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Light filtering by epidermal flavonoids during the resistant response of cotton to *Xanthomonas* protects leaf tissue from light-dependent phytoalexin toxicity

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Lacinilene C
Quercetin-3-*O*-β-glucoside
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ABSTRACT

2,7-Dihydroxycadalene and lacinilene C, sesquiterpenoid phytoalexins that accumulate at infection sites during the hypersensitive resistant response of cotton foliage to *Xanthomonas campestris* pv. *malvacearum*, have light-dependent toxicity toward host cells, as well as toward the bacterial pathogen. Adaxial epidermal cells surrounding and sometimes covering infection sites turn red. The red cells exhibited 3–4-fold higher absorption at the photoactivating wavelengths of sunlight than nearby colorless epidermal cells. Red epidermal cells protected underlying palisade mesophyll cells from the toxic effects of 2,7-dihydroxycadalene plus sunlight, indicating a role for epidermal pigments in protecting living cells that surround infection sites from toxic effects of the plant's own phytoalexins. A semi-quantitative survey of UV-absorbing substances extracted from epidermal strips from inoculated and mock-inoculated cotyledons indicated that the principal increase in capacity to absorb the photoactivating wavelengths was due to a red anthocyanin and a yellow flavonol, which were identified as cyanidin-3-*O*-β-glucoside and quercetin-3-*O*-β-glucoside, respectively.

1. Introduction

Red pigmentation is often associated with plant disease resistance (Hipskind et al., 1996; Kangatharalingam et al., 2002; Kumar et al., 1997; Punyasiri et al., 2005), and there is some evidence that anthocyanins can contribute to resistance (Gandikota et al., 2001; Hipskind et al., 1996). The most obvious sign of the hypersensitive resistant response in upland cotton (*Gossypium hirsutum* L.) leaves and cotyledons to *Xanthomonas* infection (provided that the inoculum density is low enough that the entire tissue does not die) is a dark red flush (Rowlan et al., 1991). Its intensity is correlated with the level of bacterial blight resistance (Kangatharalingam et al., 2002). Epidermal cells near infection sites turn red, beginning with the subsidiary cells of the stomatal complex (Kangatharalingam et al., 2002). When plants in the field are infiltrated with dilute inocula, highly resistant cotton lines develop small, round, dark lesions (Brinkerhoff et al., 1984). We show here that these lesions consist of a center of hypersensitively necrotic cells surrounded and sometimes covered by red epidermal cells.

Another characteristic of the resistant response of cotton foliar tissue to *Xanthomonas* is accumulation of sesquiterpenoid phytoalexins in the mesophyll cells closest to microcolonies of the bacterial pathogen (Pierce and Essenberg, 1987; Pierce et al., 1996). These phytoalexins are light-activatable: when exposed to ultraviolet light, they generate free radicals, can inactivate enzymes and nick DNA, and are more toxic to the bacterial pathogen than in the dark (Steidl, 1988; Sun, 1987; Sun et al., 1989). Zeringue reported that two of the phytoalexins, lacinilene C **1** (LC) and lacinilene C 7-methyl ether **2** (LCME), are also toxic to the plant *Lemna minor* L. (lesser duckweed) cultured with a light/dark cycle (Zeringue, 1987). In this work, we tested whether LC **(1)** and its

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precursor, 2,7-dihydroxycadalene **3** (DHC) have light-dependent toxicity to the cotton leaf's own mesophyll cells and found that they do.

Since anthocyanins, which are the principal red pigments of plants, and other flavonoids can function as antioxidants or as scavengers of light-generated free radicals (Laughton et al., 1989; Tsuda et al., 1994), one might speculate that the red flush provides antioxidants to protect the plant tissue from the phytoalexin-generated free radicals. However, tissue distributions of the compounds are inconsistent with this idea: the red pigment is concentrated in the vacuoles of epidermal cells (Kangatharalingam et al., 2002), whereas the phytoalexins are in the underlying palisade and spongy mesophyll tissue (Essenberg et al., 1992). One of us (Sun) proposed that the red epidermal flush functions instead to protect healthy mesophyll tissue surrounding infection sites by acting as a filter or "sunscreen", absorbing the active wavelengths of sunlight. This role would be similar to that of the flavonoids that have been shown to protect plant DNA from ultraviolet-induced damage (Schmitz-Hoerner and Weissenboeck, 2003; Stapleton and Walbot, 1994). Localization of sunscreen flavonoids in the adaxial epidermis has been shown in barley (Schmitz-Hoerner and Weissenboeck, 2003) and in three woody species (Bidel et al., 2007). In the latter study, it was found that epidermal absorbance at 375 nm resulted exclusively from flavonols and flavones (Bidel et al., 2007).

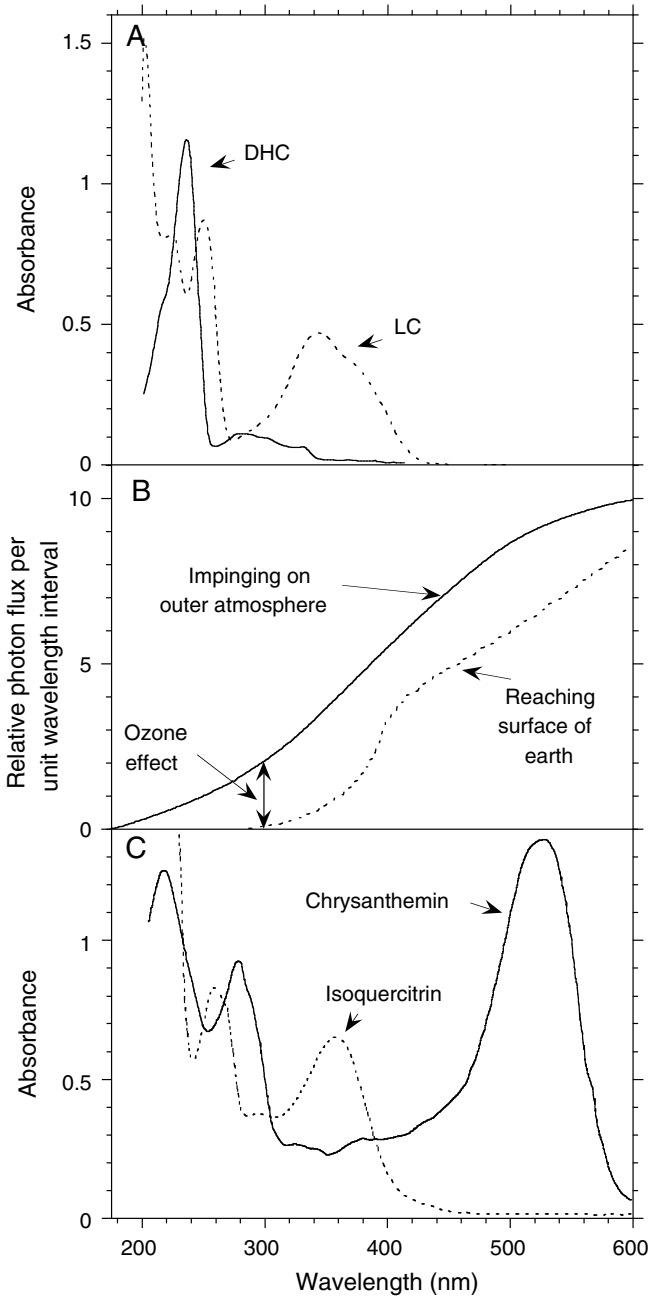


Fig. 1. Spectral properties of sesquiterpenoid phytoalexins and flavonoids from *Xanthomonas*-inoculated cotton leaves and of sunlight. (A) Absorption spectra of sesquiterpenoid phytoalexins: 2,7-dihydroxycadalene (**3**) (7.9 μM in MeOH) λ^{MeOH} nm (log ε): 237 (4.861), 331(3.51) (Essenberg et al., 1982, 1990); lacinilene C (**1**) (37 μM in MeOH) λ^{MeOH} nm (log ε): 251 (4.067), 343 (3.805) (Essenberg et al., 1990). (B) Wavelength distributions of the sun's photons incident on the earth's atmosphere and its surface. (Data are re-plotted from p. 172 of (Nobel, 1970); used by permission.) (C) Absorption spectra of flavonoids: chrysanthemin (**4**) (39 μM in MeOH with 1% HCl to keep the anthocyanin in its acidic form) $\lambda^{MeOH\ (1\%\ HCl)}$ nm (log ε): 282 (4.38), 331 (3.65) 528 (4.54) (this work); isoquercitrin (**5**) (40 μM in MeOH) λ^{MeOH} nm (log ε): 258 (4.29), 331 (4.04), 360 (4.21) (this work).

The range of wavelengths that would need to be absorbed to protect cotton tissue from its own phytoalexins can be predicted from the known absorption spectra of the phytoalexins and from the spectrum of sunlight. UV–visible absorption spectra of the two most potent light-activatable phytoalexins of cotton foliage, DHC (**3**) and LC (**1**), are shown in Fig. 1A. DHC (**3**) is several-fold more toxic to *Xanthomonas campestris* pv. *malvacearum* (*Xcm*) than LC (**1**) in the light, as well as in the dark (Essenberg et al., 1990; Steidl, 1988; Sun, 1987; Sun et al., 1989). The ultraviolet wavelengths that are most intensely absorbed by these compounds are, however, essentially absent from sunlight that reaches the earth's surface due to their absorption by ozone in the stratosphere (Fig. 1B). Therefore, the wavelengths of sunlight capable of activating DHC (**3**) and LC (**1**) are centered about their longer wavelength absorption maxima at 331 nm and 343 nm, respectively, and span the range from the shortest wavelength of incident sunlight ≈290 nm (Fig. 1B), to the longest wavelength absorbed by the phytoalexins, ≈420 nm (Fig. 1A). Thus UV-A radiation (315–400 nm) would be the most damaging, although UV-B (290–315 nm) radiation would also contribute. Further objectives of this study were to determine whether red epidermal cells of cotton leaves absorb light of those wavelengths more than colorless cells do, and, if so, to determine whether the red cells can protect underlying mesophyll cells from the toxic phytoalexins and to identify pigment(s) that are responsible for the increased absorption by red cells.

2. Results and discussion

2.1. Development of red pigment in epidermal cells near infection sites in resistant leaves

The pinpoint-sized lesions induced by dilute inocula of *Xcm* in field-grown, highly resistant cotton lines OK1.2 and WbM(0.0) are the results of a hypersensitive resistant response (Brinkerhoff et al., 1984; Essenberg et al., 1979). When they were examined with a microscope, epidermal cells surrounding and sometimes over the infection sites were found to be red (Fig. 2A). When examined with fluorescence optics (Fig. 2B), the lesion centers exhibited the yellow-green fluorescence that has been shown to be principally due to the sesquiterpenoid phytoalexins LC (**1**) and LCME (**2**) (Essenberg et al., 1992).

We have observed red epidermal cells as early as 40 hpi (Kangatharalingam et al., 2002). In this study under field conditions, the red rings were more than half-formed by 5 dpi and were complete or nearly so by 10 dpi.

The "parasol" of red epidermal cells always extended beyond the cluster of dead cells and overlay the neighboring, surviving palisade mesophyll cells. Although the phytoalexins are most concen-

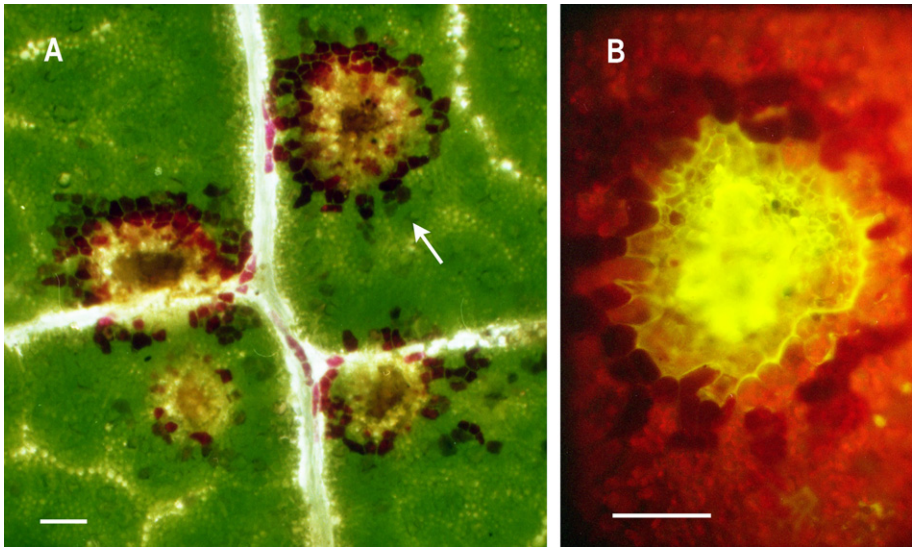


Fig. 2. Sites of resistant responses in a leaf of cotton line OK1.2 to *Xanthomonas campestris* pv. *malvacearum* in the field. Fresh tissue four weeks post-inoculation with *ca* 2×10^6 cfu ml^{-1} of *Xcm*; (A) Transmitted white light micrograph showing four sites of attempted infection, with red epidermal cells surrounding the areas of hypersensitive resistant responses; and (B) Fluorescence (>520 nm) from the site indicated with an arrow in (A) when viewed with blue excitation (460–485 nm). The yellow–green autofluorescence is mainly due to sesquiterpenoid phytoalexins LC (**1**) and LCME (**2**) concentrated at the infection site (Essenberg et al., 1992), and red fluorescence is due to chlorophyll in surrounding healthy tissue. Scale bars = 100 μm.

trated in the dead cells (Pierce and Essenberg, 1987), an efflux experiment showed that they can diffuse outward, and the fluorescence of LC (**1**) and LCME (**2**) is visible in the cell walls of living cells adjacent to the dead cells [(Essenberg et al., 1992) and Fig. 2B]. A ^{13}C-pulse-labeling study suggested that surviving cells which do not undergo the hypersensitive response even carry out some phytoalexin biosynthesis that continues after hypersensitive cell death is complete (Górski et al., 1995). Thus the living cells adjacent to the infection site are exposed to the phytoalexins.

2.2. Light-dependent toxicity of sesquiterpenoid phytoalexins to leaf tissue

When healthy OK1.2 leaves were infiltrated with solutions of DHC (**3**), toxicity was manifested several days later by scattered Evan's-blue-stainable palisade cells (Fig. 3) or, with relatively high DHC (**3**) concentrations, by tissue collapse. Plants maintained in continuous darkness after infiltration exhibited less damage, and plants infiltrated simply with the solvent (5% aq. MeOH) exhibited little damage under either light or dark conditions. In a similar experiment, LC (**1**) also showed light-dependent toxicity: healthy OK1.2 leaves infiltrated with 0.21 mM LC (**1**) and incubated 72 h in a normal light/dark cycle were found to have on average 36 dead cells/mm^2, significantly more ($P < 0.05$) than with 0 mM LC (**1**) (i.e., 5% aq. MeOH) in the light/dark cycle, or with 0.21 mM LC (**1**) in the dark, or with 0 mM LC (**1**) in the dark (averages: 10, 3, and 0.3 dead cells/mm^2, respectively). These data should not be interpreted to show that LC (**1**) has greater toxicity to cotton leaves than DHC (**3**), since the two phytoalexins were not tested in the same experiment, and sensitivities of leaves to the phytoalexins varied from experiment to experiment, perhaps due to variations in light intensity and condition of the plants. On one occasion, 0.2 mM DHC (**3**) was observed to cause more damage than 0.2 mM LC (**1**) did in the above experiment. What was consistently observed was that treatment with either phytoalexin in a light/dark cycle resulted in more cell killing than the three control treatments.

During the natural hypersensitive response of resistant cotton leaves to *Xcm*, DHC (**3**) and LC (**1**) have been observed to attain local concentrations of 1-7 mM at infection sites (Pierce et al., 1996).

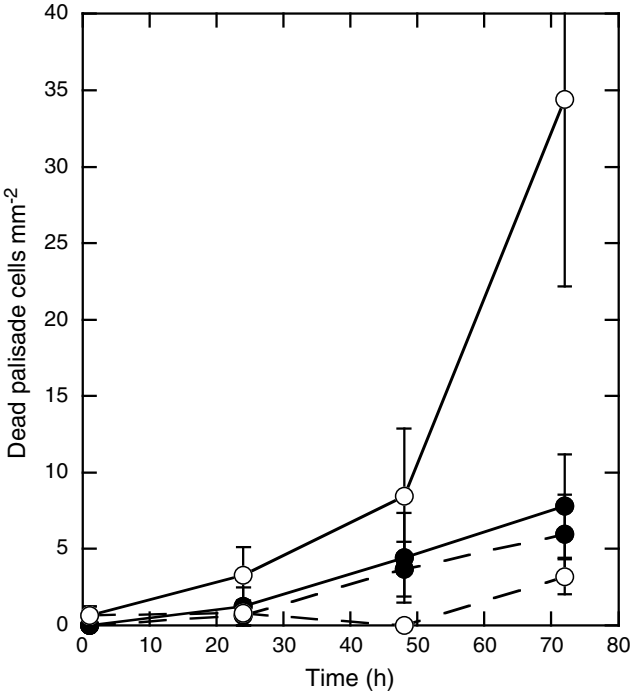


Fig. 3. Killing of leaf mesophyll cells by exposure to the sesquiterpene phytoalexin 2,7-dihydroxycadalene (**3**) (DHC) and light. Healthy leaves of OK1.2 *G. hirsutum* were infiltrated with 0 mM (dashed lines) or 0.58 mM (solid lines) DHC (**3**) in MeOH–H_2O (5:95, v/v) (4 leaves/treatment), and the plants were incubated in a growth chamber in a normal light/dark cycle (○) or in continuous darkness (●). Dead cells were detected by Evan's blue staining. Points show arithmetic means ± S.E of six to nine 0.41-mm^2 fields. At 72 h, the value for DHC (**3**) in the light/dark cycle was significantly higher than values for the other three treatments ($P < 0.05$).

The phytoalexins are probably diluted from these concentrations as they diffuse from the hypersensitively responding infection centers into surrounding healthy tissue (Essenberg et al., 1992). Although the 0.2 mM–0.5 mM solutions of DHC (**3**) and LC (**1**) whose phytotoxicity is reported in the paragraph above were certainly altered by transpiration or absorption of the infiltration

fluid and by absorption of the phytoalexins into cells, their concentrations were probably within the naturally occurring ranges. We conclude that the living cells surrounding infection sites in resistant cotton leaves are probably exposed to phytotoxic levels of phytoalexins.

The cell killing by exogenous DHC (**3**) and LC (**1**) required two to three days (Fig. 3 and data not shown). It is unlikely that the phytoalexins were stable in the leaf tissue for that entire period. On one occasion, we infiltrated leaves with 1.0 nmol DHC (**3**)/cm^2, then extracted and quantitated it at 24 h. Twenty-one percent was recovered from a leaf maintained in the dark and 9% from a leaf maintained in the light/dark cycle. The leaf cell killing, which is only detected after 48–72 h, may be the result of damage by light-activated phytoalexins which only after a delay results in cell death or, alternatively, may be the result of damage by phytoalexin in a light-independent process that makes the cell vulnerable to subsequent, lethal effects of light. We think the first of these mechanisms is more probable. In reactions with purified components, DHC (**3**) has been shown to cause photo-activated nicking of DNA and inactivation of enzymes (Sun et al., 1989). UV radiation at the absorbance maxima of DHC (**3**) was more effective than radiation of 400, 500, or 600 nm wavelengths in promoting DHC (**3**)-induced nicking DNA, and the DNA nicking was inhibited by scavengers of reactive oxygen species and of free radicals (Sun et al., 1989). Therefore, damage to vital leaf cellular components by sunlight-activated phytoalexins seems the likely cause of the plant cell killing observed here.

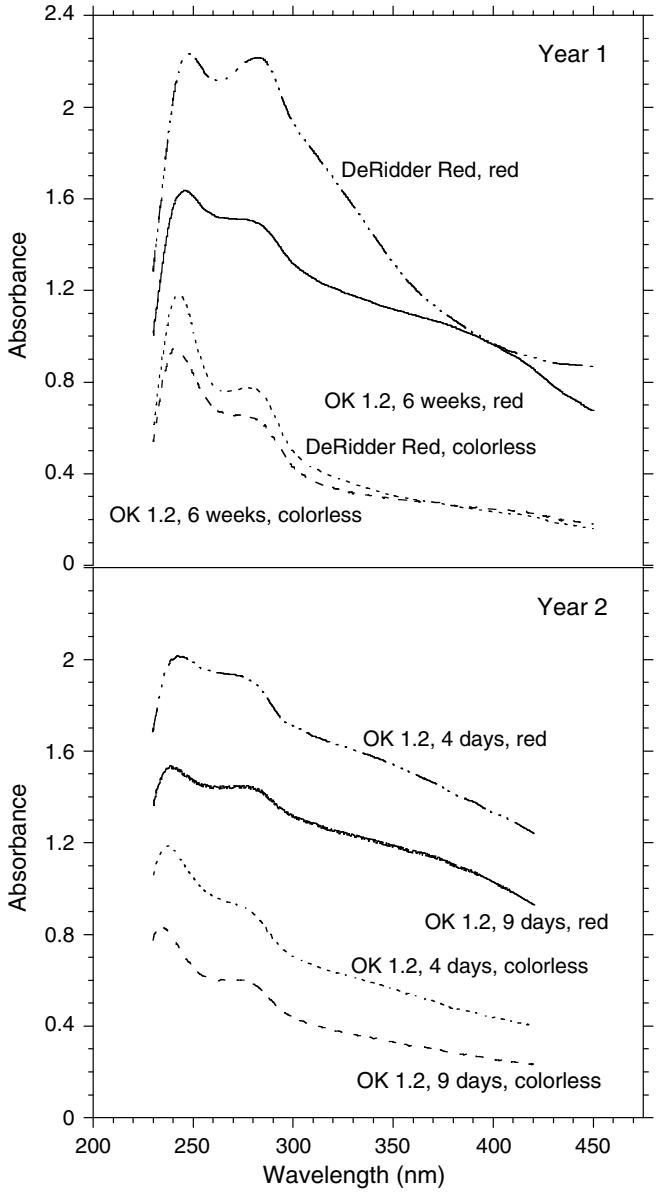


Fig. 4. Microspectrophotometry of red and colorless cells in the adaxial epidermis of field-grown cotton plants in two summers. (A) Year 1. (B) Year 2. Leaves of bacterial blight-resistant OK1.2 plants were spray-inoculated with *X. campestris* pv *malvacearum* (inoculum concentration was *ca* 2×10^6 cfu/ml), and leaves were harvested for analysis after various periods as indicated in the figure. De Ridder Red plants were not inoculated. Each line is the mean of spectra from ten epidermal cells. To compensate for variations in thickness of the specimens, spectra were taken on red and colorless cells within 2–8 cell diameters from each other.

2.3. Microspectrophotometry of epidermal strips

Foliage leaves of bacterial blight-resistant line OK1.2 cotton plants growing in an Oklahoma field were inoculated with *Xcm* by infiltration. Inoculated areas turned dark red during the days that followed. Non-inoculated leaves of the red-leafed cultivar De Ridder Red were also examined to learn whether this line could provide model plants for a test of the photoprotective efficacy of the red cells. When viewed under the microscope, the adaxial epidermal cell layers of both inoculated OK1.2 and non-inoculated De Ridder Red leaves were mosaics of red and apparently colorless cells, similar to those illustrated in (Kangatharalingam et al., 2002). (Approximately 20–50% of epidermal cells were red.) Pieces of the adaxial (upper) epidermal cell layer were peeled from the leaves, and UV–visible absorption spectra of red and of colorless cells were recorded (Fig. 4). Spectra recorded in two different years and at different times after inoculation differed somewhat in shape. However, at the relevant wavelengths, 290–420 nm, all leaf samples showed three- to four-fold higher absorbance by red cells than by colorless cells. The absorbance values of red cells were high, between 0.8 and 2.2. When converted to percent transmittance, the absorbance data of Fig. 4 indicate that at the λ_{max} of DHC (**3**), 331 nm, red epidermal cells transmitted only 6–14% as much of the incident sunlight as colorless cells (Table 1).

The answer to the question posed in the Introduction is that red epidermal cells of inoculated resistant cotton leaves do absorb incident light several-fold more strongly than colorless cells throughout the absorption range of the light-activatable phytoalexins, absorbing about 95% of the incident light (Fig. 4 and Table 1).

2.4. Protection of leaf tissue from phytoalexin toxicity by red epidermal cells

The presence of a mosaic of red and colorless epidermal cells in healthy, non-inoculated leaves of De Ridder Red and the similarity

Table 1
Spectral properties of the adaxial epidermis of field-grown cotton plants[a]

Cotton line and sample description[b]	Cell type	A_{331}	$\%T_{331}$	Ratio $\%T_{red}/\%T_{colorless}$
Year 1				
De Ridder Red	Red	1.54	2.9	0.06
	Colorless	0.35	45	
OK1.2, six weeks	Red	1.18	6.6	0.14
	Colorless	0.32	48	
Year 2				
OK1.2, four days	Red	1.60	2.5	0.10
	Colorless	0.61	25	
OK1.2, nine days	Red	1.23	5.9	0.13
	Colorless	0.36	44	

[a] Each value is the mean from ten cells.
[b] De Ridder Red was not inoculated. For OK1.2, times after inoculation are indicated; see Fig. 4 for details.

of these cells' absorption spectra to those of red and colorless epidermal cells in inoculated OK1.2 leaves (Fig. 4) indicated that De Ridder Red leaves could be used to test the photo-protective effect of the red cells toward exogenous phytoalexin.

Leaves of De Ridder Red growing in the field were infiltrated with 0, 0.58, or 0.69 mM DHC (**3**). Seven days later, the frequency of dead palisade cells per overlying red epidermal cell was found to be significantly lower than the frequency of dead cells per overlying colorless epidermal cell in leaves that had received each of the three infiltrations (Fig. 5). Similar experiments were performed two other times outdoors and twice in the growth chamber. The frequency of dead palisade cells per red epidermal cell was in all experiments lower than the frequency per colorless epidermal cell.

The data of Fig. 5 for 0 mM DHC (**3**) show that infiltration with 5% aq. MeOH had a small toxic effect and that the red epidermal cells protected against that also.

2.5. Extraction and identification of pigments

The broad, rather featureless absorption spectra of the leaf cells shown in Fig. 4 suggest the presence of multiple pigments. The pigments were extracted from leafy cotyledons of resistant cotton plants, rather than their leaves, because of the ease with which cotyledons can be infiltrated with inoculum. The resistant responses of cotton leaves and cotyledons to *Xcm* are phenotypically similar (Essenberg et al., 1992; Pierce and Essenberg, 1987; Pierce et al., 1996). For extraction of the red pigment, we used WbM*gl*, a glandless derivative of WbM(0.0) (Davis and Essenberg, 1995), to avoid contamination from the anthocyanin that is present in the lysigenous glands. When acidic methanol/water (80:20, v/v) extracts were fractionated by reversed phase chromatography on octyldecylsilane followed by adsorption chromatography on cross-linked polyvinylpyrollidone, a single red pigment, R1, was isolated. Its UV–visible absorption, mass, and NMR spectra agreed with published data for cyanidin-3-*O*-β-glucoside (**4**) (Andersen, 1988; Francis, 1982; Van Calsteren et al., 1991). Its ^{1}H–^{1}H COSY and ^{1}H–^{13}C HETCOR spectra established all connectivities and correlations expected for this compound. We concluded that R1 is the common anthocyanin of leaves, cyanidin-3-*O*-β-glucoside (**4**) (chrysanthemin). It has previously been identified as the only anthocyanin of cotton buds and flowers (Hedin et al., 1967) and found to increase in leaves in response to the insecticide methomyl (Parrott et al., 1983).

Chrysanthemin (**4**) has an absorption maximum at 282 nm (Fig. 1C), corresponding to one of the maxima in the red cell spectra (Fig. 4). However, the phytoalexins' absorption maxima of 331 and 343 nm lie in an absorption valley for chrysanthemin (**4**) – the shape of its absorption spectrum suggests that it is not the principal chromophore responsible for the absorption exhibited by the red epidermal cells at those wavelengths. Apparently one or more other chromophores are present.

A semi-quantitative survey was made of the pigments present in the epidermal cell layer of inoculated OK1.2 cotyledons. To obtain enough epidermal tissue for this survey, the abaxial (lower) epidermis was examined, because it is easier to excise from cotyledons than the adaxial epidermis. Since the epidermal layer that turns red is the one exposed to direct light, the cotyledons (still attached to their plants) were turned upside down after inoculation to expose the abaxial epidermis to direct light and were held in that position until harvest.

Epidermal strips of both *Xcm*-inoculated and mock-inoculated cotyledons were extracted with petrol and then with acidic methanol. Water was added to the methanol extract, the methanol was evaporated, and the water was extracted with hexane and then with EtOAc. Absorption spectra of all extracts were recorded. The principal absorption intensities at 331 and 343 nm were in the red, residual aqueous fraction (which on the basis of its spectrum evidently contained another near-UV-absorbing material, Y1, in addition to chrysanthemin (**4**)) and in the EtOAc extract. The EtOAc-extracted material was subjected to size-exclusion and normal-phase chromatography, yielding two fractions with 331 and 343 nm absorption, Y2 and Y3. However, of these three yellow, UV-A-absorbing pigments, only Y3 exhibited higher absorbance in the extract from *Xcm*-inoculated tissue than in the extract from mock-inoculated tissue (Table 2). When subjected to normal-phase HPLC, Y3 exhibited the same retention time as authentic isoquercetrin (**5**).

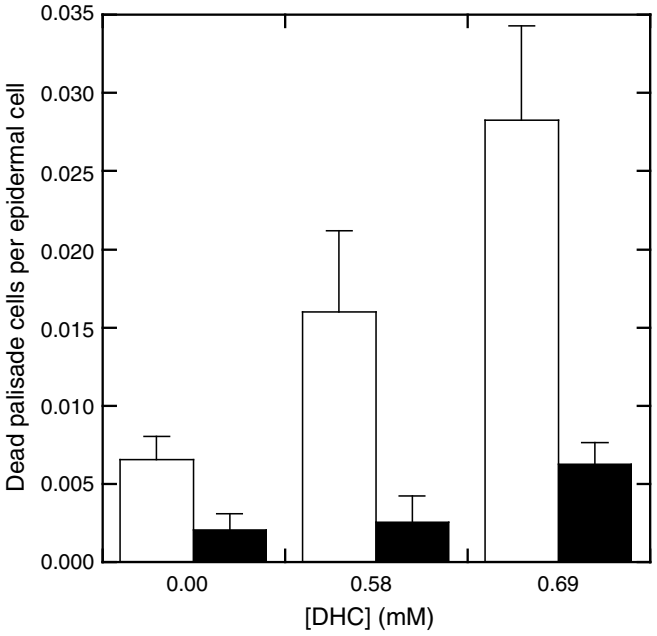
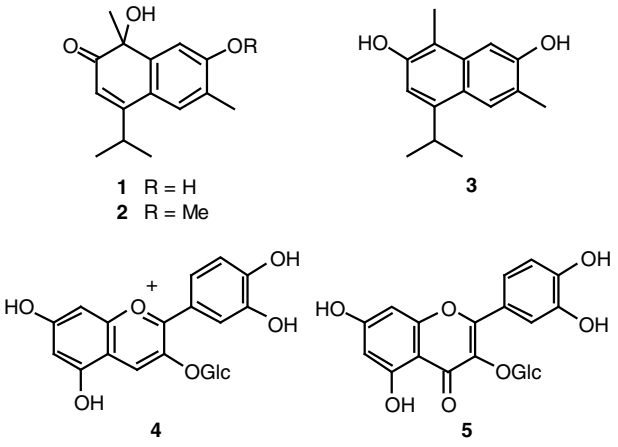


Fig. 5. Protection of leaf mesophyll cells from toxic effect of 2,7-dihydroxycadalene (**3**) and sunlight by overlying red epidermal cells. Bars show frequency of dead palisade mesophyll cells per over-lying colorless (open bars) or red (dark bars) epidermal cell. Healthy leaves of De Ridder Red *G. hirsutum* growing in the field were infiltrated with 0 and 0.58 mM or 0 and 0.69 mM 2,7-dihydroxycadalene (**3**) in MeOH–H_2O (5:95, v/v) (3 leaves per pair of treatments). At 7 days, leaves were harvested, and dead cells were detected by Evan's blue staining. Bars show arithmetic means ± S.E of 33 (0 mM), 14 (0.58 mM), or 27 (0.69 mM) 0.41-mm^2 fields. Frequency of dead cells was significantly less under red epidermal cells (0 and 0.69 mM, $P < 0.01$; 0.58 mM, $P < 0.025$).



The isolation was scaled up to obtain pigment quantities sufficient for identification, starting with whole cotyledons. A pig-

ment with the chromatographic and spectral characteristics of Y3 was isolated. Comparison of its UV–visible absorption and one- and two-dimensional NMR spectra with those of an authentic sample and with reported data (Jurd, 1962; Slimestad and Hostettmann, 1996) demonstrated that fraction Y3 contained the flavonol glycoside quercetin-3-*O*-β-D-glucoside (**5**) (isoquercitrin) plus another, unidentified component. (The sample did not ionize well during electrospray ionization mass spectral analysis, but did yield low-intensity $m/z = 462.9$ ions like those of standard isoquercitrin (**5**).) In contrast to the red anthocyanin chrysanthemin (**4**), the yellow flavonol glycoside isoquercetrin (**5**) absorbs strongly at 331 and 343 nm (Fig. 1C). Thus, isoquercitrin (**5**) is a candidate for the pigment that absorbs at those wavelengths in red epidermal cells (Fig. 4). It has also been observed to increase in cotton leaves in response to infection with *Verticillium dahliae* (Howell et al., 1976). Gould and Lister, in reviewing functions of the various kinds of flavonoids in plants, concluded that the flavonols and flavones are "highly effective UV filters" (Gould and Lister, 2006).

The observed absorption spectra of the red epidermal cells differ from those of chrysanthemin (**4**) and isoquercetrin (**5**) at the very shortest wavelengths: absorption by the red cells appears to fall below 240 nm (Fig. 4), whereas absorption by both flavonoids rises below that wavelength (Fig. 1C). However, sensitivity of the microspectrophotometer is low near its short-wavelength limit of 230 nm, and we think this disparity is not real.

2.6. Cellular concentrations of flavonoids

Estimates of the concentrations of chrysanthemin (**4**) and isoquercitrin (**5**) in the red epidermal cells were made, using absorbance values of Fig. 4 and the known molar extinction coefficients of these two identified pigments (see Fig. 1). The path-length of light through foliage leaf epidermal cells was determined by microscopic examination of cross-sections of fresh leaves to be, on average, 25 μm. We made the simplifying assumption that the higher absorbance in red cells at the 282 nm peak was entirely due to chrysanthemin (**4**), since it was the only pigment isolated that had an absorption maximum near that wavelength and was the only fraction whose A_{282} was much higher from inoculated epidermal strips than from mock-inoculated strips. Since both inoculated and control epidermal samples contained similar amounts of the unidentified yellow pigments Y1 and Y2, we assumed that the difference in absorbance at 331 nm between red and colorless cells was due to isoquercitrin (**5**) produced in response to infection. These rather simplistic assumptions led to the upper estimates of concentration increases of chrysanthemin (**4**) and isoquercitrin (**5**) in the red epidermal cells that are given in Table 3.

The estimated cellular concentration differences of Table 3 may be compared with the differences in extracted amounts reported in Table 2. The values in Table 3 are 10- to 20-fold higher. This contrast is not surprising, given that: (1) the inoculated leaf epidermal strips from which Table 2 data were derived were a mosaic of colorless as well as red cells; (2) the plants used for Table 2 were from a growth chamber, where less intense anthocyanin color is observed than in field conditions under sunlight, where the plants used for Table 3 were grown; (3) some losses of flavonols would have occurred during isolation.

The upper estimates of cellular concentrations of both pigments (Table 3) were in the range 14–43 mM. Although we place little reliance on the precision of these estimates, these concentrations are easily high enough for co-pigmentation, i.e., reversible formation of a complex, to be occurring between the anthocyanin and the flavonol glycoside (Osama, 1982). Co-pigmentation can result in shifts of absorption maxima, usually to longer wavelengths, increases in absorption intensity, and stabilization of the anthocyanin against oxidation (Brouillard et al., 1997).

3. Concluding remarks

The red flush which develops in cotton foliage during a resistant response to *Xcm* involves more than the absorption of green light that gives the leaves red color – there is also increased broad absorption throughout the UV–B and UV–A ranges of sunlight. Although all pigments responsible for this increase have not been identified, chrysanthemin (**4**) and isoquercitrin (**5**) were identified as the principal UV-absorbing compounds enriched in epidermal tissue of bacterial blight-resistant cotton cotyledons in response to *Xcm* infection. In De Ridder Red, the red-leafed cotton cultivar shown here to have both red and colorless epidermal cells with absorption spectral characteristics similar to those of inoculated resistant cotton leaves, this filtering of the highest-energy photons of sunlight by the epidermal cell layer protected the underlying palisade mesophyll cells from damage by the leaf's own photoactivatable phytoalexin 2,7-dihydroxycadalene (**3**). Thus, accumulation of UV-absorbing flavonoids in epidermal cells surrounding bacterial infection sites protects the living mesophyll cells that surround the dead cell cluster of the hypersensitive resistant response from the sunlight-dependent toxicity of the sesquiterpenoid phytoalexins produced during that response.

4. Experimental

4.1. General

For micrography, disks of fresh leaf tissue were mounted in water and examined using a Nikon Optiphot microscope. Fluorescence was excited with a 100 W mercury lamp; filters were 460–485 nm (excitation) and >520 nm (emission). UV–visible absorption spectra were recorded with a Zeiss UMSP80 microspectrophotometer with a xenon illuminator on epidermal strips mounted in water on a quartz slide under a quartz cover slip. The FAB-MS of chrysanthemin (**4**) was determined with thioglycerol as solvent matrix and with ionization by Ce ions at 35 kV. For EI-TQ-MS analysis in the negative ion mode, isoquercetrin (**5**) in MeOH was mixed with 0.2–0.3 part of aq. 20 mM NH_4OAc; electrospray ionization was at -3.8 kV. For chrysanthemin (**4**), 400 MHz ^1H and ^1H–^1H COSY NMR spectra were recorded in CD_3OD/0.02 M DCl; 400 MHz ^{13}C and ^1H–^{13}C HETCOR spectra were recorded in CD_3OD/1.0 M CD_3CO_2D. Tetramethylsilane was internal standard. For isoquercetrin (**5**), 600 MHz ^1H, ^{13}C, ^1H–^1H DQCOSY, HMQC, and HMBC NMR spectra were recorded in CD_3OD. DHC (**3**) was synthesized by Stipanovic and Steidl (Stipanovic and Steidl, 1986). Lacinilene C (**1**) [predominantly *R* (Essenberg et al., 1990)] was extracted from *X. campestris* pv. *malvacearum*-inoculated OK1.2 cotyledons as a component of the phytoalexin extract described in (Abraham et al., 1999) and isolated by HPLC as in (Essenberg et al., 1990). Standard isoquercetrin (**5**) was from Sarsyntex, Merignac, France.

4.2. Plants, bacteria, inoculation and harvest of plant material

G. hirsutum plants were the glandless line WbM*gl* (Davis and Essenberg, 1995) and the glanded lines OK1.2 (Pierce and Essenberg, 1987) and WbM(0.0) (Essenberg et al., 1982), all of which are highly resistant to bacterial blight incited by *X. campestris* pv. *malvacearum,* and De Ridder Red, an obsolete cultivar whose leaves, stems, flowers, and bolls turn red with continued exposure to light due to the *R1* gene [R. Kohel, personal communication and (Percy and Kohel, 1999)]. Plants were grown in a growth chamber

Table 2
Quantitation of anthocyanin and other UV-absorbing pigments extracted from epidermal strips taken from *Xanthomonas*-inoculated and mock-inoculated plants of cotton line OK1.2

Fraction or compound	Tissue source	λ_{max} (nm)	Absorbance[a]		Concentration[b] (μmol/g fr. wt)
			331 nm	λ_{max}	
Aqueous (chrysanthemin + Y1 mixture)[c] attributed to:					
Chrysanthemin (**4**)	*Xcm*-inoculated	528	0.07	0.36	1.2
Chrysanthemin (**4**)	mock-inoculated	528	0	0	0
Y1	*Xcm*-inoculated	325	0.36	0.36	
Y1	mock-inoculated	325	0.46	0.46	
EtOAc (components resolved):					
Y2[d]	*Xcm*-inoculated	322	0.48	0.49	
Y2[d]	mock-inoculated	322	0.52	0.56	
Y3[d] (Isoquercitrin (**5**))	*Xcm*-inoculated	360	0.35	0.47	3.4
Y3[d] (Isoquercitrin (**5**))	mock-inoculated	360	0.11	0.13	0.98

[a] Pigments extracted from 0.084 g fr.wt, dissolved in 10 ml solvent.
[b] Concentrations determined with ε at λ_{max} listed in the table. See legend to Fig. 1 for ε values.
[c] Solvent was H_2O (1 mM HCl). Assuming the A_{528} was all due to chrysanthemin (**4**), A_{331} of 0.07 in the *Xcm*-inoculated sample could be attributed to chrysanthemin (**4**) and the remaining A_{331} to Y1. Similarly, the value for A_{325} attributed to Y1 was obtained by subtraction of the A_{325} attributable to chrysanthemin (**4**).
[d] Solvent was MeOH–H_2O (7:3).

(Pierce et al., 1993) or in a field at the Oklahoma State University Agronomy Station near Perkins, OK. Inocula of *X. campestris* pv. *malvacearum* strain 3631 (Pierce et al., 1993), of *ca* 2×10^6 cfu ml^{-1} for the micrographs of Fig. 2 and microspectra of Fig. 4, or *ca* 5×10^6 cfu ml^{-1} for the remaining work, were prepared as previously described (Pierce and Essenberg, 1987) and infiltrated from needleless syringes into entire cotyledons of growth-chamber-grown seedlings (6–8 days post-emergence) (Pierce and Essenberg, 1987) or into areas of *ca* 5 mm diameter on young, well-expanded foliage leaves of 6-week-old field plants. Mock-inoculation was with sterile saturated $CaCO_3$ solution.

Epidermal strips were peeled from leaves and cotyledons with fine forceps. Their excision from cotyledons was aided by infiltration with a 0.4 mg ml^{-1} solution of pectic enzymes [Macerase from Sigma, desalted as in (Pierce and Essenberg, 1987)] 12–60 min before peeling. The strips were immediately immersed in water and either mounted for microspectrophotometry, which was performed within 48 h, or frozen prior to extraction.

Cotyledons to be extracted were harvested 3–6 days post-inoculation and frozen in liquid N_2.

4.3. Treatment of leaves with phytoalexins and analysis of cell death

To avoid photodecomposition, solutions of sesquiterpene phytoalexins were prepared under UV-deficient illumination from General Electric FO612/GO gold fluorescent lamps. DHC (**3**) and LC (**1**) were dissolved in MeOH-H_2O (5:95, v/v); their concentrations were determined by dilution and UV spectrophotometry. The solns were infiltrated from needleless syringes into young, well-expanded leaves, still attached to plants. Field-grown plants (6–13 weeks old) were infiltrated in the field from foil-wrapped syringes. Chamber-grown plants (4–6 weeks old) were infiltrated under gold fluorescent lamps. For treatment with phytoalexin plus light, infiltrated plants were immediately replaced in the growth chamber in which they had been grown with a 14-h light/10-h dark cycle (Pierce et al., 1993). During the 10-h period of each day when all lamps were on, photosynthetic photon flux density was approx. 6×10^2 μmol s^{-1} m^{-2} at foliage leaf level and 5×10^2 μmol s^{-1} m^{-2} at cotyledon level. Plants to be treated with phytoalexin in the dark were placed in the same growth chamber, but inside two nested cardboard boxes with panels cut away so that air flowed freely past the plants from the floor vents, but light did not enter. Temperatures within the inner box differed from those in the main growth chamber by less than 1 °C.

Leaf samples for analysis were excised with an 11-gauge needle (1-mm diam.) fitted to a water-filled syringe. They were ejected into a mesh bag, which was immersed in Evan's blue stain (0.75% in water) at room temp. Vacuum was drawn with a pump for 30 s, and the flask was clamped off for 3.5 min. Vacuum was then slowly released through a finely drawn-out glass pipet. The samples were rinsed twice in 400 ml H_2O. Samples that had been treated with DHC (**3**) were mounted in water and examined with a Nikon Optiphot microscope; Evan's blue-stained cells per 10X or 20X field were counted. Samples that had been treated with LC (**1**) were photographed with 35-mm slide film through the microscope. The images were projected onto paper, and Evan's blue-stained cells were counted. Mean numbers of dead cells per mm^2 were compared by the unequal variance *t*-test (Ruxton, 2006).

4.4. Fractionation procedures

The red pigment was isolated from frozen, inoculated WbM*gl* cotyledons by the following procedure. Frozen tissue (150 g or 100 g) was homogenized in MeOH–H_2O (80:20, v/v), 0.1% HOAc (7 ml g^{-1}) at 0, centrifuged, decanted, then homogenized a second time in 4 ml g^{-1} of the same solvent. The combined extracts were concentrated by rotary evaporation and filtered through glass

Table 3
Estimated differences in concentration of chrysanthemin (**4**) (Cn-3-G) and isoquercitrin (**5**) (Qu-3-G) between red and colorless cells in the adaxial epidermis of leaves from field-grown cotton plants[a]

Cotton line	Treatment[b]	Cell type	A_{282}	Δ[Cn-3-G] (mM)	A_{331}	Δ[Qu-3-G] (mM)
Year 1						
DeRidder Red	None	Red	2.21	24	1.54	43
		Colorless	0.76		0.35	
OK1.2	42 dpi	Red	1.50	14	1.18	31
		Colorless	0.64		0.32	
Year 2						
OK1.2	4 dpi	Red	1.87	17	1.60	36
		Colorless	0.87		0.61	
OK1.2	9 dpi	Red	1.43	14	1.23	32
		Colorless	0.57		0.36	

[a] Each value is the mean from ten cells. Concentration differences were calculated using a path length of 25 μm, based on measurements of adaxial epidermal thickness in leaf cross-sections.
[b] DeRidder Red was not inoculated. For OK1.2, days post-inoculation (dpi) are indicated; see Fig. 4 for details.

wool. Extract from 50 g tissue was applied to an open column of octadecylsilane (6 g) that had been poured in MeOH, then rinsed with H_2O. The flow-through fluid was discarded, and the pigment was eluted with MeOH and acidified to 0.1% HOAc. It was concentrated by evaporation, its solvent was adjusted to MeOH:H_2O:HOAc (26:74:0.026), and it was fractionated (3–4 g fr. wt equivalent per run) by HPLC at room temp. on a 10 mm i.d. × 25 cm octadecylsilane column. Elution was at a flow rate of 3 ml min^{-1} with a program of solvent A = MeOH:H_2O:HOAc (10:80:10) and solvent B = MeOH:H_2O:HOAc (60:30:10): 0–7 min solvent A; 7–20 min, linear gradient from 0% to 30% solvent B; 20–23 min, linear gradient to 100% solvent B; 23–37 min, solvent B. Detection was by eye; the red pigment eluted at MeOH–H_2O (17.5:82.5, v/v) and was collected. It was concentrated by rotary evaporation. The red pigment was resolved from several colorless, UV-absorbing compounds by chromatography on polyvinyl pyrrolidone (Wrolstad and Struthers, 1971). An open, 25–mm i.d. × 41 cm column was poured in 0.1% aq. HCl; the sample was applied in the same solvent; elution was with MeOH containing 0.1% HCl. Detection was at 280 nm and 528 nm; the colorless compounds eluted first; the red pigment eluted much later as a single peak. Yield was 99 mg.

Pigments were isolated from epidermal strips as follows. All procedures were at ambient temp. Frozen strips (84 mg fr. wt from inoculated or mock-inoculated OK1.2 cotyledons) were homogenized with petroleum ether (10 ml), centrifuged, decanted, then homogenized a second time with another 5 ml of the same solvent. The residue was extracted with MeOH containing 0.1% HCl (2.5 ml × 2). The combined MeOH extracts were filtered through paper. Water (2.5 ml) was added, MeOH was evaporated, and H_2O (2.5 ml) was added. The sample was extracted with hexane (3 × 5 ml) and then with EtOAc (3 × 5 ml). The EtOAc extracts were combined, evaporated, and subjected to size exclusion chromatography on Sephadex LH20 (1 cm i.d. × 48 cm) in MeOH:H_2O (70:30) at 0.5 ml min^{-1} with detection of A_{254}. Absorption spectra of all fractions, including the petroleum ether and water extracts, were recorded. The principal LH20 peak exhibiting A_{331} and A_{343} (Y3) was pooled and evaporated to dryness. It was dissolved in MeOH containing 0.1% HCl and subjected to HPLC on a 3.9 mm i.d. × 300 mm octadecylsilane column (10 μm particle diameter). Elution was at a flow rate of 1 ml min^{-1} with a program of solvent A = CH_3CN:H_2O:CF_3CO_2H (5:95:0.05) and solvent B = CH_3CN:CF_3CO_2H (100:0.05): 0–10 min, linear gradient from 5% to 15% solvent B; 10–20 min, isocratic 15% solvent B; 20–45 min, linear gradient from 15% to 80% solvent B; 45–54 min, isocratic 80% solvent B. Detection was by A_{215}. Authentic isoquercetrin (**5**), as well as Y3 from both inoculated and mock-inoculated epidermal strips, eluted at 20.4 ± 0.1 min.

The infection-induced Y3 pigment was isolated from lyophilized, inoculated OK1.2 cotyledons. Lyophilized tissue (6.1 g dry wt) was ground to a powder in liquid N_2. The powder was subjected to the same series of extractions as described above for epidermal strips, although with 6X greater solvent volumes. Extract from 2 g dry wt per run was subjected to chromatography on Sephadex LH20 (1.5 cm i.d. × 30 cm). The Y3-containing peaks were combined and were subjected in two aliquots to normal-phase chromatography on silicic acid (325 mesh, 20 mm i.d. × 35 cm) with mobile phase $CHCl_3$:EtOAc:HCOOH (20:70:10) and detection at 360 nm. Yield was 10.3 mg.

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