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On the properties of fluorescing compounds in guard and epidermal cells of *Allium cepa* L.

G. Weissenböck[1], H. Schnabl[2], H. Scharf[3] and G. Sachs[1]

[1] Botanisches Institut der Universität Köln, Gyrhofstrasse 15, D-5000 Köln 41,
[2] Institut für Landwirtschaftliche Botanik der Universität Bonn, Meckenheimer Allee 176, D-5300 Bonn 1, and
[3] Carl Zeiss, D-7082 Oberkochen, Federal Republic of Germany

Abstract. Onion guard cells, in contrast to those of *Vicia* and *Pisum*, do not require an alkaline treatment in order to fluoresce. Fluorescing compounds of *Allium cepa* L. were characterized using in-vivo microspectrophotometry; furthermore, in-vitro chemical analysis for epidermal tissue, intact guard and epidermal cells, and isolated guard-cell protoplasts was performed. The emission intensity (λ_{max} 520 nm) decreased when intact onion guard cells were excited with 436-nm light, but increased (λ_{max} 470 nm) when excited at 365 nm. This photodecomposition at 436 nm is typical of flavins or flavoproteins whereas an increase in fluorescence intensity with excitation at 365 nm may be explained by the presence of other substances. The presence of flavins could not be unambiguously confirmed from these results. Indeed, the absorption spectra of the vacuolar area of guard cells did not show the peak at 445 nm which is characteristic for flavins. Furthermore, there was no decrease of absorption at the excitation wavelengths of 440 and 330 nm. Since spectral data indicate the presence at high amounts of flavonoids in guard and epidermal cells, this may reduce the sensitivity for the detection of flavins in guard cells. Using thin-layer chromatography and high-performance liquid chromatography together with hydrolytic procedures, flavonol glycosides with kaempferol and quercetin as aglycones substituted with sulphate and glucuronate were identified. Further studies on guard-cell metabolism should consider the presence of flavonoids in stomata of onion and other plants.

Key words: Absorption spectrum (in vivo; flavin, flavonol) – *Allium* – Emission spectrum (in vivo) – Epidermis (fluorescente) – Flavin – Flavonol – Guard cell (fluorescene).

Abbreviations: GCP = guard-cell protoplast; HPLC = high-performance liquid chromatography; TLC = thin-layer chromatography

Introduction

Guard cells of onion leaves (*Allium cepa* L.) autofluoresce green when irradiated with blue light (Zeiger and Hepler 1979). This type of fluorescence seems to be a specific property of the *Allium* species, since guard cells of other plants including *Vicia faba* and *Pisum sativum* (Zeiger and Hepler 1979; Schnabl et al. 1986; Weissenböck et al. 1986) fluoresce green only when treated with ammonia or other chemicals which increase the intracellular pH (Zeiger 1980, 1981).

The fluorescing compounds of *Vicia* and *Pisum* have been described as flavonol glycosides in studies using isolated guard-cell protoplasts (GCPs) and, in the case of *Vicia*, using in-vivo microspectrophotometric and in-vitro measurements (Vierstra et al. 1982; Weissenböck et al. 1984, 1986; Schnabl et al. 1986).

For comparison, we extended these analyses to *Allium cepa*. Since the spectral properties of the fluorescing chromophore(s) of *Allium* are in accordance with the emission characteristics of flavins or flavoproteins (at 520 nm), this class of substances had been suggested to be the major fluorescing pigment in their guard cells. Zeiger and coworkers concluded that flavins and flavoproteins may be associated with the tonoplast and thus play an important role as primary blue-light photoreceptors by generating an electrochemical gradient (Zeiger and Hepler 1979; Zeiger 1983).

However, flavonoids may also be present in the cells (compare Schnabl et al. 1986). It is possible

to distinguish between these two classes of pigments by spectroscopic techniques: flavonoids do not appreciably absorb at wavelengths longer than 400 nm, while flavins show a peak at 445 nm (Ghisla et al. 1984). Furthermore, flavins are characterized by a fast photodecomposition (50% within 1 min; Zeiger and Hepler 1979), whereas the decay of flavonoids under these conditions is negligible (Kaneta and Sugiyama 1971; Schnabl et al. 1986).

The aim of the present investigation was to characterize the fluorescing compound(s) of *Allium cepa* by in-vivo as well as in-vitro measurements of epidermal tissue and isolated GCPs.

Material and methods

Plant material. Ten-day-old plants of *Allium cepa*, (cv. Wintersteckzwiebeln, gelbe Senshyu; Fa. Küpper, Eschwege, FRG), were grown from bulbs in peat moss in a growth chamber at 18° C on a 15 h light/9 h dark regime. Irradiance was of 95 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (HQS-lamps; Osram, München, FRG). For preparative isolation of flavonoids, plants were grown for four weeks at 21° C on a 13 h light/11 h dark regime.

Epidermis and protoplast isolation. Epidermal strips were obtained by peeling the epidermis from fully expanded leaves. Guard-cell protoplasts were prepared (Schnabl et al. 1978) and purified by published techniques (Schnabl 1981).

Detection of flavins in guard cell protoplasts. To determine whether or not flavins are detectable in onion guard cells, their protoplasts were isolated from epidermal peels and extracted according to Ghisla et al. (1984). The green autofluorescence is retained during isolation procedures (Zeiger and Hepler 1979). Epidermal peeling was carried out in dim light, followed by the isolation of GCPs in darkness. Purified GCPs (10^4–10^5 protoplasts per isolation) were suspended in a minimal volume of 10 mM phosphate buffer, pH 7.0, homogenized, centrifuged (5 min at $5000 \cdot g$) and re-extracted two times. The UV-VIS absorption spectrum was recorded from the clear supernatant after dilution with CH_3OH.

Flavonoid extraction and characterization. For flavonoid analysis of GCPs the same extracts were used as for the detection of flavins. Epidermal tissue was extracted in 80% CH_3OH as described earlier (Weissenböck et al. 1984). Absorption spectra (200–500 nm, UVIKON 810, Kontron, Eching, FRG) were recorded using crude and purified fractions, adding diagnostic shift reagents according to Mabry et al. (1970). Flavonoid compounds were characterized by polyamide column chromatography (Popovici et al. 1977; Schulz et al. 1985), thin-layer (TL) electrophoresis (Markham 1982), acid and enzymatic hydrolysis, preparative thin-layer chromatography (TLC) on cellulose "Avicel" (Macherey & Nagel, Düren, FRG) and spectral analysis of purified compounds (compare Schulz et al. 1985; Weissenböck et al. 1984, 1986). Flavonoids were detected on TLC plates under UV-light (354 nm) using standard reagents, NH_3 vapor, $AlCl_3$ and "Naturstoffreagenz" (2-aminoethyldiphenylboric acid ester; Weissenböck et al. 1984).

Solvents used for TLC (v/v) were: (i) 5% aqueous CH_3COOH; (ii) chloroform: acetic acid 3:2, water saturated (CAW), for glycosides; (iii) CAW 2:1, water saturated; (iv) Forestal, water, CH_3COOH, 32 vol% HCl 10:30:3; (v) 15% CH_3COOH; for aglycones.

Electrophoresis of flavonoids was performed on cellulose TL "Avicel" using 0.1 M CH_3COONa/HCOOH buffer, pH 2.2, according to Markham (1982).

For high-performance liquid chromatography (HPLC), 20 µl of extract, diluted with water to 50% CH_3OH, were injected onto an HPLC column (250 mm long, 4.6 mm diameter; ZORBAX C8; Du Pont, Bad Nauheim, FRG) and developed with a gradient of 2 to 10% acetonitrile in water (1% phosphoric acid) within 8 min, 10 to 18% of the same solvents within 13 min, (exponent −2 of the gradient controller). The liquid chromatograph, gradient controller, UV-absorbance detector set at 340 nm and integrator were as described by Weissenböck et al. (1984). Quantitative calculations were performed using kaempferol 3-rhamnosylglucoside (nicotiflorin; Roth, Karlsruhe, FRG) as an external standard (Weissenböck et al. 1984). Flavonol aglycones were also purchased from Roth; for their separation see Weissenböck et al. (1984).

Microscopy and microscopic spectrophotometry. For microscopical analysis, including cytophotometry of intact guard and epidermal cells, the epidermal peels were kept in darkness before use. Cells were viewed and analysed in the absence and presence of 0.05% NH_4OH in distilled water as described earlier (Schnabl et al. 1986).

Microscopy and in-vivo spectrophotometry, recording fluorescence and absorbance spectra of the cells (vacuolar area), were carried out with the Universal Microscope Spectral Photometer UMSP 80 from Zeiss, Oberkochen, FRG. The equipment and the conditions for measuring spectral data have been described in detail by Schnabl et al. (1986).

Acidic and enzymatic hydrolysis. Flavonoid compounds were partially or completely hydrolysed with 1 N HCl (100° C, up to 3 h). Hydrolysates were spotted onto TLC plates or injected onto the HPLC column and analysed for the presence of intermediates and products using reference substances (see Weissenböck et al. 1984). Intermediates of the acid treatment were hydrolysed enzymatically with β-D-glucuronidase (type H-3 AF) from *Helix pomatia* (Sigma, Heidelberg, FRG) as described by Schulz et al. (1985).

Results

In-vivo emission spectra. When flavins or flavoproteins are irradiated with short-wavelength light, an autofluorescence emission maximum at 520 nm and a photobleaching effect is expected (Ghisla et al. 1984; Ninnemann 1980). When vacuolar areas of *Allium cepa* guard cells were exposed to λ 436 nm, which is close to the characteristic long-wavelength peak of flavins (445 nm), the decay of fluorescence intensity at 520 nm was 50% within 3 min (Fig. 1). The fluorescence at 680 nm is caused by chlorophyll which decomposes more rapidly. In contrast, no photodecomposition was observed when guard cells were excited at 365 nm, near the second absorption peak of flavins (370 nm). However, an increase in fluorescence intensity, by a factor of 2 to 3, was detected within 10–15 min, and an appreciable hypsochromic shift

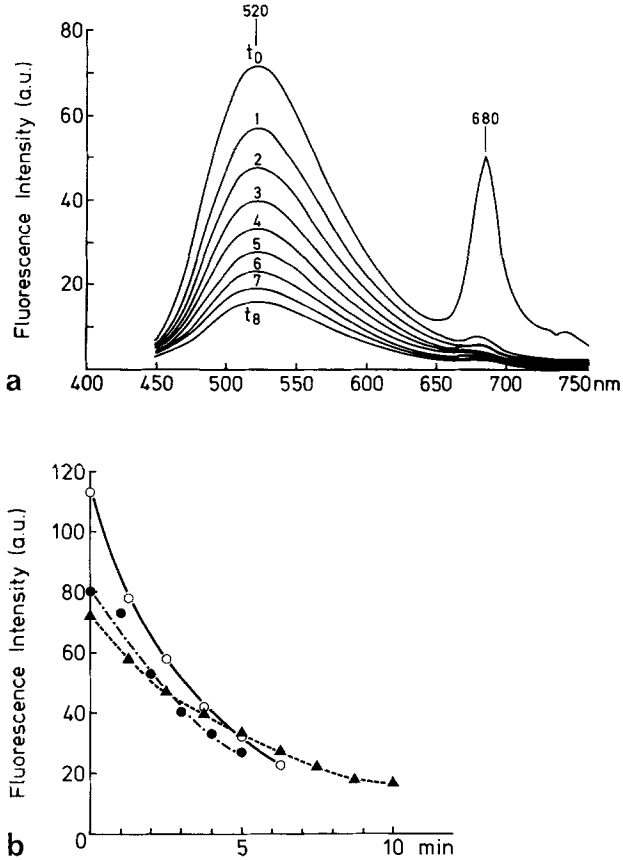


Fig. 1. a Typical emission spectrum and time-dependent spectral characteristics (t_0–t_8, 0 min up to 10 min) of the green autofluorescence (λ_{max} 520 nm; excitation at 436 nm) of vacuolar areas of onion guard cells. The peak at 680 nm is caused by chlorophyll from chloroplasts present in the circular measuring field of 50 µm. **b** Decrease in fluorescence intensity at 520 nm of guard cells during time, up to 10 min, of continuous excitation at 436 nm; three independent measurements with freshly prepared epidermal tissue

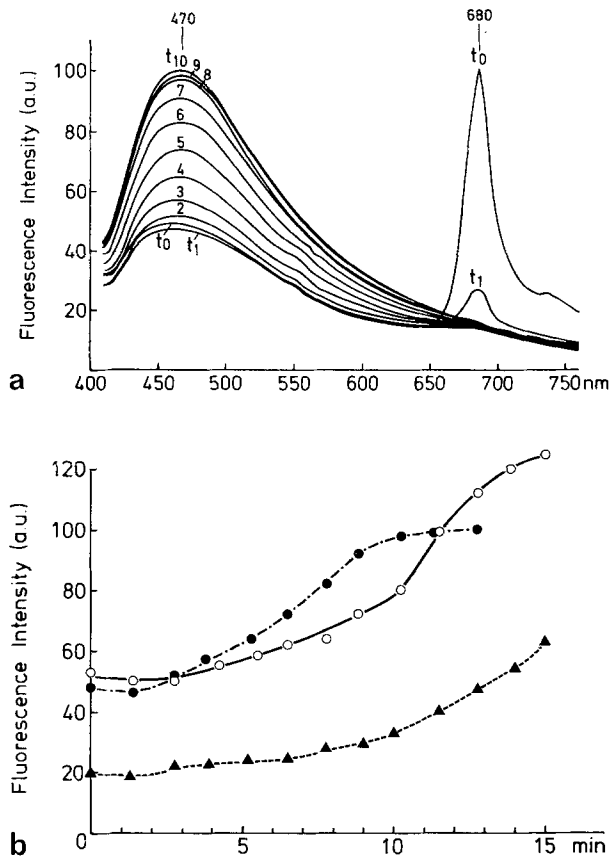


Fig. 2. a Typical emission spectrum and time-dependent spectral characteristics (t_0–t_{10}, 0 min up to 15 min) of the green autofluorescence (λ_{max} 470 nm; excitation at 365 nm) of vacuolar areas of onion guard cells. Emission at 680 nm is caused by the chlorophyll (see **Fig. 1**). **b** Increase in fluorescence intensity at 470 nm of guard cells during time, up to 15 min, of continuous excitation at 365 nm; three independent measurements with freshly prepared epidermal tissue

of the emission spectrum is observed with λ_{max} 520 to 470 (Fig. 2).

When 0.05% NH_4OH was added to intact guard cells, a bathochromic shift from 470 nm to 540 nm occurred. This was accompanied by an increase in fluorescence intensity of approx. threefold within 20 min of exposure (excitation 365 nm; Fig. 3a, c).

Epidermal cells not treated with alkali showed no green fluorescence when excited either at λ 436 or at λ 365 nm (not shown). When they were exposed to ammonia an immediate intense green fluorescence appeared with an emission maximum at 540 nm. Measurements of the time-dependence of this induced fluorescence showed only a slight increase during 15 min (Figs. 3b, c).

From Figs. 1 and 2 we may conclude that flavins are either absent from guard cells or, when excited with 365 nm light, their decrease in fluorescence intensity at 520 nm is compensated by an increase at 470 nm as a consequence of the presence of another class of compounds.

In-vivo absorption spectra. Figure 4 represents the absorption spectrum of intact *Allium* guard cells with λ_{max} at 265 and 325 nm. Absorption peaks at 370 and especially at 445 nm, typical of flavins, were not detectable even when the sensitivity of the spectrophotometer was strongly increased (Fig. 4, insert). Furthermore, continuous illumination of guard cells at 330 nm up to 8 min did not cause any decrease in the absorbance intensity. These data indicate that no measurable amounts of flavins or flavin-like material are present in guard cells of *Allium cepa* although the characteristic autofluorescence at 520 nm (see Fig. 1) was

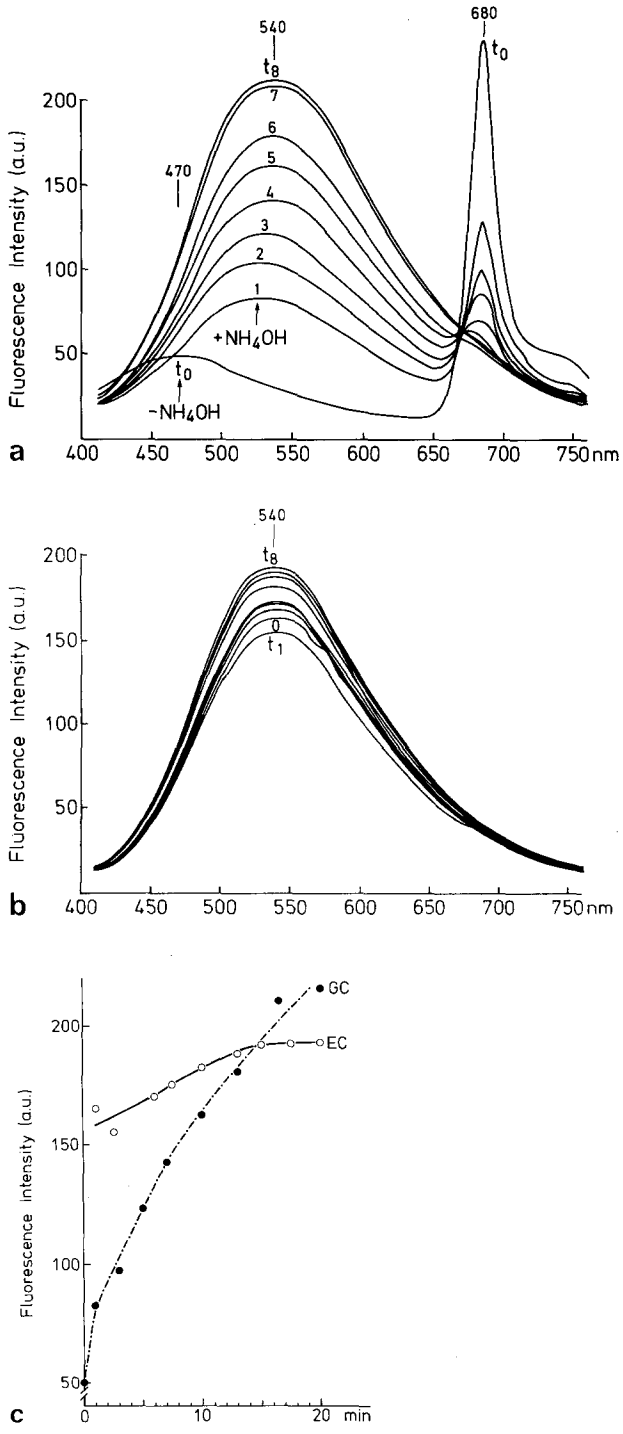


Fig. 3. a Time-dependent spectral characteristics of the green fluorescence of the vacuolar area of onion guard cells. Autofluorescence with λ_{max} at 470 nm ($=t_0$, $-NH_4OH$; see **Fig. 2**) shifted to λ_{max} at 540 nm after the addition of ammonia ($+NH_4OH$) during 20 min of exposure. Time intervals (t_1 through t_8, from 1 to 20 min) and calculations are shown in (**c**) for the guard cells (*GC*). **b** Alkaline-induced ($+NH_4OH$) fluorescence at λ_{max} 540 nm of epidermal cells (*EC*) during time (t_1–t_8, from 1 to 20 min) as calculated in (**c**). Continuous excitation was at 365 nm for both cell types; independent measurements with freshly prepared epidermal tissue

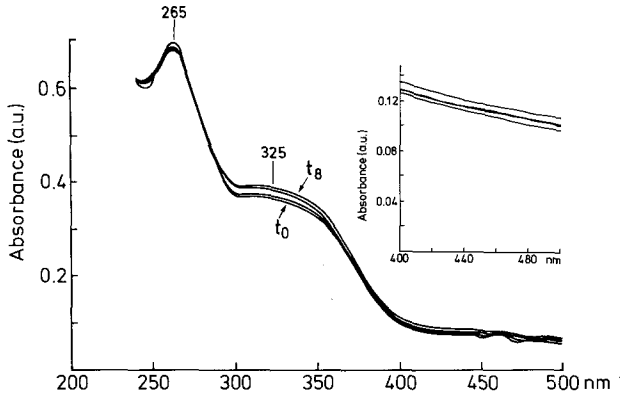


Fig. 4. Absorption spectra of the vacuolar area of onion guard cells during continuous irradiation with 330-nm light, recorded from t_0 through t_8 (0–8 min). *Insert*: recordings of absorptivity at increased sensitivity of the spectrophotometer (0–8 min); independent measurements with freshly prepared epidermal tissue. Circular measuring field = 6.3 µm

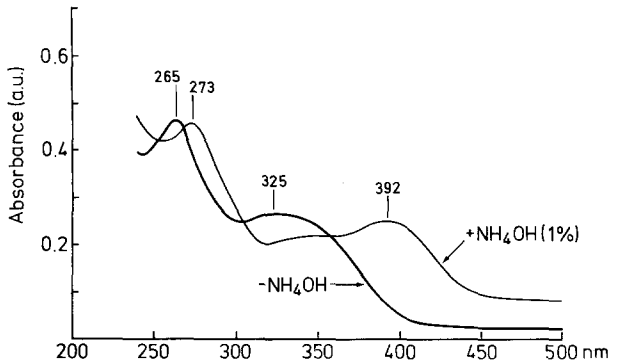


Fig. 5. Absorption spectra of the vacuolar area of onion guard cells before and after treatment with NH_4OH

present at the time when the illumination was started. The spectrum in Fig. 4 indicates the presence of flavonoid-like material in high concentration.

When 1% NH_4OH, a flavonoid-ionizing agent, was added, a 67-nm bathochromic shift of the long-wavelength band was observed (Fig. 5). The absorbance was stable during the experimental time of approx. 10 min. When epidermal cells are compared with guard cells, identical maxima are obtained, indicating the presence of the same type of flavonoid(s) in both cell types (Fig. 6). Crude 80% methanolic extracts of epidermal peelings consisting of guard and epidermal cells gave, within the experimental error, identical results (Fig. 7). The alkali shift of this spectrum was the same as the intact cells (compare Table 1 with Fig. 5). When diagnostic shift reagents were added for further analysis (Mabry et al. 1970), characteristics typical of flavonoids were found (Table 1).

Absorption spectra of crude extracts of GCPs

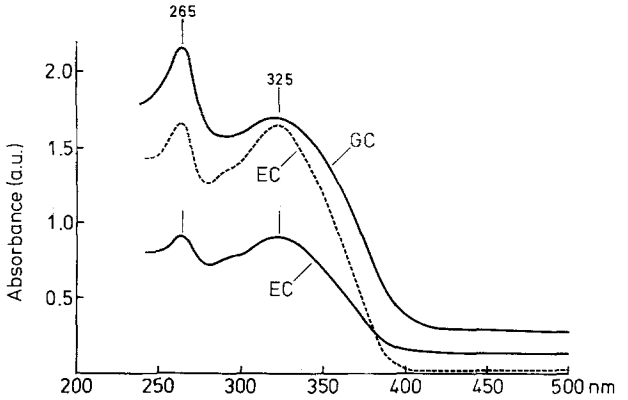


Fig. 6. Absorption spectra of vacuolar areas of onion guard cells (*GC*) and epidermal cells (*EC*). For *EC* (---), same cell with higher sensitivity. Circular measuring field = 6.3 μm for *GC*, 24 μm for *EC*

Table 1. UV spectral data of crude methanolic extracts of *Allium* epidermal peels. Values are given in nm; sh = shoulder

CH_3OH	267	298 sh	326	
CH_3ONa	275	391		
$AlCl_3$	270	299	337	392 sh
$AlCl_3/HCl$	270	298	332	392
CH_3COONa	266	297	330	365 sh 400 sh
CH_3COONa/H_3BO_3	266	296 sh	332	

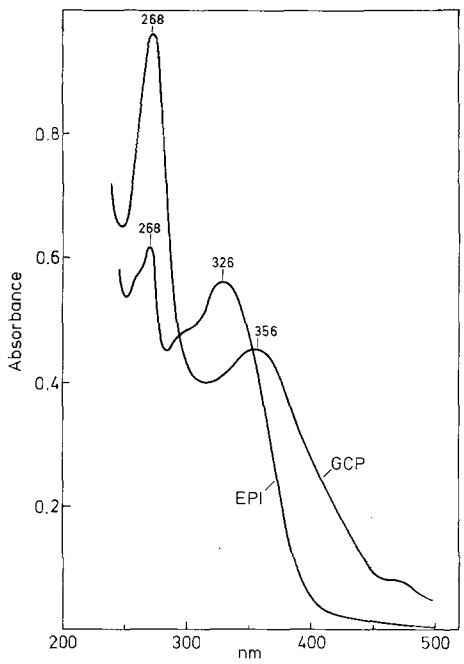


Fig. 7. Absorption spectra of methanolic crude extracts from peeled epidermal tissue (*EPI*) and from isolated *GCPs* of *Allium cepa*

showed, without adding reagents, a 30-nm bathochromic shift of the long-wavelength peak (Fig. 7). This is apparently caused by the presence of phosphate buffer used in order to detect flavins (Gishla et al. 1984). After purification of the GCP extracts on polyamide columns, the same absorption maxima as for epidermal tissue were recorded (268 and 326 nm in methanol).

In GCP extracts, no appreciable peak at 445 nm typical of flavins could be detected, and no photodecomposition of the absorbance was observed during a 30-min period of excitation at either 440 or 330 nm.

Flavonoids of epidermal tissue and isolated guard-cell protoplasts. On the basis of several properties, flavonoid constituents of onion epidermal tissue as well as of GCPs may be characterized as flavonol glycosides of high polarity (water solubility), with kaempferol and quercetin being the aglycones, substituted with sulphate and – or glucuronide moieties. Characteristics may be summarized as follows:

(i) On polyamide column chromatography all compounds showed high retentions and were not eluted with H_2O and CH_3OH; the subsequent addition of 0.01% NH_4OH to CH_3OH led to an immediate elution of a broad peak without separation of the individual flavonoids, (see Schulz et al. 1985) indicating compounds with acidic groups.

(ii) On thin-layer electrophoresis all flavonoids migrated towards the anode at pH 2.2 (R_f approx. 0.3) which is a known property of sulphated flavonoids (Markham 1982). There were two deep-purple bands under UV (354 nm) light, which turned yellow-greenish after fuming with NH_3 vapor. This behaviour indicates flavonoids substituted in position 3.

Since it has not been possible so far to separate and purify the various flavonoid components using classical preparative techniques (TLC; column chromatography), crude extracts of epidermal tissue as well as of GCPs were subjected to partial and complete acidic and enzymatic hydrolysis. Acid hydrolysis took place within 5 min (1 N HCl, 100° C) resulting in two major flavonoids (compounds 1, 2) and the major aglycones quercetin (compound 3) and kaempferol (compound 4; Table 2). Compounds 1 and 2 were hardly further hydrolyzed during a subsequent 2-h period. The presence of sulphate after acid hydrolysis was indicated by the appearance of a white precipitate on the addition of 2M $BaCl_2$ ($BaSO_4$, see Markham 1982).

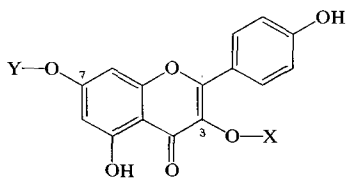
The stability of compounds 1 and 2 (Table 2) towards acid treatment indicates that they may be

Table 2. Onion flavonoids from epidermal tissue and GCP extracts separated on TLC, solvent 2, after acid hydrolysis of crude extracts. y = yellow (green) fluorescing; or = orange fluorescing

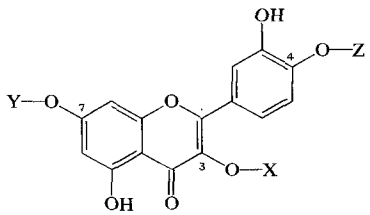
Compound no.	R_f	Color under UV-light (354 nm) Naturstoffreagenz	
		−	+
4	0.49	y	y
3	0.18	y	or
2	0.16	y	y
1	0.06	y	or

glucuronides (Harborne 1965). Thus, they were treated with β-glucuronidase for 2 h at 37° C. The reaction was terminated by adding methanol and the mixture was subjected to TLC and HPLC analysis. Compound 1 yielded quercetin, and compound 2 yielded kaempferol (70–80% yield compared to t_0, when enzymatic hydrolysis was started; yield estimation by HPLC). Glucuronic acid was identified according to Schulz et al. (1985); in addition, other unknown sugars were detected. There were some minor flavonoids yielding kaempferol, quercetin and an unknown aglycone after β-glucuronidase treatment.

Our results strongly indicate that compounds 1 and 2 are 7-O-glucuronides of quercetin and kaempferol respectively. They show yellow autofluorescence under UV and there is almost no shift of the short-wavelength band after the addition of sodium acetate (see Table 1). From the interpretation of spectral data according to Mabry et al. (1970), coupled with the results of hydrolysis and colour behaviour on TLC, the following basic structures of onion flavonoids are suggested:



kaempferol-type flavonols



quercetin-type flavonols

X, Y, Z = various substituents such as sugars, sulphate, glucuronic acid

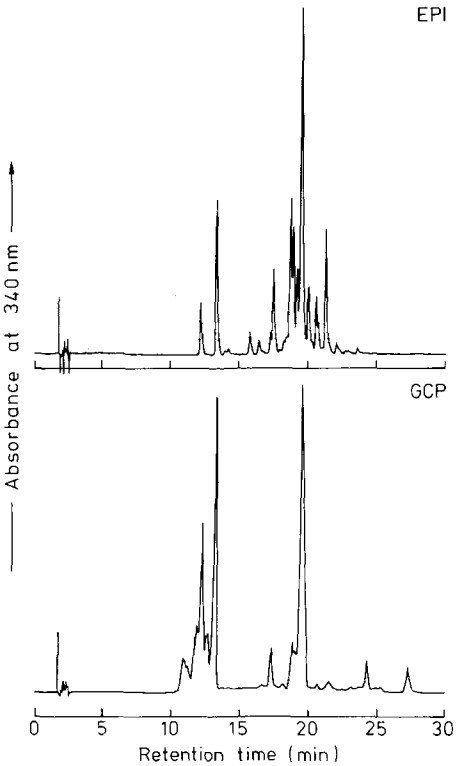


Fig. 8. Analysis of crude extracts of epidermal peelings (*EPI*) and of isolated *GCP* from *Allium cepa* by HPLC

Further analysis will be necessary for structural elucidation of individual compounds.

Flavonoid constituents of epidermal and GCP extracts could be partly separated by HPLC (Fig. 8); they show similar basic patterns with differences in their distribution which apparently reflect characteristics of the two cell types, guard cells and epidermal cells. It should be noted that no flavonoid aglycones were present and no phenylpropanoids could be detected in the extracts by HPLC, detection at 300 and 260 nm, polyamide column chromatography, and thin-layer electrophoresis.

The total flavonoid content of GCPs was estimated by HPLC at 340 nm to be 95 nmol $\cdot 10^{-6}$ protoplasts (average of n = 6). This is in agreement with the total flavonoid contents of isolated GCPs from *Vicia faba* and *Pisum sativum*, where 85 and 82 nmol $\cdot 10^{-6}$ protoplasts, respectively, were determined (Weissenböck et al. 1984; 1986).

Discussion

Results shown in Fig. 1 confirm earlier observations by Zeiger and Hepler (1979) and Palevitz et al. (1981) that autofluorescing compounds of the epidermis are restricted to guard cells of *Allium*

cepa. This phenomenon seems to be typical of guard cells of the genus *Allium*. The emission characteristics of this green fluorescence is in accordance with spectral properties of flavins or flavoproteins with λ_{max} at 520 nm (see Zeiger and Hepler 1979; Zeiger 1980).

We confirmed that fluorescence intensities measured at 520 nm show photodecomposition characteristic of flavins when guard cells were exposed to 436 nm; this is close to the long-wavelength absorption maximum of flavins at 445 nm (Ghisla et al. 1984). Under our conditions we measured a 50% decrease in intensity within 3 min, whereas Zeiger and Hepler (1979) and Palevitz et al. (1981) obtained the same value within 1 min.

However, when flavins are present, photobleaching should also be detectable at an excitation wavelength of 365 nm which is close to the absorption maximum of 370 nm. This expectation could not be confirmed in our measurements since fluorescence intensity did not decrease but instead increased (Fig. 2). It is not known yet whether the autofluorescence observed with λ_{max} 470 nm (excitation at 365 nm) resulted from the same compounds showing an emission λ_{max} of 520 nm (excitation at 436 nm; compare Figs. 1 and 2), or, rather, is caused by the presence of other substances.

Therefore, we may conclude that flavins are either absent in guard cells or the decrease in fluorescence intensity of flavins is concealed by other substances showing a strong increase with time (excitation at 365 nm; Fig. 2).

At present it is not clear whether or not the increase observed without ammonia in *Allium* guard cells (Fig. 2) is a result of the presence of flavonoids. When NH_4OH was added, there was an alkaline-induced bathochromic shift of the emission spectrum (λ_{max} 470→540 nm; Fig. 3a) followed by an increase in fluorescence intensity which was stronger compared to that without alkali (see Fig. 2). Accordingly, in a previous paper we showed that the alkaline-induced increase in fluorescence intensity is typical of flavonol glycosides (kaempferol 3,7-O-glycosides) present in guard cell vacuoles of *Vicia faba* (Schnabl et al. 1986), but the differences in the emission maxima (520 nm *Vicia*, 540 nm *Allium*) may result from the specific characteristics of the vacuolar medium.

The UV-VIS absorption spectrum of flavins shows maxima at 266, 370 and 445 nm (Ghisla et al. 1984; Ninnemann 1980). However, the absorption spectrum of *Allium* guard cells is not consistent with these characteristics (Figs. 4–6). It should be noted that cells from which these spectra were obtained still showed their typical green fluorescence when the spectral analysis was started. Thus, a complete photodestruction of the 445-nm peak seems to be improbable.

When guard cells are compared with epidermal cells, identical UV-absorption spectra typical of flavonoids were recorded in vivo and in vitro (Figs. 5–7), indicating that there are similar basic patterns of flavonol glycosides for both cell types (see Fig. 8). As to the guard-cell-specific approx. threefold increase in emission intensity during 20 min of excitation at 365 nm (Fig. 3), there are several possible interpretations of this phenomenon and a detailed discussion of comparable findings with *Vicia faba* guard and epidermal cells was given in Schnabl et al. (1986). To us, the most plausible explanation at present is that an intracellular alkalinization, continuously increasing with time, is induced by the ammonia treatment. Since this treatment may be coupled to a damage of cell membranes the increase in fluorescence intensity is probably a consequence of the properties of the various flavonol glycosides present in guard-cell vacuoles. When *Allium* flavonols were isolated from epidermal tissue or from GCPs, they appeared deep purple without any fluorescence on TLC under 354-nm UV light and they turned yellow-greenish immediately after fuming with NH_3 vapor. When the epidermal cells show a very rapid increase in fluorescence intensity within seconds after alkali treatment, this may be the result of a facilitated penetration of ammonia which leads to fluorescence of vacuolar flavonols (Fig. 3).

The presence of flavonol glycosides in guard and epidermal cells may be connected with the general role of those compounds as screens for UV radiation. At present, however, a possible, yet-unknown role of flavonols in the mechanism of stomatal action has to be considered (Weissenböck et al. 1984, 1986; Schnabl et al. 1986). Furthermore, the occurrence and involvement of flavins or other non-flavonoid light-sensitive photoreceptor molecules should be carefully reexamined.

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