patterns in flowers are attributed in part to nonuniform distribution of epidermal flavonoids

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Abbreviations: CCD, charge-coupled device; UV-A, 320–400 nm; UV-B, 280–320 nm; visible, 400–700 nm.

(Thompson et al., 1972). However, the geometry of epidermal cells, as well as the pigment they contain, could influence the absorption of UV and visible radiation by the epidermal tissues of leaves and flowers. Work with visible light has demonstrated that leaf epidermal cells, which are generally domed, can act individually as miniature lenses that can focus direct light on small patches of mesophyll at intensities several times that of the ambient irradiance (Haberlandt, 1928; Martin et al., 1989; Poulson and Vogelmann, 1990; Myers et al., 1994; Vogelmann et al., 1996). Similar to leaves, flower petals often have large, lens-like, conical-papillate epidermal cells, which protrude from the petal surface (Kay et al., 1981). In flowers, the intense absorption of both UV (Brehm and Krell, 1975) and visible light (Noda et al., 1994) has been linked to the shape of these strongly domed cells, and in both cases the flattened cells are reported to absorb the light less strongly. This difference in absorption between conical-papillate and flattened cells has several possible sources. First, the conical-papillate cells are taller, and a beam of light would have a longer pathlength within the flavonoid-containing vacuole. Second, lens-like epidermal cells would also bend light rays through refraction, thus further increasing the pathlength. Third, the lens-like properties of these cells might focus light directly on the vacuolar pigments.

Although previous studies (Brehm and Krell, 1975; Noda et al., 1994) have implicated epidermal cell shape in influencing absorption of both visible and UV light, this effect has not been quantified. The separate effects of epidermal cell shape and pigmentation have been difficult to identify because these parameters often vary together (Baagøe, 1977; Levy, 1978; Rieseberg and Schilling, 1985; Steiner, 1990). Here we examine the effects of epidermal cell shape and pigmentation on the optical properties of plant tissue with the following goals: (a) to quantify focusing of light by papillate and flattened epidermal cells, (b) to measure the transmittance of papillate and flattened epidermal cells, (c) to separate the effects of pigment content and cell shape on absorption by individual cells and by the epidermis as a whole, and (d) to assess the effects of cell shape and pigmentation on the optical properties of intact tissues.

To accomplish these goals, we needed to vary both cell shape and pigment content, and the *Mixta*⁺ and *mixta*⁻ lines of *Antirrhinum majus* provided an excellent model system for this purpose. This gene, expressed only in flower lobes, controls epidermal cell shape. In the normal, *Mixta*⁺ line, the adaxial epidermis of the corolla contains strongly conical-papillate cells; the *mixta*⁻ line carries a single-gene mutation, which causes these epidermal cells to be only slightly domed (Noda et al., 1994). To alter pigment content we used supplemental UV light, which stimulates synthesis of flavonoid pigments in epidermal cells of *Antirrhinum* corollas (Fritze et al., 1991) and leaf and flower epidermises of a variety of other plants (Caldwell et al., 1983; Flint et al., 1985; Lois, 1994; Liu et al., 1995).

MATERIALS AND METHODS

Seeds for the *Mixta*⁺ and *mixta*⁻ lines of *Antirrhinum majus* were kindly provided by Dr. Cathie Martin (John

Innes Centre, Norwich, UK). Plants were grown under greenhouse conditions in 4-inch pots and a loam:peat:Perlite mixture (1:1:1, v/v; Persolite Products, Florence, CO) fertilized with 17–6–10 controlled-release fertilizer pellets (Grace Sierra Horticultural Products, Milpitas, CA). During the growth period plants received supplemental lighting from 1000-W Sylvania Metalarc lamps on a 12-h photoperiod. To alter the floral pigment content plants received either low or high exposures of UV radiation (Fig. 1) daily for 5 h in the middle of the photoperiod. UV light was provided by four UVB-313 lamps (Q-Panel, Cleveland, OH) for each treatment. For the low-UV treatment, one layer of Plexiglas (2.9 mm) and one layer of cellulose diacetate (0.8 mm, Grafix Clear Acetate supplied by National Architectural and Engineering, Denver, CO) blocked essentially all UV radiation, so that plants received negligible integrated biologically effective UV-B irradiance daily (calculated based on Caldwell's [1968] generalized plant action spectrum normalized at 300 nm). For the high-UV treatment, one layer of cellulose diacetate blocked UV-C radiation (less than 280 nm), and plants received 12.0 kJ m⁻² d⁻¹ integrated biologically effective UV-B irradiance. The UV fluence rate was measured using a model 742 spectroradiometer (Optronic Laboratories, Orlando, FL) calibrated against a 1000-W tungsten-filament quartz-halogen standard lamp (Optronic Laboratories, traceable to the National Institute of Standards and Technology, Gaithersburg, MD) and was checked for wavelength accuracy using known mercury emission lines. Cellulose diacetate was replaced as necessary to maintain these UV exposures (Adamse and Britz, 1992; Middleton and Teramura, 1993).

Under these conditions, the pink, anthocyanin-containing flowers of both lines began to appear in about 3 months. *Antirrhinum* flowers are bilaterally symmetrical, with two upper and three smaller lower lobes. Using the two upper lobes for these experiments, we generally measured the optical properties of one lobe and extracted flavonoids and related compounds from a matching region in the other lobe.

Focusing of Visible Light by Epidermal Cells

Tissues were viewed using an IMT-2 inverted microscope (Olympus), and images were captured using a CCD

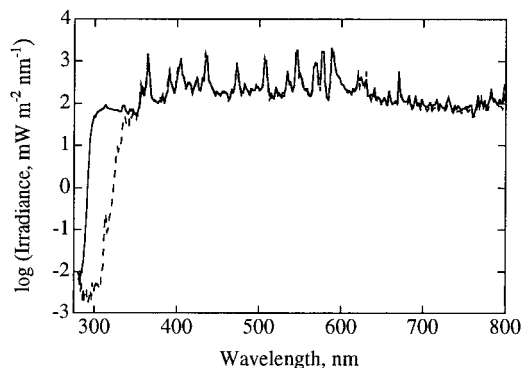


Figure 1. Spectra of low- (---) and high-UV (—) light conditions under which plants were grown, as described in "Materials and Methods."

camera (CH270 camera head, CE200A 16/40 camera electronics unit, AT200 controller board, 35-mm shutter; all from Photometrics, Tucson, AZ), with a 16-bit resolution of dynamic range, cooled to -110°C using liquid nitrogen and PMIS image-processing software (Photometrics).

Epidermal tissues, obtained either by peeling or by making a shallow, oblique cut through petals with a razor blade, were placed on a microscope slide that had been covered with 2% (w/v) agarose (type VII, Sigma) in water. The agar allowed us to view the patterns of light below the plane of the epidermis in a medium with a similar refractive index to the tissue. To reduce evaporation the slide was covered with a humidified chamber consisting of one-half of a plastic Petri dish that was lined with moistened filter paper.

Collimated light from the microscope lamp was filtered as necessary through red (peak wavelength 680 nm, maximum transmittance 37%, bandwidth 5 nm), green (peak wavelength 530 nm, maximum transmittance 40%, bandwidth 6 nm), or blue (peak wavelength 430 nm, maximum transmittance 47%, bandwidth 6 nm) interference filters (Corion Instrument, Waltham, MA). Even with incident light levels reduced by these filters, the CCD camera could capture an image with exposures of 0.1 to 0.5 s. To determine the depth of the focal spots, exposures were taken using white, red, green, or blue light while focused on the tops of the cells, and then the exposure series was repeated using the calibrated focus knob to focus at known depths through the tissue and agar. To calculate focal intensification factors, each image was divided by a background image (taken without tissue) using the PMIS software.

Extraction and Measurement of Flavonoids and Related Compounds

Discs 8 mm in diameter were placed in 1 mL of acidified methanol (methanol: H_2O :HCl, 79:20:1, v/v) in a 1.5-mL microfuge tube and homogenized with a Teflon pestle. Extracts were stored frozen (-80°C) for up to 1 week, then clarified by centrifugation (Eppendorf model 3200 microfuge), and diluted to 3 mL. Spectra of the extracts (190–820 nm) were obtained with an 8452 diode array spectrophotometer (Hewlett-Packard).

Imaging UV Penetration through Epidermal Tissues

Because UV light was not transmitted through the optical system of the Olympus IMT-2 microscope, we placed tissue samples on a fluorescent, calcofluor-containing film to translate the patterns of UV light penetration into a visible image, which could pass through the microscope and be captured by the CCD camera system, although it was too dim to view clearly by eye. The film was cast on a microscope slide as described previously (Day et al., 1993), except that we covered the calcofluor layer with a different hydrophobic barrier, which was necessary to prevent leaching of the calcofluor into the tissue. After the calcofluor layer dried, we dipped the slides in a 2% solution of polyvinyl chloride in tetrahydrofuran, then in a 0.8% solution of polystyrene in toluene, and finally in the polyvinyl

chloride solution again, allowing the slides to dry after each coating. Tissue samples were prepared as described previously and placed on a droplet of water on a coated microscope slide. The droplet was removed, leaving only a thin film of water between the tissue and the film, and the sample was protected from further drying with a humidified chamber.

UV light was provided by a 150-W xenon-arc lamp (Bausch and Lomb, Rochester, NY) and passed through a quartz collimating lens into a monochromator (model 77200; Oriel, Stratford, CT) that was set at the desired wavelength. The small amount of spectrally impure, stray light from the monochromator degraded the images of the UV transmission patterns; therefore, light from the monochromator was passed through an interference filter (312 nm, maximum transmittance 25%, bandwidth 12 nm; 362 nm, maximum transmittance 11%, bandwidth 10 nm; both from Corion Instrument) matched to the wavelength set on the monochromator, either 312 or 362 nm. A quartz fiber-optic bundle (1.0-mm diameter) then carried the light to less than 1 mm above the sample.

Images were obtained from healthy cells (identified as turgid cells retaining anthocyanin), which had settled directly onto the fluorescent film. Exposures of 5 min generally provided good images that showed two-dimensional position information for the relative intensity of UV after it had penetrated the epidermis. To translate photon counts from the CCD camera to %T, where T = transmittance, we divided each image by a background image that was obtained from an adjacent portion of the film without tissue. Absorbance was calculated from %T ($A = -\log[\%T/100]$). Transmittance of UV light could only be measured at one image plane, the interface between the epidermis and fluorescent film.

Optical Properties of Intact Petals

Measurements were made using an integrating sphere 10 cm in diameter, coated internally with white reflectance coating (Eastman Kodak), which provides uniformly high, diffuse reflectance throughout the spectral region of interest. Light was provided by the 150-W xenon-arc lamp, and spectra (160–830 nm, 0.65 nm resolution) were recorded by the Photometrics CCD camera (cooled to -112°C), which was attached to a SpectraPro 275 spectrograph (Action Research, Action, MA) and coupled to a computer running CCD9000 spectral acquisition software (Photometrics). A 1.0-mm diameter quartz fiberoptic cable carried light from the integrating sphere to the spectrograph. For measurements of reflectance and transmittance, samples of petal tissue were mounted with the adaxial surface facing the light over the appropriate ports (12.5-mm diameter) in the integrating sphere. Reflectance spectra were calibrated using a 10% reflectance standard (Labsphere, North Sutton, NH) as the primary reference.

Preliminary experiments with a helium-neon laser (632.8 nm) as a light source demonstrated that there was enough stray light within the spectrograph to interfere with measurement of the relatively low amounts of UV light. The stray light gave background counts in regions of the UV

spectrum where there was no laser output. Although stray light did not significantly affect the results in the visible region of the spectrum, in the UV region, lamp output and reflected or transmitted light from the tissue were both relatively low, and stray light produced artifactually high reflectance and transmittance values. To reduce stray light, we placed a visible light-blocking, UV-transmitting filter (Schott UG-5, Melles Griot, Irvine, CA) in the light path when acquiring data for the UV portion of reflectance and transmittance spectra. Then, each spectrum was built from data collected with (280–390 nm) and without (391–750 nm) the UG-5 filter in the light path. The filter, however, did not eliminate the stray light problem entirely, so a further correction was necessary. Counts in the short-wave UV region of the spectrum were due almost entirely to stray light, so for each spectrum we found the wavelength (about 245 nm) with the minimum number of counts and subtracted this number of counts across the spectrum.

Because our integrating sphere was a single-beam instrument, our measurements were subject to substitution error (Wendlandt and Hecht, 1966). We characterized the substitution error of the sphere using 10 and 100% reflectance standards (Labsphere) and made appropriate corrections to the transmittance and reflectance spectra. Corrections for substitution error increased with increasing %R, where R = reflectance, or %T and were less than 5% for measurements reported here.

RESULTS

These experiments were designed to characterize the differences in the optical properties of *Mixta*⁺ and *mixta*[−] flowers and to determine how much of the difference is attributable to epidermal cell shape and how much to pigmentation. We measured the focusing characteristics, which depend on cell shape, and the pigmentation of the two lines to help interpret the differences we might see in UV penetration through epidermal tissues and in optical properties of discs from intact petals.

Focusing of Visible Light by Epidermal Cells

The conical-papillate cells of the *Mixta*⁺ line and the flattened, although still slightly domed, cells of the *mixta*[−] line of *Antirrhinum* focused visible light quite strongly (Fig. 2). Red light, which was not absorbed by the anthocyanin pigment in the vacuole, gave the most focal intensification, whereas green light, which was absorbed strongly by the anthocyanin, showed little or no focal intensification (Fig. 3). White light, which included absorbed and nonabsorbed wavelengths, and blue light, which was weakly absorbed, produced intermediate focal patterns.

Differences in epidermal focusing between the two lines are evident in both the magnitude of focal intensification and in the depth and size of the brightest focal spot (Figs. 2 and 3). In three replicate experiments for each line, *Mixta*⁺ cells produced a smaller, more intense focal spot than *mixta*[−] cells (Fig. 2). For *Mixta*⁺ cells, focal intensification was 3.3 to 4.5 × ambient, whereas for *mixta*[−] cells it was only 2.1 to 2.7 × ambient. *Mixta*⁺ cells produced

maximal focal intensification at a depth of 45 to 52 μm, well within the 50- to 60-μm-tall cells. In contrast, *mixta*[−] cells produced maximal focal intensification at a depth of 60 to 75 μm, below the 25 μm-thick epidermis, in the mesophyll tissue.

Pigment Content

Anthocyanin and presumably other flavonoids in the *Antirrhinum* petals are found in the adaxial and abaxial epidermal cells. An earlier investigation (Noda et al., 1994) of the *Mixta*⁺ and *mixta*[−] lines of *Antirrhinum* reported no significant difference in pigmentation between the two, but under our growth and assay conditions we found clear differences in the amount of flavonoid extractable from the two lines (Fig. 4) and tested the significance of differences with Student's *t* tests on data from each of the wavelengths marked with an error bar. When plants were grown under low-UV conditions, *mixta*[−] flowers contained significantly less flavonoid than *Mixta*⁺ flowers (*P* < 0.01 at 278 nm; *P* < 10^{−5} for 312, 322, 362, and 530 nm). When plants were grown under high UV, differences between pigmentation in flowers from *Mixta*⁺ and *mixta*[−] lines were smaller, but still significant (*P* < 0.01 for 312 and 322 nm, *P* < 0.05 for 312, 362, and 530 nm). As expected, the high-UV treatment was correlated with an increased flavonoid production in both *Mixta*⁺ and *mixta*[−] lines (*Mixta*⁺: *P* < 0.05 for all five wavelengths tested; *mixta*[−]: *P* < 0.05 for 278 and 312 nm, *P* < 0.01 for 322, 362, and 530 nm). *Mixta*⁺ flowers that developed under low-UV conditions contained only slightly more pigment than the *mixta*[−] flowers that developed under high-UV conditions, and the difference was only significant at 312 nm (*P* < 0.05). Some individual high-UV, *mixta*[−] flowers contained levels of pigment comparable with the low-UV, *Mixta*⁺ flowers; this was important because it allowed a comparison of the optical properties of conical-papillate and flattened cells with similar pigmentation.

UV Penetration through Epidermal Tissues

Both UV-A at 362 nm and UV-B at 312 nm penetrated most through cell wall and nonvacuolar cytoplasmic regions of the epidermal peels (Fig. 5). The image plane for these cells was on the fluorescent film, at the base of the cells. This was below the plane of maximum focal intensification of visible light for *Mixta*⁺ cells and above it for *mixta*[−] cells. Although bright focal spots under the center of the cells were evident at this depth for all wavelengths of visible light tested and for both *Antirrhinum* lines (data not shown), no such focal spots were evident for either UV wavelength. Evidently the strong absorption of UV light by pigment in the central vacuole eliminated the focusing effect.

To separate the effects of epidermal cell shape and pigmentation on UV penetration through the epidermis, we plotted maximum absorbance (under the vacuolar region) at 312 and 362 nm of individual epidermal cells against absorbance of extracted flavonoids at these wavelengths, and then we compared the regression lines obtained for the

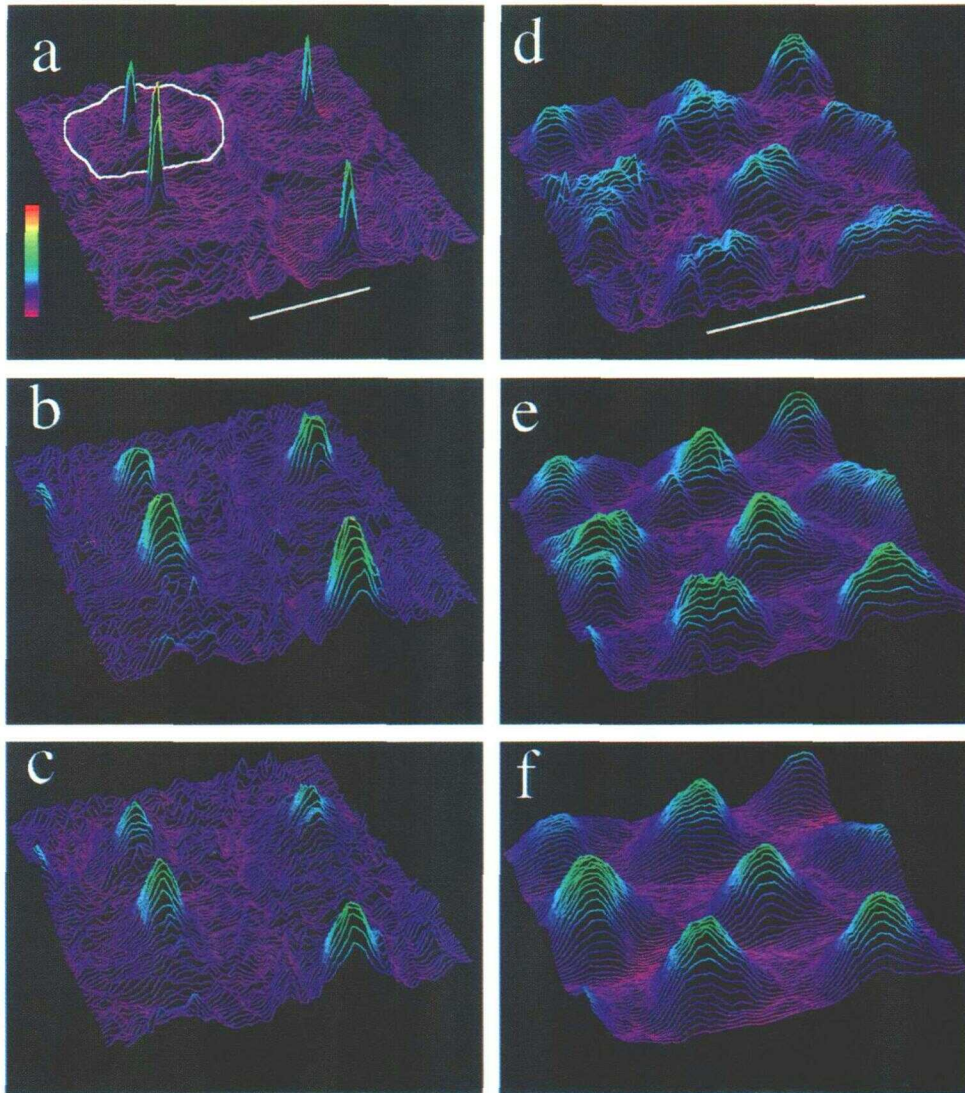


Figure 2. Three-dimensional representations of patterns of light (680 nm) at 45 μm (a and d), 60 μm (b and e), and 75 μm (c and f) below the cell surface of adaxial epidermis from *Mixta*⁺ (a–c) and *mixta*[–] (d–f) flowers. Color scale bar applies to a to f and shows intensification factors relative to incident light levels. Purple = 0; red = 5 times incident light. White scale bars = 30 μm . A single focal spot is produced by each cell and is represented here by a peak. Cell walls transmit relatively little light of this wavelength, so the walls and the areas under the walls appear as purple valleys. A single cell is outlined in white (a) for clarity.

Mixta⁺ and *mixta*[–] samples (Fig. 6a). The ordinate value of each point represents the average of 10 to 20 absorbance measurements (for 10 to 20 individual cells) in one sample; the abscissa value represents the corresponding absorbance of flavonoids extracted from a matching section of tissue taken from the opposite upper lobe of the same flower. All regression lines in Figure 6 are significant ($P < 0.01$). A comparison of the regression lines in Figure 6a indicates that there was a significant difference between maximum UV absorption by individual *Mixta*⁺ and *mixta*[–] cells with similar pigment content ($P < 0.01$) (Neter and Wasserman, 1974). Further analysis of the data in Figure 6a indicates that about 50 to 75% of the difference in maximum absorption by individual *Mixta*⁺ and *mixta*[–] epidermal cells can be attributed to the differences in cell shape and arrangement.

We also measured the overall absorption by epidermal peels, including absorbance by wall, cytoplasmic, and vacuolar regions of the cells examined for Figure 6a, and correlated it with absorbance of extracted flavonoids as described above. Overall absorption by epidermal peels from *Mixta*⁺ and *mixta*[–] corollas with similar pigmentation was not significantly different (Fig. 6b, $P > 0.5$).

Optical Properties of Intact Petals

Flowers from *Mixta*⁺ and *mixta*[–] plants grown under similar conditions were clearly distinguishable by eye. *Mixta*⁺ flowers are a darker pink (Noda et al., 1994), and the difference seen visually was quantified by reflectance measurements. A t-test comparing *Mixta*⁺ and

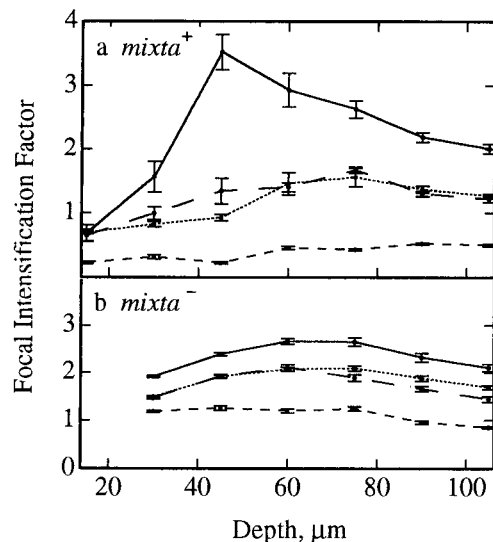


Figure 3. Focal intensification factors for *Mixta*⁺ (a) and *mixta*⁻ (b) epidermal focusing of white (long dashes), 680 nm (solid lines), 530 nm (short dashes), and 430 nm (dotted lines) light at various depths beneath the cell surface. Error bars represent SES. Data are from a single representative peel for each line; $n = 5$ cells for *Mixta*⁺ and 20 cells for *mixta*⁻.

mixta⁻ reflectance for each of the wavelengths marked with error bars in Figure 7 indicated that *Mixta*⁺ flowers reflect significantly less light than *mixta*⁻ flowers at 430 ($P < 0.05$) and 530 nm ($P < 0.001$) but that differences in the UV and red spectral regions are not significant. Transmittance (Fig. 7b) of *Mixta*⁺ petals was significantly lower than for *mixta*⁻ petals at 430, 530, and 680 nm ($P < 0.01$). Differences in calculated absorbance ($\%A = 100 - \%T - \%R$; Fig. 7c) correspond to the measured differences in reflectance and transmittance, with the *Mixta*⁺ flowers having a significantly higher absorbance at 362 ($P < 0.05$), 430, and 530 nm ($P < 0.001$).

To determine how much differences in pigmentation and epidermal cell shape contributed to the observed differences in reflectance, and thus in appearance, we compared absorption of extracted flavonoid pigments with measured reflectance of intact petals at selected visible and UV wavelengths (Fig. 8). For each line large changes in petal reflectance resulted from small changes in absorbance of extracted flavonoids when flavonoid absorbance was low, but when absorbance of extracted flavonoids was high, overall petal reflectance changed only slightly with increasing pigment content. We fitted hyperbolic curves to the *Mixta*⁺ and *mixta*⁻ data separately. Each curve shows how pigmentation affects petal reflectance with cell shape held constant, and a comparison of the two curves gives an indication of the contribution of cell shape to the observed differences in petal reflectance. The two hyperbolic fits are significantly different ($P < 0.01$) (Neter and Wasserman, 1974). Where there is low flavonoid absorbance, the two fit curves differ by 5.7%, and they converge to within 1.7% at wavelengths where flavonoid absorbance is high. Further analysis of the data in Figures 7a and 8 for 530 and 550 nm indicated that about 50 to 70% of the difference in reflectance

seen in Figure 7a is attributable to differences in cell shape and the remainder to differences in pigmentation. This region, which showed the most significant difference in %R between *Mixta*⁺ and *mixta*⁻ lines, is also the region of maximum anthocyanin absorbance of visible light, but note that extracted flavonoids have intermediate absorbance here (0.4–0.7).

DISCUSSION

The *Mixta*⁺ gene encodes a protein with sequence homology to the DNA-binding domain of myb-related proteins and may be involved in the transcriptional control of epidermal cell shape (Noda et al., 1994). Noda et al. (1994) also noted a similarity between the Mixta protein and the regulatory products of the *cl* and *p* loci of maize, which exert transcriptional control over enzymes involved in flavonoid biosynthesis (Grotewold et al., 1991), but they found no difference in pigmentation between the two lines. Subsequent work in the same laboratory has shown that transcript levels for several biosynthetic enzymes involved in flavonoid biosynthesis are similar in *Mixta*⁺ and *mixta*⁻ lines (C. Martin, personal communication). In contrast, under our growth conditions, we found highly significant differences in flavonoid content between *Mixta*⁺ and *mixta*⁻ lines that accompany the previously reported difference in epidermal cell shape. There is variability in pigmentation between individual plants, and if the Mixta protein indeed does not influence flavonoid biosynthesis, the difference in pigmentation we observe might be explained if the *mixta*⁻ seed we obtained came from a pale-colored individual.

The difference in the shape of the epidermal cells of the *Mixta*⁺ and *mixta*⁻ corollas has consequences for the optical properties of petals. We have described the differences

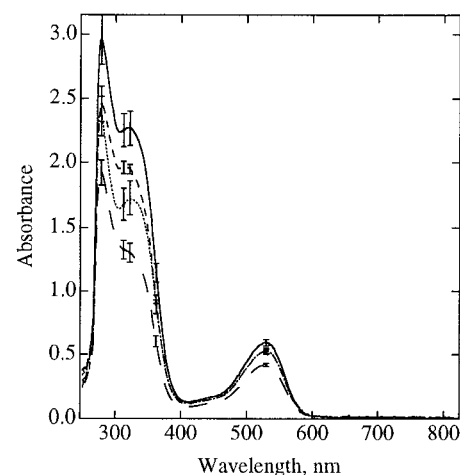


Figure 4. Absorbance of extracts from *Antirrhinum* flowers from plants grown under various conditions: *Mixta*⁺, high UV (solid line); *Mixta*⁺, low UV (short dashes); *mixta*⁻, high UV (dotted line); *mixta*⁻, low UV (long dashes). Spectra are averages of 11 to 17 individual spectra, and error bars at selected wavelengths represent SES.

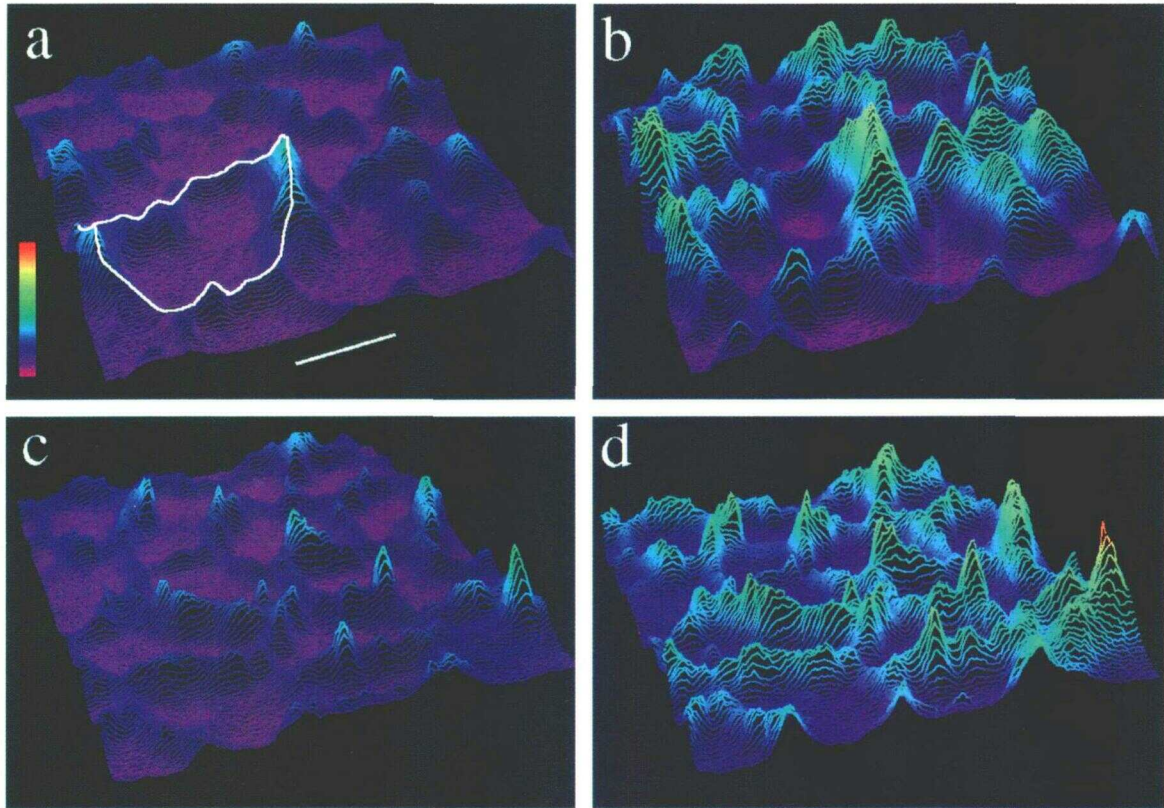


Figure 5. Penetration of 312 nm (a and c) or 362 nm (b and d) light through epidermal peels from *Mixta*⁺ (a and b) and *mixta*⁻ (c and d) flowers. Color scale bar applies to a to d and shows %T, where T = transmittance, relative to incident light levels. Purple = 0%T; red = 80%T. White scale bar = 30 μ m. The ridges representing relatively high levels of UV penetration are located under the walls and peripheral cytoplasm of the epidermal cells. The purple valleys lie under the center of each cell, through which little UV light penetrates. A single cell is outlined in white (a) for clarity.

between *Mixta*⁺ and *mixta*⁻ lines in epidermal focusing (Figs. 2 and 3), penetration of UV light through individual epidermal cells (Figs. 5 and 6), and reflectance and transmittance by petals (Figs. 7 and 8) and will discuss each of these.

Epidermal Focusing

The differences in epidermal focusing are expected and reasonable. The 50- to 60- μ m-tall, conical-papillate *Mixta*⁺ epidermal cells, which have a relatively small radius of curvature, focus light more strongly, in a smaller spot, and at shorter focal length than the slightly domed *mixta*⁻ cells. The magnitude of focal intensification that we observed (2.1- to 4.5-fold for red light) is common in leaves. In fact, leaves of some species (*Medicago*, *Zea*, and *Impatiens*) can concentrate light 15- to 20-fold via focusing (Vogelmann et al., 1996). Differences in pigmentation between the two lines should not affect this comparison because red light is not absorbed significantly by the floral pigments in either line. Although papillate epidermal cells have frequently been observed in flowers and the possible effects of these cells on the optical properties of petals have been discussed (Exner and Exner, 1910; Brehm and Krell, 1975; Kay et al., 1981), to our knowledge this is the first time it has been

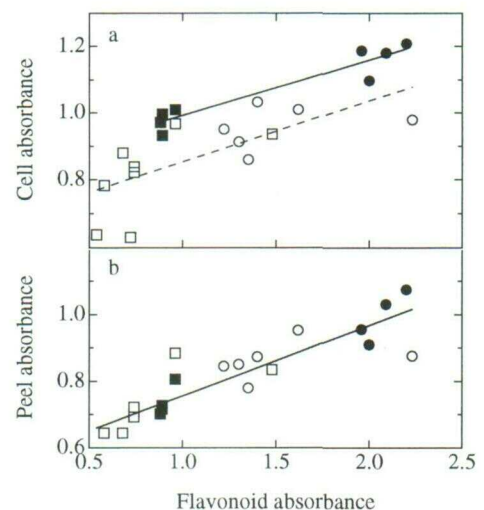


Figure 6. Correlation between average maximum absorbance of epidermal cells (a) or overall absorbance of epidermal peels (b) with the corresponding absorbance of extracted flavonoids at 312 nm (●, ○) or 362 nm (■, □). Data are shown for *Mixta*⁺ flowers (●, ■) grown under low-UV conditions and for *mixta*⁻ flowers (○, □) grown under both low- and high-UV conditions. Linear regression lines for *Mixta*⁺ (—) and *mixta*⁻ (---) are shown separately in a, and a single regression line is shown in b. See text for details.

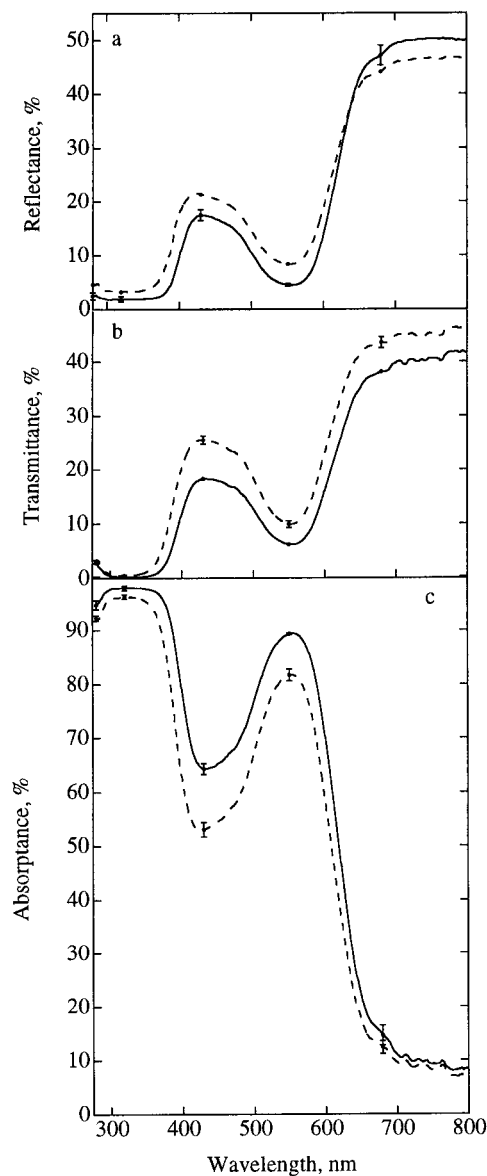


Figure 7. Reflectance (a), transmittance (b), and absorbance (c) spectra of discs from *Mixta*⁺ (—) and *mixta*[−] (---) petals. All plants were grown under low-UV conditions. Spectra are averages of five to six individual spectra, and error bars at selected wavelengths represent *SES*.

focal intensification and focal length for epidermal cells from flowers.

UV Penetration through the Epidermis

About 50 to 75% of the difference in absorption of UV light by individual *Mixta*⁺ and *mixta*[−] epidermal cells can be attributed to differences in cell shape, an effect caused by the differences in pathlength or focal length. The pathlength of a ray proceeding straight through the middle of a *Mixta*⁺ epidermal cell is 50 to 60 μm ; in *mixta*[−] petals it is only about 25 μm . Clearly, this 2-fold difference in pathlength could have an effect on absorption of light. In ad-

dition, the bending of light by refraction, as it is focused by epidermal cells, increases pathlength, and this effect would be more pronounced in *Mixta*⁺ than *mixta*[−] cells. Focal length of the epidermal cells would alter light absorption because it would affect the proportion of light passing through the pigmented central region of epidermal cells as compared with the unpigmented, peripheral (wall and cytoplasmic) regions. The tall *Mixta*⁺ epidermal cells, with their relatively short focal length, concentrate light within their pigmented vacuoles, where it is more likely to be absorbed. The short *mixta*[−] epidermal cells, with a larger radius of curvature and longer focal length, direct a smaller proportion of the light through the pigmented vacuole; the focal plane for *mixta*[−] epidermal cells is within the unpigmented mesophyll.

The difference observed for maximum absorption of UV light by individual epidermal cells was small, corresponding to a difference in transmittance of 5% or less, in spite of the pronounced differences in pathlength and focusing between the two lines. This is probably because UV absorption was very high even in the *mixta*[−] epidermal cells; they are effectively black to UV light. Thus, optimizing absorption in the *Mixta*⁺ cells by increasing pathlength or focusing UV on the vacuole would have a minimal effect.

Although there was a significant difference between the maximum UV absorbance by central regions of individual *Mixta*⁺ and *mixta*[−] epidermal cells, the overall absorbance by epidermal peels, including both the central and peripheral regions of cells, was not significantly different for the two lines. This result is reasonable because differences in pigmentation and cell shape between the two lines are most pronounced in the central regions of the cells, whereas most UV light penetrates the epidermis through the peripheral regions of the cells.

Up to 50% of incident UV-B at 312 nm was transmitted through the epidermal cell periphery, whereas virtually no UV-B penetrated through the central regions (Fig. 5). Thus, potential cellular sites for UV-B damage within the epidermis include the adaxial and periclinal regions of the plasmalemma and cytoplasm. In the underlying tissues, potential sites for damage are limited to those portions of cells

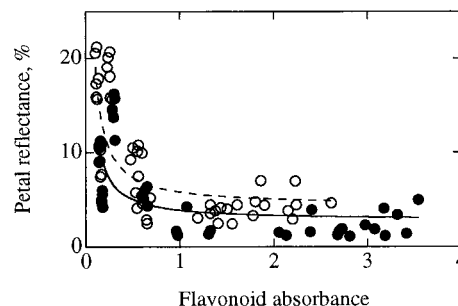


Figure 8. Relationship between reflectance of *Mixta*⁺ (●) and *mixta*[−] (○) petals and the corresponding absorbance of extracted flavonoids. Data for petal reflectance were taken from those used to construct Figure 7; data for 280, 290, 312, 362, 400, 480, 530, 550, and 570 nm were combined. Hyperbolic fits to the data are shown for *Mixta*⁺ (—) and for *mixta*[−] (---). See text for details.

that are located beneath the epidermal periclinal cell walls that serve as channels for UV-B penetration.

Optical Properties of Intact Petals

Differences in the optical properties of discs from intact petals of *Mixta*⁺ and *mixta*⁻ lines are consistent with our observations of epidermal peels. In the UV, where absorbance by pigments in the central vacuole is very high, there are no significant differences in reflectance or transmittance between the two lines. Again, if absorption by the epidermis is very high in *mixta*⁻ cells, increased pigmentation and focal effects of the *Mixta*⁺ cells would not increase absorption significantly. In contrast, there were significant differences in the transmittance and reflectance of *Mixta*⁺ and *mixta*⁻ petals in the blue and green, where flavonoid absorbance was intermediate and increasing absorbance by increasing pathlength or optimizing focus would have a measurable effect.

The results reported here on UV penetration through the epidermis and intact petals have implications for penetration of UV light into leaves as well as flowers. The role of epidermal flavonoids as protectants from damage by UV-B is well established (see the introduction). Our results suggest that the job of protection could be performed equally well by flattened or papillate epidermal cells, provided they like *Antirrhinum* contain high levels of flavonoids. With most of the UV-B penetration occurring through the peripheral regions of the cell, tissues underlying either domed or flattened epidermal cells would be subjected to similar hot-spots of potential UV damage corresponding to these regions. However, if the flavonoid level and absorption of UV-B were lower, such that significant penetration occurred through the central regions of the cell, epidermal cell shape could influence the amount of protection provided to underlying tissues, and positioning the absorbing pigment in domed or papillate epidermal cells could confer an advantage.

Our results, which show little effect of cell shape on UV reflectance from intact *Antirrhinum* petals, are in contrast to an earlier report linking papillate shape of flower epidermal cells to high UV absorption in petals (Brehm and Krell, 1975). It is difficult to compare the two studies directly, however, because different techniques were used to characterize flavonoid content. In the earlier paper, which focused mainly on the localization of flavonoid pigment, the presence or absence of flavonoids was determined using fluorescence microscopy, color changes of flavonoids under alkaline conditions, and paper chromatography. In this study we quantified the relative amount of extracted flavonoids spectrally, so we were able to correlate absorbance of extracted flavonoids at specific wavelengths with the optical properties of petals at those same wavelengths. It is possible that the *Antirrhinum* petals contain more UV-absorbing pigment than the petals examined by Brehm and Krell (1975) so that effects of cell shape are less pronounced. Also, Brehm and Krell noted a concentration of UV-absorbing pigment in the tips of the papillate cells, and this localization could affect the UV absorption characteristics of the cells.

Absorption of visible light is generally lower than absorption of UV light in both leaves and flowers, and with some notable exceptions, the absorbance of visible light by leaf epidermal cells is negligible. However, visible pigmentation in the epidermis of flowers is commonplace and important for attracting pollinators (Kevan, 1983). Of course, at wavelengths at which absorption is negligible (e.g. 680 nm for *Antirrhinum*), epidermal cell shape should make little difference. But at wavelengths with intermediate absorbance, cell shape can affect visible light penetration and appearance of the organs, and this could affect visual cues presented to pollinators. Increased absorbance by the epidermis that is associated with positioning the pigment in papillate epidermal cells leads to more saturated appearance of flower color in *Antirrhinum*. This effect on saturation may have importance to pollinator behavior. One interpretation of floral UV patterns is that UV absorption increases color saturation and that bees use color saturation as an orientation cue (Lunau, 1992). Removing UV rays from the reflected light would make the remaining (visible) wavelengths that the bees see more spectrally pure, and hence the color would appear more saturated. Increased efficiency of absorption by visible pigments such as anthocyanin could have the same effect—removing some wavelengths from the reflected light, increasing color saturation, and perhaps providing stronger orientation cues for pollinators.

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