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Kaempferol 3-*O*-Galactoside, 7-*O*-Rhamnoside is the Major Green Fluorescing Compound in the Epidermis of *Vicia faba*[1]

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ABSTRACT

The vacuoles of lower epidermal strips from *Vicia faba* exhibit an intrinsic green fluorescence when incubated in alkaline buffers. Using an alkaline-induced absorbance change as a spectrophotometric assay, the major pigment responsible for this fluorescence was isolated and identified as the flavonoid: kaempferol 3-*O*-galactoside, 7-*O*-rhamnoside. The aqueous absorption maxima were 394 and 341 nanometers at pH 10.0 and 6.0, respectively, with a pKa of 8.3 and the fluorescence emission maximum was 494 nanometers at pH 10.0. The *in vivo* concentration was estimated to be between 3 and 10 micromolar. The absorption spectrum of this flavonoid is different from the action spectrum for stomatal opening indicating that this compound is not the photoreceptor pigment for the blue light response of *Vicia faba* guard cells.

Plants control much of their water loss and gas exchange by regulation of their stomatal aperture through the swelling and shrinking of the two guard cells surrounding the stoma (4, 12). Guard cell size and hence, stomatal aperture, is influenced by several environmental factors, one of which is light. Action spectra indicate the involvement of two distinct photoreceptor pigment systems in guard cell photophysiology; one requires high light intensities and is sensitive to both red and blue light (photosynthetic pigments), while the other is effective at much lower light intensities and is sensitive only to wavelengths less than 520 nm (2, 5, 9, 14, 15). Action spectra for this blue light response resembles those measured for other blue light-sensitive responses, with peaks at 380 and 450 nm (2, 9, 15), implying that a similar type of photoreceptor pigment may be involved. Experiments from the majority of other blue light-sensitive responses indicate that the chromophore is most likely a flavin (11, 13).

Zeiger and Hepler (21, 22) discovered that the blue light-responsive guard cells of *Allium* exhibit an intrinsic green vacuolar fluorescence. More recently, epidermal strips from *Vicia faba* also have been reported to display the same type of intrinsic green fluorescence after incubation with alkaline buffers (19, 20). Based on the assumption that a flavin is involved in photoperception in guard cells and the knowledge that flavins are highly yellow-green fluorescent, it was suggested that this intrinsic fluorescence is mediated by a flavin and that this flavin is involved in the blue light response of guard cells (19, 20).

Using the ability of *V. faba* epidermal strips to exhibit green fluorescence after alkalinization as a spectrophotometric assay, we attempted to identify the pigment(s) responsible. The principal goal was whether this fluorescence is indeed flavin-mediated and possibly linked to guard cell photophysiology.

MATERIALS AND METHODS

V. faba (cv. Improved Long Pod, from Lagomarsine Seeds, Inc., Sacramento, CA) were grown in a peatlike soil mixture and watered once a week with ½ Hoagland solution. Growth conditions consisted of a 16-h light period (light intensity was 40 w m^{-2}) at 23°C. The relative humidity was 70%. Epidermal strips were obtained by peeling off the lower epidermis from young fully expanded leaves. Care was taken to avoid mesophyll contamination.

Absorption spectra of a single leaf epidermal strip were measured using a vertical cuvette in conjunction with a single beam spectrophotometer similar to that described by Davis *et al.* (3). The epidermal strip (~100 mm^2) was washed twice with distilled H_2O, placed at the bottom of the cuvette, and a 50 mm^2 circular window placed on top of the sample. Absorption spectra were recorded with and without the addition of 1 drop of 1 N NH_4OH and a difference spectrum obtained by computer-assisted subtraction.

Absorption spectra of crude extracts or partially purified compounds were measured using a Cary 15 spectrophotometer. Uncorrected fluorescence excitation and emission spectra were determined using an Aminco Bowman (Model H-8202) spectrophotofluorometer. Samples were made alkaline by the addition of several drops of 1 N NH_4OH or 0.5 M MeONa[4] to the aqueous and methanol extracts, respectively.

Isolation of fluorescing compounds was performed by two dimensional descending paper chromatography. Epidermal strips from approximately 20 leaves were immersed immediately after peeling in methanol. The strips were washed twice with methanol, the combined extracts dried *in vacuo*, redissolved in methanol, and stored at −20°C. This solution was spotted onto washed Whatmann No. 3 chromatographic paper (23 × 27 cm) and developed in the first dimension with *t*-butanol:acetic acid: H_2O (3:1:1) (TBA) and in the second dimension with 15% acetic acid (7). Fluorescent spots, observed under UV light with and without exposure to NH_3 vapors, were cut out of the chromatogram and eluted with methanol.

The fluorescing compound was tentatively identified by its absorption spectra in several solvents (methanol ± MeONa, methanol + $AlCl_3$ ± HCl, and methanol + sodium acetate ± H_3BO_3) as described by Mabry *et al.* (7). The aglycone of the fluorescing compound, obtained after sugar hydrolysis, was identified by

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[4] Abbreviations: MeONa, sodium methoxide; TBA, *t*-butanol:acetic acid:H_2O (3:1:1).

comparison of its absorption spectra in methanol ± MeONa and its chromatographic behavior with flavonoid standards. Sugar hydrolysis was accomplished by dissolving the purified compound in 0.1 N trifluoroacetic acid and heating for 30 min at 120°C in an autoclave. The aglycone was then extracted with diethyl ether and purified by two dimensional paper chromatography (see above). The aglycone was co-chromatographed with flavonoid standards using six different TLC development systems: precoated cellulose plates (Merck) developed with 15% acetic acid, 40% acetic acid, and *t*-butanol:acetic acid:H_2O (3:1:1) or precoated polyamide-6 plates (Macherey-Nagel) developed with $CHCl_3$:methanol:methylethyl ketone:acetone (20:10:5:1), benzene:methylethyl ketone:methanol (60:26:14), and toluene:petroleum ether:methyl ethyl ketone:methanol (60:26:7:7) (7).

The hydrolyzed sugars were reduced and acetylated according to the procedure of Albersheim *et al.* (1) and identified and quantified by GLC as described by Meinert and Delmer (8). Sugar linkage composition were determined by combined GC-MS of permethylated alditol acetates (8).

Tentative location of the two sugars on the flavonoid was accomplished using partial hydrolysis with β-galactosidase (7). The flavonoid was incubated at pH 7.3 overnight at 37°C with a few crystals of β-galactosidase and the resulting hydrolyzed flavonoid isolated by two-dimensional descending paper chromatography (see above). The remaining attached sugars were identified after trifluoroacetic acid hydrolysis of the flavonoid by GLC as described above.

β-Galactosidase, kaempferol, and quercetin were purchased from Sigma.

RESULTS

As described previously (19), leaf epidermal strips from *V. faba* exhibit an intrinsic green fluorescence when incubated in alkaline buffers. We also observed here that alkaline conditions concomitantly induce a faint green color in such epidermal strips. When the absorption spectrum of a single epidermal strip (~20 μm thick) was measured (Fig. 1), an increase in absorbance (~0.1 *A*) at 394 nm was detected after the addition of NH_4OH. The difference spectrum (± NH_4OH) exhibited a maximum at 398 nm. In addition, aqueous extracts from epidermal strips exhibited a concomitant shift in fluorescence with an increase in pH, with the extracts becoming green-fluorescent (504 nm emission maximum) after the addition of NH_4OH (pH 10.0) (Fig. 2).

Using the NH_4OH (or MeONa)-induced absorbance change as a spectrophotometric assay, the fluorescing compound(s) was purified and isolated. Paper chromatograms of crude methanol extracts displayed several fluorescing compounds under UV light with one prominent purple fluorescing spot (R_F; TBA, 0.65; 15% acetic acid, 0.74) which became intensely yellow-fluorescent after exposure to NH_3 vapor. An absorption spectrum in methanol of this fluorescing compound, purified by two-dimensional paper-chromatography (Fig. 3), displayed maxima at 265 and 347 nm which shifted to 272 and 402 nm upon the addition of MeONa. The fluorescence excitation spectra match closely the absorption spectrum of the compound (± MeONa) with the uncorrected fluorescence emission maximum shifted from 421 to 514 nm after addition of MeONa.

The absorption and fluorescence spectra of this compound when dissolved in aqueous buffers (10 mM Mes/Hepes) was found to be highly pH-dependent (Fig. 4). An increase in pH from 6.0 to 10.0 shifted the absorbance maximum from 341 to 383 nm. The difference spectrum at pH 10.0 minus pH 6.0 had a maximum at 393 nm and a minimum at 316 nm. The difference spectrum maximum of the purified compound (393 nm) was very close to the maximum obtained with the crude extracts (398 nm). The fluorescence emission maxima of this compound also shifted to longer wavelengths, from 423 nm at pH 6.0 to 494 nm at pH 10.0.

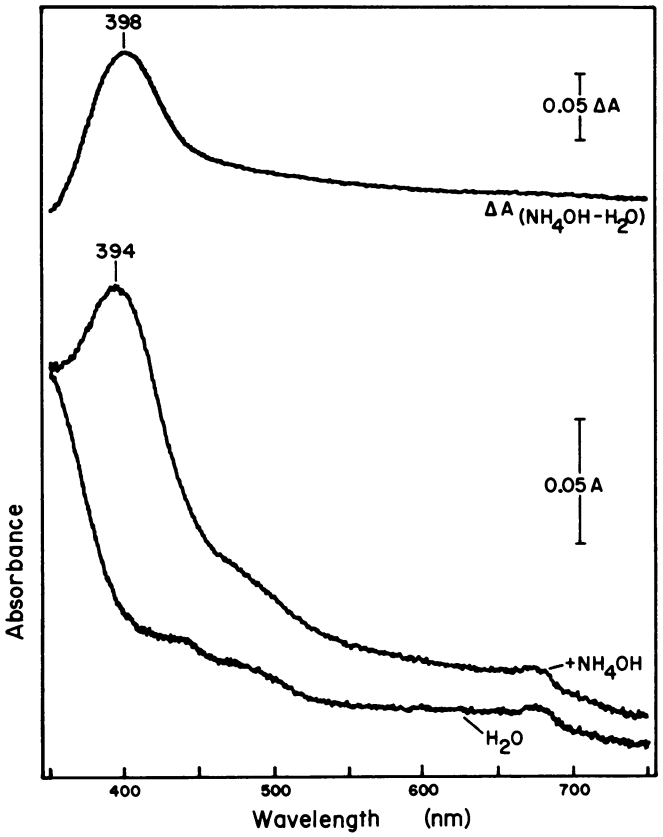

FIG. 1. Absorption and difference spectra (with or without the addition of NH_4OH) of a single leaf epidermal strip from a *V. faba* leaf.

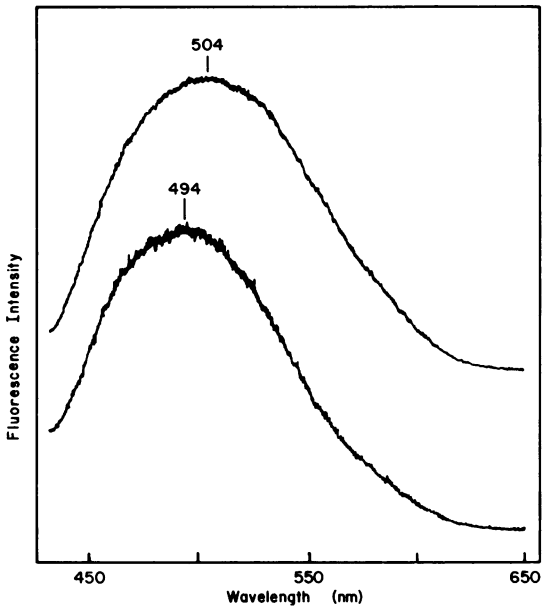

FIG. 2. Aqueous fluorescence emission spectra (uncorrected) of the crude extract from *V. faba* epidermal strips (top) and of the fluorescing compound purified from such extracts (bottom) in 10 mM Mes/Hepes (pH 10.0).

More importantly, the fluorescence emission spectrum of the purified compound (Fig. 2) was similar to that for the crude extracts (emission maxima were 494 and 504 nm, respectively). Other fluorescing compounds were isolated from crude extracts and probably were responsible for the spectral differences between

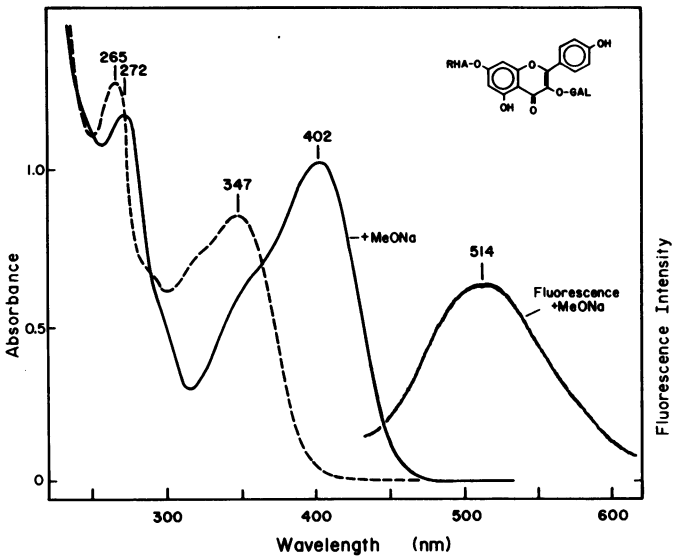

FIG. 3. Absorption and fluorescence spectra of the fluorescing compound isolated from *V. faba* epidermal strips in methanol, with and without sodium methoxide. Tentative structure of the compound is; kaempferol 3-*O*-galactoside 7-*O*-rhamnoside.

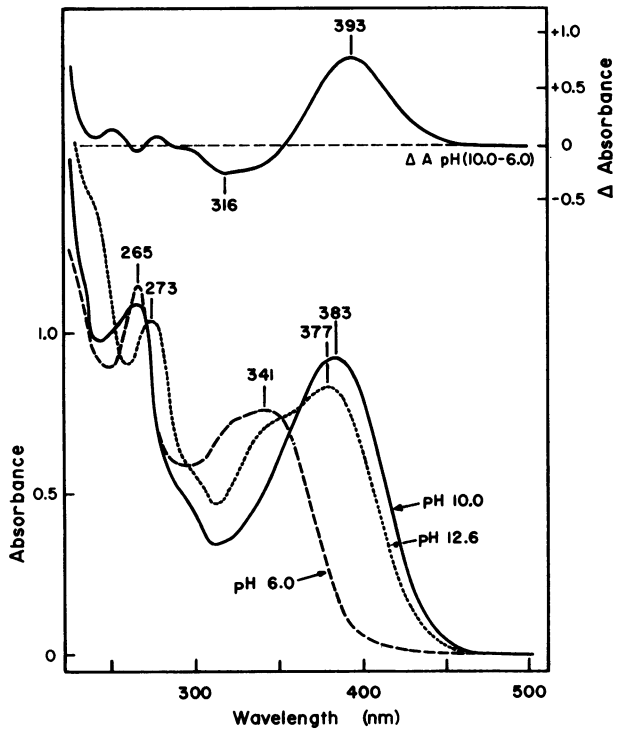

FIG. 4. pH dependence of the aqueous absorption spectrum of the fluorescing compound isolated from *V. faba* epidermal strips. (Upper) Difference in the absorption spectrum of the compound at pH −10.0 minus that at pH −6.0.

the crude extracts and the purified flavonoid, but they were found in trace quantities in comparison with this compound and were generally pH insensitive.

Using the difference in A between 393 and 316 nm quantitatively, the pKa of the fluorescing compound was spectrophotometrically determined (Fig. 5). Only slight changes in $A_{(393-316)nm}$ occur below pH 6.8. Between pH 7.0 and 9, there is a dramatic increase in $A_{(393-316)nm}$ as would be expected, with an estimated pKa of 8.3. Above pH 10, the $A_{(393-316)nm}$ began to decrease (Fig. 5), indicating that a second pKa exists above pH 11.5.

The fluorescing compound was determined to be a flavonoid by its characteristic absorption spectra in various solvent systems (see above [7]). Mild hydrolysis of the fluorescing compound in 0.1 N trifluoroacetic acid produced a new yellow fluorescing substance (R_F; TBA, 0.73, 15% acetic acid, 0.02) indicating that the flavonoid is glycosylated. The aglycone was identified as kaempferol by absorption spectra (± MeONa [7]), and by chromatography with purified kaempferol in six different TLC development systems (see above). Sugars obtained after hydrolysis of this compound were identified by comparison of the GC retention times of the alditol acetates with standards (8). Only rhamnose and galactose were found in significant quantities with a molar ratio of approximately 1 to 1. Combined GC-MS of the permethylated alditol acetates (8) indicated that both rhamnose and galactose were terminally linked, as determined by the characteristic fragmentation patterns (6), to the flavonoid aglycone. Based on the kaempferol glycoside's characteristic absorption and fluorescence spectra (± MeONa [7]), the sugars were linked to the 3 and 7 hydroxyl groups of the aglycone. The enzyme β-galactosidase was then used to remove the galactose moiety selectively (7). The absorption and fluorescence spectra of the partially digested compound (R_F; TBA, 0.30, 15% acetic acid, 0.19) was consistent with the galactose being linked to the 3 position. The green fluorescing substance from *V. faba* epidermal strips was therefore tentatively identified as kaempferol 3-*O*-galactoside, 7-*O*-rhamnoside.

DISCUSSION

From these results, we conclude that a majority of the alkaline-induced intrinsic green fluorescence of *V. faba* epidermal peels is due to a glycoside of the flavonoid, kaempferol. Definitive proof would come from *in vivo* fluorescence spectroscopy but such attempts by us were unsuccessful. We find in using fluorescence microscopy that both guard and epidermal cells exhibit such fluorescence. This fluorescence, however, is not as easily detected in epidermal cells because it is less intense and because most epidermal cells are destroyed in the process of peeling (as judged by vital staining). Qualitatively, both types of cells displayed a similar fluorescent color and exhibited the same response to alkaline buffers. Based on an extinction coefficient of E_{394nm}—2 × 10^4 l mol^{-1} cm^{-1} for similar kaempferol glycosides (18) and the magnitude of the alkaline-induced absorbance change from single epidermal peels, assuming a homogeneous distribution of the

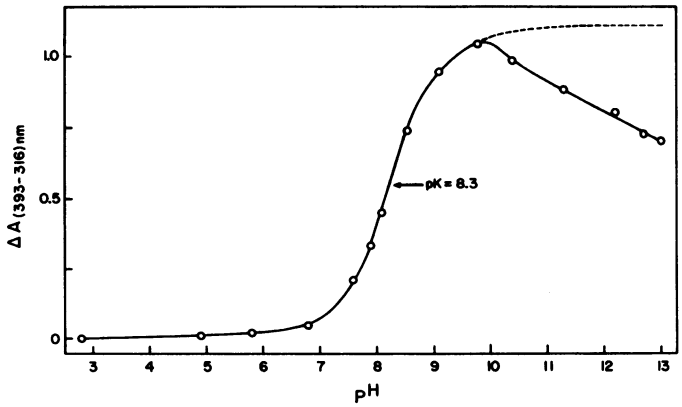

FIG. 5. pKa of the fluorescing compound as determined by pH-induced absorbance changes. The compound's absorption spectrum at pH 2.8 was substracted from the absorption spectra of the compound dissolved in solutions of varying pH, and the difference in absorbance ($A_{393nm} - A_{316nm}$) plotted as a function of pH.

flavonoid, we estimated the *in vivo* concentration of this flavonoid to be between 3 and 10 mM. Because we did not attempt to separate guard cells from epidermal cells, it was not possible to determine the flavonoid concentration in each cell type.

Guard cell photophysiology does appear to be regulated by a blue light-absorbing photoreceptor pigment, possibly a flavoprotein. However, the conclusion that this alkaline-induced intrinsic fluorescence in *V. faba* is related to this pigment is unsubstantiated because: (a) This fluorescence is not restricted to the guard cells. (It has been suggested that because guard cells displayed this fluorescence [19–22] and because they are directly responsive to blue light [5, 21] the two phenomena might be related.); (b) On theoretical grounds, one would not expect the photoreceptor pigment to fluoresce *in vivo* because photoreceptor pigments are generally only weakly fluorescent *in vivo* enabling them to be highly efficient (16); (c) Flavins, in particular, do not display similar pH-induced absorbance and fluorescence shifts for the pH ranges found to be effective here (between pH 6 and 10 [17]); and (d) The absorption spectrum of this flavonoid is not similar to the reported action spectrum for stomatal opening in this species (5, 9). Thus, this flavonoid may be eliminated as a potential candidate for the photoreceptor pigment regulating stomatal opening.

Intrinsic guard cell fluorescence has also been reported to occur in *Allium* (21, 22). In contrast to *Vicia*, this fluorescence does not require incubation in alkaline buffers and does appear to be restricted only to the vacuoles of guard cells. It is possible that the fluorescence in *Allium* is also due to a flavonoid with a lower pKa and/or that *Allium* vacuoles have an inherently higher pH. Using recent methods described for the isolation of large quantities of guard cells (10), it should be possible to identify the fluorescing pigment from *Allium* as well.

In conclusion, the alkaline-induced intrinsic fluorescence in epidermal strips is not due to a flavin, but primarily due to the flavonoid kaempferol 3-*O*-galactoside, 7-*O*-rhamnoside, and consequently, not related to guard cell photophysiology. However, this fluorescence may well have practical applications for guard cell physiology. It should be possible to detect perturbations in guard cell vacuolar pH (especially pH values above 6.5) by monitoring either fluorescence or absorbance changes of this flavonoid. For example, Zeiger (19, 20) has reported that fusicoccin will also induce vacuolar guard cell fluorescence probably by stimulating proton export out of the vacuole and therefore raising the pH. This flavonoid might also be useful as a convenient specific marker in the isolation of intact vacuoles from isolated *Vicia* guard cells.

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