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# Cell wall sited flavonoids in lisianthus flower petals

Kenneth R. Markham[a,*], Ken G. Ryan[a], Kevin S. Gould[b], Geoff K. Rickards[c]

[a]*NZ Institute of Industrial Research and Development, P.O. Box 31310, Lower Hutt, New Zealand*
[b]*School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand*
[c]*School of Biological Sciences, Victoria University of Wellington, Private Bag 600, Wellington, New Zealand*

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**Abstract**

Flavonoids are considered to be located predominantly in the vacuoles of epidermal cells and in the cuticular wax of terrestrial plants. However, recent reports have suggested that flavonoids may also reside elsewhere in the cells of green leaves. In the present study of lisianthus flower petals, it is demonstrated that ca. 30% of the whole petal flavonol glycosides are located in the cell wall. These flavonol glycosides are distinguished from the vacuolar glycosides in that they lack acylation. Evidence from light and confocal microscopy studies is corroborated by HPLC analyses of isolated protoplasts and cell wall digests, these having been produced by enzymic treatment of epidermal peels. This is the first report of the occurrence of flavonoids in petal cell walls, and it describes novel methodology for such studies. 

*Keywords:* *Eustoma grandiflorum*; Lisianthus; Petals; Cell wall flavonoids; CLSM; HPLC

## 1. Introduction

Flavonoids are a class of phenolic natural products which are virtually ubiquitous in land-based green plants (Markham, 1982). They are known to have a diverse range of functions in plants ranging from UV protection, coloration, free radical scavenging and defence against bacteria, fungi, insects, etc., to influence upon pollination, pollen tube growth, nodule formation and allelopathy (Harborne, 1994). Thus, the location or compartmentalisation of these compounds within plants is of significance with respect to their function. For example, flavonoids bound into the cell wall would unlikely be involved in functions outside of this tissue. Furthermore, their very existence could be overlooked because they may not even be extractable by traditional means.

There is now a considerable body of evidence which suggests that plant flavonoids are synthesised on the endoplasmic reticulum (Stafford, 1990; Hrazdina, 1992). They are subsequently translocated to a variety of specific sites, and in particular to the cuticles and vacuoles of epidermal cells (Stafford, 1990; McClure, 1975). Limited evidence also suggests that flavonoids may be compartmentalised in or on a variety of tissues and organelles, including the nucleus and cytoplasm (Hutzler et al., 1998; Grandmaison and Ibrahim, 1996; Ibrahim, 1992). However, the existence or otherwise of flavonoids in cell walls seems to have attracted little attention. Thus, scant reference is made to this issue in any of the five major review volumes on flavonoids published since 1962 (Harborne, 1994).

Deposition of flavonoids within the cell wall was originally proposed to account for the poor extractability of flavonoids from some plant material, or to account for the size of the attached polysaccharide moiety (Markham, 1972). An example of the former is the isolation of kaempferol-3-*O*-glucoside from cell wall material of Norway spruce, *Picea*

* Corresponding author. Tel.: +64-4-569-0577; fax: +64-4-596-0055.
*E-mail address:* k.markham@irl.cri.nz (K.R. Markham).

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*abies* (Strack et al., 1988). Although some of this flavonoid was extractable with methanol, much larger amounts were liberated when pre-extracted plant material was treated with hot alkali. In this case the flavonoid seemed not to be associated with the enzymically produced pectin or hemicellulosic components, but rather with the (Bjorkman) lignin. In the needles of a number of other gymnosperms, light microscopy studies of $AlCl_3$-induced flavonoid fluorescence in sections, and thin-layer chromatographic analyses of peels, have indicated that the constituent biflavonoids are confined to the cuticle and cutinised regions of the cell wall (Gadek et al., 1984). A cell wall location has also been proposed for the partially methylated flavonol glucosides of *Chrysosplenium americanum* (Ibrahim et al., 1987) on the basis of immunofluorescence and immunogold localisation. More recently, Hutzler et al. (1998) have presented evidence obtained by confocal laser scanning microscopy (CLSM) in support of the existence of a cell wall location for some flavonoids in Norway spruce needles. In that work flavonoids were detected via the fluorescence induced by diphenylboric acid 2-aminoethyl ester (Naturestoffreagenz A or NA). Overall however, most of the evidence for the existence of flavonoids in plant cell walls has been indicative or observational with little support from actual isolation work. Rare too are reports of the structural identification of purported cell wall associated flavonoids.

In the course of studying the pigment interactions that contribute to flower colour at the cellular level, it has become evident that the location of different flavonoid types within the petal could be a significant factor in limiting the extent of possible inter-flavonoid interactions. The lisianthus (*Eustoma grandiflorum*) cultivars under study exhibit a limited range of flower colours comprising essentially only white, pink, magenta and purple. Although the gross colour differences are due to variation in the types and levels of the anthocyanins accumulated, the accompanying eightfold excess of flavonol glycosides is also thought to play a role in flower colour through copigmentation with the anthocyanins (Markham and Ofman, 1993). High pressure liquid chromatography (HPLC) analysis has shown that the flavonol glycoside profile is the same for all cultivars. Whilst light microscopy examination reveals that the anthocyanins are located in the vacuoles of the epidermal cells, it does not reveal the location of the colourless flavonol glycosides which have been assumed to accompany the anthocyanins in the vacuole. The study reported here provides *direct* evidence for the location of a substantial proportion of these flavonol glycosides in the lisianthus petal cell wall. A novel approach is described which has led to the detection, isolation and identification of these cell wall associated flavonoids for the first time.

## 2. Results

### 2.1. Microscopy and microspectroscopic studies

In flower colour experiments designed to study the effect on colour of changing various parameters, anthocyanins and the full flavonol glycoside complement have been mixed in ratios defined from whole petal extracts, and at the appropriate pH, in attempts to reconstitute the original flower colour in vitro (e.g., Markham and Ofman, 1993; Bloor, 1997, 1999). However, the assumption that all flavonol glycosides are available to interact with the anthocyanins in the cell vacuole was brought into question as a result of our microspectroscopic studies on individual cells from purple lisianthus petals. The UV absorption spectra of adaxial epidermal cells showed broad maxima in the normal region for flavonol-3-glycosides of about 350 nm. In contrast, spectra obtained from the cell wall region of the same cells exhibited absorption maxima in the 380–393 nm region (Fig. 1). This absorption is considered to represent that of the constituent flavo-

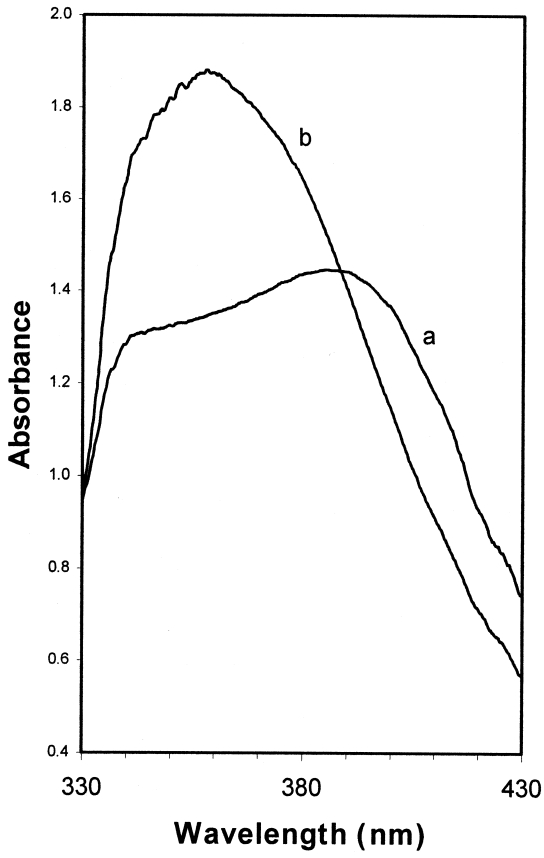



Fig. 1. Typical UV–Vis absorption spectra of lisianthus (line #54) adaxial epidermal cell wall and petal macerate, (a) before and (b) after, addition of 10% HOAc, as measured on a modified Zeiss compound microscope (pH change: ca. 5.3 (macerate) to ca. 2.5).

nols, since it shifted to the 350–360 nm region upon the addition of 10% HOAc to either intact tissue or a tissue macerate (Fig. 1). The spectral shift suggests that these flavonols are interacting in some way with other cell wall components, this interaction being nullified by acidification.

In order to isolate compartmentalised flavonol glycosides, it was necessary first to establish the tissue regions in which they reside. To this end, transverse sections of white lisianthus petals stained with NA were examined by conventional epifluorescence microscopy. At the excitation wavelength of 450 nm, flavonol glycosides fluoresce yellow or yellow-green and were clearly visible only in the epidermal cells. Within these cells, significant yellow fluorescence appeared to develop with time, both in the thick cell walls and in the intact vacuoles following infusion with NA. However, with this method, precise localisation of staining is complicated by the presence of out-of-focus background blur.

CLSM images of NA treated epidermal cells also revealed fluorescent staining in the epidermal cell walls. The stain was of uniform intensity across the cell wall, although there was a brighter region lining the inner surface (Fig. 2). It was not possible to resolve whether this originated from the cell wall itself or from the thin strand of cytoplasm surrounding the vacuole. Some fluorescence was also present in strands of cytoplasm within the cells but there was no staining in the vacuole of these cells. NA also modified the bright autofluorescence observed in unstained cuticles, intensifying the fluorescence and making it yellower. Staining was also observed in cell walls of some mesophyll cells. However, this was of lower intensity and may represent the contents of ruptured epidermal cells smeared there during section preparation.

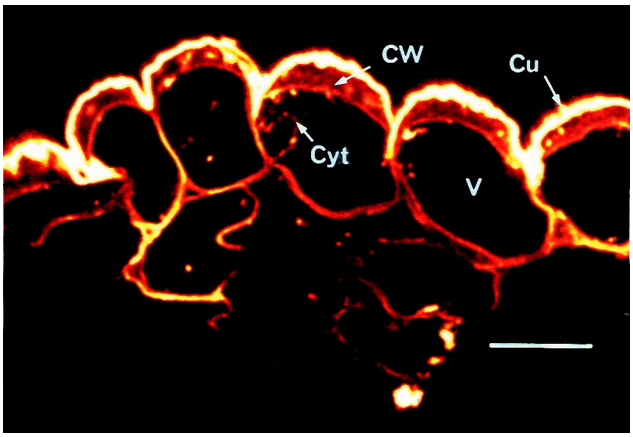



Fig. 2. Epidermal cells of white lisianthus (Wakasaki) petals stained with NA, as viewed by CLSM (scale: 20 μm). Flavonoid presence is indicated by orange coloration which is particularly evident throughout the thick upper wall of the epidermal cells (CW = cell wall; Cu = cuticle; Cyt = cytoplasm; V = vacuole).

### 2.2. Tissue type isolation and flavonoid studies

Flavonoids resident in the various tissue types referred to above were extracted in a series of steps. Lipophilic cuticular components (fraction 5) were obtained from intact white petals by washing with cold diethyl ether. Flavonoids in other relevant tissues were obtained from adaxial epidermal peels (with attached

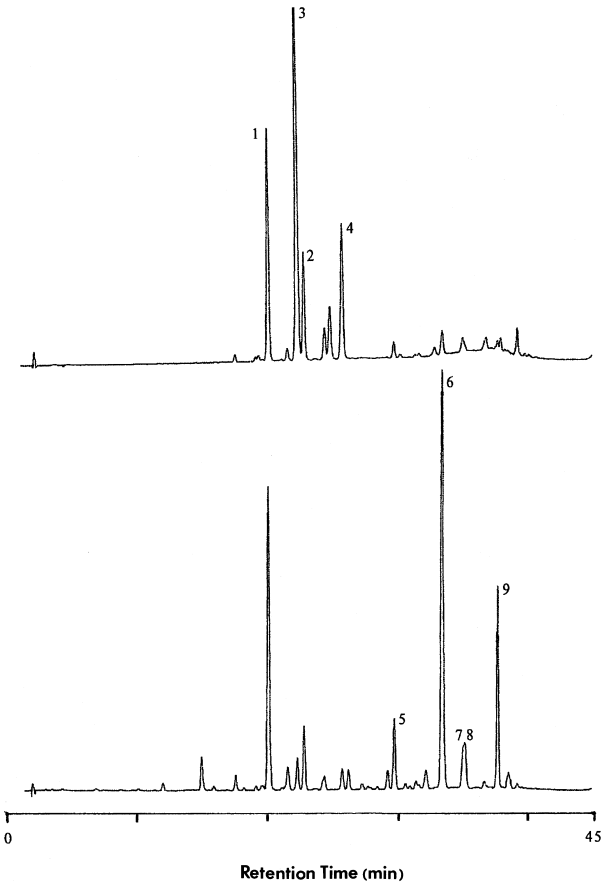



Fig. 3. HPLC profiles (352 nm) of cell wall located flavonoids (fraction 2, upper trace) and protoplast flavonoids (fraction 3, lower trace): **1** — kaempferol-3-*O*-[6-*O*-rhamnosylgalactoside]-7-*O*-rhamnoside, **2** — isorhamnetin-3-*O*-[6-*O*-rhamnosylgalactoside]-7-*O*-rhamnoside, **3** — kaempferol-3-*O*-[6-*O*-rhamnosylgalactoside], **4** — isorhamnetin-3-*O*-[6-*O*-rhamnosylgalactoside], **5** — kaempferol-3-*O*-[6-*O*-rhamnosyl-4-*O*-*E*-caffeoylgalactoside]-7-*O*-rhamnoside, **6** — kaempferol-3-*O*-[6-*O*-rhamnosyl-4-*O*-*E*-*p*-coumaroylgalactoside]-7-*O*-rhamnoside, **7** — isorhamnetin-3-*O*-[6-*O*-rhamnosyl-4-*O*-*E*-*p*-coumaroylgalactoside]-7-*O*-rhamnoside, **8** — kaempferol-3-*O*-[6-*O*-rhamnosyl-4-*O*-*E*-ferulylgalactoside]-7-*O*-rhamnoside, **9** — kaempferol-3-*O*-[6-*O*-rhamnosyl-4-*O*-*Z*-*p*-coumaroylgalactoside]-7-*O*-rhamnoside. The upper trace represents an injection volume of 4 μl and the lower an injection volume of 2 μl. Retention times (in minutes) are detailed in Section 4.

mesophyll) which were washed to liberate adhering mesophyll constituents (fraction 1) and then treated with a cellulase/pectolyase enzyme mix to yield cell wall digest (fraction 2) and protoplasts (fraction 3). Products from both white and purple lisianthus petals were obtained in this manner. Cell wall hydrolysates, protoplast contents (obtained after sonication in ethanol), mesophyll and cuticle washings, together with reference extracts from untreated petals, were analysed by HPLC.

Consistent with earlier findings (Markham and Ofman, 1993; Asen et al., 1986), the whole petal extract HPLCs (352 nm) revealed the presence only of flavonol glycosides, 66% of which were monoacylated with *p*-coumaric and other hydroxycinnamic acids. The protoplast contents (fraction 3) from both white and purple petals gave flavonol profiles similar to this, with the acylated derivatives comprising 68% of the total (Fig. 3). In contrast, the cell wall hydrolysates (fraction 2) from both petal types contained only non-acylated flavonol glycosides (Fig. 3). Of the four major flavonol glycosides detected in fraction 2, two were the triglycosides reported previously from whole petal extracts, namely kaempferol- and isorhamnetin-3-*O*-[6-*O*-rhamnosylgalactoside]-7-*O*-rhamnosides, **1** and **2**, respectively (Asen et al., 1986; Markham and Ofman, 1993). The other two, **3** and **4**, were shown to be artifacts derived from these by enzyme hydrolysis. Thus, a 60-h exposure of the epidermal peels to the enzyme mix produced only **3** and **4**. The major of these was shown to be kaempferol-3-*O*-[6-*O*-rhamnosylgalactoside] (**3**) from its absorption spectrum, retention time, and by cochromatography with an authentic sample (e.x., *Cymbidium* flowers). The other was assigned the analogous isorhamnetin-3-*O*-[6-*O*-rhamnosylgalactoside] (**4**) structure on the basis of its distinctive absorption spectrum and retention time, which indicated that it too lacked a 7-linked sugar. In situ therefore, the cell wall sited flavonoids are considered to be the unacylated triglycosides **1** and **2**. The distinctive absence of acylated derivatives in the cell wall digest was shown not to be a result of enzyme action, by subjecting the protoplast contents to the protoplasting enzyme mix for a longer period. After 60 h, the percentage of acylated flavonol glycosides remained unchanged at 68%.

Another feature distinguishing cell wall sited flavonoids from protoplast sited flavonoids was the much higher proportion of isorhamnetin glycosides in the former. The ratio of isorhamnetin to kaempferol glycosides in the cell wall fraction (fraction 2) was determined by integration of the relevant (see Section 4) HPLC peaks as about 1:2, whereas in the protoplasts the ratio was only about 1:7.

The level of flavonoids associated with the petal cell wall was unexpectedly high. Integration of the flavonol glycoside peaks in the HPLC analyses of the enzyme digest (fraction 2) and the protoplast contents (fraction 3) indicated that about 30% of the petal flavonoids reside within the cell wall. Most of the remaining 70% is found within the protoplast, as levels in the cuticular layer and in the mesophyll are negligible by comparison.

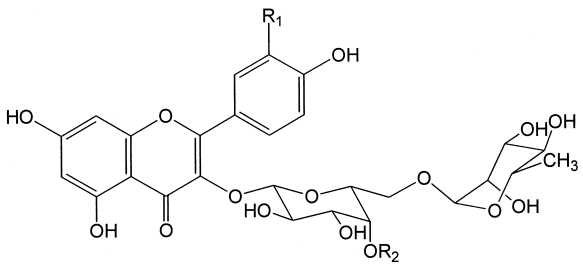
The cuticle and the mesophyll fractions (fractions 5 and 1, respectively) were both found to be very low in flavonoids. HPLC analysis of the cuticular wash revealed no hydroxycinnamic esters that might account for the cuticular autofluorescence observed by epifluorescence microscopy. Only a low level presence of the flavonoids found in whole petal extracts was detected. The mesophyll washings likewise contained low levels of the glycosides found in the protoplasts, but it is possible that they originate from epidermal protoplasts ruptured in the workup process. If indeed the mesophyll tissue does accumulate low levels of flavonoids, the presence of the acylated glycosides not found amongst the cell wall flavonoids precludes the possibility that mesophyll flavonoids significantly contaminate the cell wall originating flavonoids.

**1** R = rhamnosyl; $R_1$ = H; $R_2$ = H: (**5**, $R_2$ = *E*-caffeoyl)
(**6**, $R_2$ = *E*-*p*-coumaroyl)
(**8**, $R_2$ = *E*-ferulyl)
(**9**, $R_2$ = *Z*-*p*-coumaroyl)

**2** R = rhamnosyl; $R_1$ = $OCH_3$; $R_2$ = H: (**7**, $R_2$ = *E*-*p*-coumaroyl)

**3** R = H; $R_1$ = H; $R_2$ = H

**4** R = H; $R_1$ = $OCH_3$; $R_2$ = H




## 3. Discussion

The clear distinction between the cell wall associated flavonols and the protoplast flavonols parallels that found by Strack et al. (1988) in Norway spruce needles. There too, the acylated flavonol glycoside was apparently confined to the vacuole and the non-acylated glycoside was recovered from cell wall material

(although the alkaline extraction procedure could have deacylated any cell wall bound acylated glycosides). The vacuolar accumulation of acylated flavone glycosides and acylated anthocyanins has been investigated by others (Hopp and Seitz, 1987; Matern et al., 1986) using isolated vacuoles, and is attributed to the existence of a specific transport system for translocation through the tonoplast. The equivalent non-acylated glycosides remained predominantly outside of the vacuole as appears to be the case in lisianthus petals and possibly also in spruce needles.

The fact that the ratio of acylated to non-acylated flavonol glycosides is much the same in the protoplast contents as it is in the whole petal extracts suggests that, as in the Norway spruce, the cell wall associated flavonols are not readily extractable. Certainly, the absorption spectra measured in vivo ($\lambda_{max}$ 380–393 nm) differ markedly from that of the isolated glycoside ($\lambda_{max}$ 350 nm in MeOH) and are thus consistent with some type of binding, for example to cell wall protein or carbohydrate. Cell wall binding is not uncommon with other phenolics, such as the hydroxycinnamic acids, which have been encountered in cell wall tissue of both monocotyledons and dicotyledons (Lozovaya et al., 1999 and references therein). Flavonoids compartmentalised in the cell wall in this way, clearly are not available to influence flower colour through copigmentation type interaction with the vacuolar anthocyanin pigments. In view of the high level and distinctive type of cell wall sited flavonoids found in the present study, this is a factor that may need to be taken into account in in vitro flower colour reconstitution experiments and in strategies designed to modify flower colour by genetic modification.

The results described above substantiate earlier, often indirect evidence for the localisation of flavonoids in plant cell walls. In addition, they provide substantial *direct* chemical evidence for the presence of flavonoids in the cell wall tissue of a flower petal for the first time.

## 4. Experimental

### 4.1. Plant material and preparation

The *Eustoma grandiflorum* lines used in this study were the purple #54 and the white Wakasaki (see Deroles et al., 1998 for further details). Epidermal peels were prepared from 10 fresh petals by applying transparent adhesive tape firmly to both the adaxial and abaxial petal surfaces and pulling the tapes apart to separate the upper and lower petal tissues by rupture of the mesophyll layer. The tape-attached adaxial epidermal tissue (with some attached mesophyll) was washed with distilled water, while being gently rubbed, to produce a "mesophyll washings" fraction (fraction 1). It was then floated face down in protoplasting solution containing 1% cellulase Onozuka R-10 (Yakult Honsha, Higashi-Shinbashi, Minatoku, Tokyo) and 0.05% Pectolyase Y23 and mannitol (to give an osmolarity reading of 600 milliosmols). The mix was incubated overnight at room temperature and then filtered through a 50 μm stainless steel mesh to yield cell wall digest (fraction 2) plus protoplasts. The protoplasts were washed in 1/10 strength VKM macro salts (Binding and Nehls, 1977) plus 17.53 g/l NaCl (wash solution), and collected by centrifugation ($100 \times g$, 5 min). The pelleted protoplasts were rinsed twice with wash solution. Isolated protoplasts were then sonicated in MeOH to liberate the contents (fraction 3), the solution evaporated, made up to 250 μl with MeOH:$H_2O$ (4:1) and centrifuged to clarify. The cell wall digest solution initially removed from the protoplasts was freed of fibre and residual protoplasts by further centrifugation. It was then passed through a C-18 Alltech (Extract clean) column under vacuum. After thorough washing with distilled water, the column was washed with MeOH to remove the flavonoid fraction. This was made up to 250 μl with MeOH:$H_2O$ (4:1). The "mesophyll washings" were cleaned up in the same manner and also made up to 250 μl. Whole petal extracts (fraction 4) were produced by extraction of ground petal tissue with MeOH:$H_2O$ (4:1) followed by centrifugation to clarify the extract. Cuticular washings (fraction 5) were obtained by dipping fresh whole petals in diethyl ether for several minutes, evaporating the solution to dryness and redissolving the residue in 250 μl of MeOH. The cleaned up and standardised fractions 1–5 were used for HPLC analysis.

### 4.2. HPLC analyses

HPLC analyses were carried out using a Waters 600E solvent delivery system, Waters 996 diode array detector, and a Jasco 851-AS intelligent sampler, results being analysed using Waters Millennium 2010 software. An injection volume of 2–4 μl (protoplast contents, whole flower extract and cell wall digest) or 20 μl (cuticular and mesophyll washings) was used with a Merck LiChrospher 100RP-18 endcapped column (5 μ, 4 × 119 mm). Elution (0.8 ml/min, 30°) was performed using a solvent system comprising solvent A (1.5% $H_3PO_4$) and solvent B (HOAc–$CH_3CN$–$H_3PO_4$–$H_2O$ (20:24:1.5:54.5)) mixed using a linear gradient starting with 80% A, decreasing to 33% at 30 min, 10% A at 33 min, and 0% A at 39.3 min. Flavonols were detected at 352 nm and hydroxycinnamic acids (searched for) at 310 nm. Retention times and on-line recorded absorption spectra for each of the major components detected are as follows: *Cell wall flavonoids*: **1**, RT = 19.95 min, $\lambda_{max}$ 265.1, 325 sh, 345.7

nm; **2**, RT = 22.74 min, $\lambda_{max}$ 255.6, 267 sh, 352.9 nm; **3**, RT = 22.21 min, $\lambda_{max}$ 265.1, 290 sh, 348.1 nm; **4**, RT = 25.72 min, $\lambda_{max}$ 255.6, 268 sh, 295 sh, 352.9 nm. *Protoplast flavonoids*: **1** and **2**, RT = 19.95 and 22.74 min (as above); **5**, RT = 29.70 min, $\lambda_{max}$ 255 sh, 265.1, 295 sh, 331.4 nm (kaempferol-7-*O*-rhamnoside-3-*O*-[6-*O*-rhamnosyl-4-*O*-caffeoylgalactoside]); **6**, RT = 33.34 min, $\lambda_{max}$ 267.4, 314.8, 350 sh.nm (kaempferol-7-*O*-rhamnoside-3-*O*-[6-*O*-rhamnosyl-4-*O*-*E*-*p*-coumaroylgalactoside]); **7**, RT = 35.09 min, $\lambda_{max}$ 255.6, 267 sh, 319.5, 355 sh.nm (isorhamnetin-7-*O*-rhamnoside-3-*O*-[6-*O*-rhamnosyl-4-*O*-*E*-*p*-coumaroylgalactoside] + possibly **8**, kaempferol-7-*O*-rhamnoside-3-*O*-[6-*O*-rhamnosyl-4-*O*-ferulylgalactoside]); **9**, RT = 37.59 min, $\lambda_{max}$ 255 sh, 265.1, 314.8, 350 sh.nm (kaempferol-7-*O*-rhamnoside-3-*O*-[6-*O*-rhamnosyl-4-*O*-*Z*-*p*-coumaroylgalactoside]?; see Asen et al., 1986). The sums of the integrals of these peaks (at 352 nm) were used in the quantification calculations for the relative levels of acylated and non-acylated flavonol glycosides, cell wall and protoplast sited flavonols, and kaempferol and isorhamnetin glycosides.

### 4.3. Microscopy

Absorbance spectra of epidermal cells from macerates and transverse and paradermal sections of lisianthus petals were obtained using a Zeiss compound microscope, modified such that all glass components were removed and a front-surfaced mirror incorporated to optimise the transmission of UV. Zeiss quartz objectives (40×) were used as condenser and objective. The light source, provided by a 100 W xenon arc, was passed through a UG5 filter (Shott) to reduce transmission of visible wavelengths and thereby improve the accuracy of UV absorbance measurements. Specimens were mounted in water on quartz microscope slides and covered with quartz coverslips. The spectral absorbance of individual cells or portions of cells was measured using an Ocean Optics spectrophotometer, connected to the microscope phototube via a fibre optic cable. A reference solution of rutin ($\lambda_{max}$ 360 nm) was used to check the accuracy of the equipment and to determine the lower wavelength limit (330–340 nm) for reliable spectral measurement.

Fresh sections of the plant material were examined for the presence of flavonols using epifluorescence microscopy with a Zeiss filterset 09 (excitation: 450 nm; beamsplitter: 510 nm; emission 520 nm) in a Zeiss Axioplan 2 microscope. Sections were then stained with a saturated solution of NA (Sigma) in 10% aqueous sucrose, and examined for yellow-green fluorescence.

CLSM of NA stained samples was carried out on a Leica TCS 4D using a 40 × /1. Ona oil immersion objective. The filter block used a 488 nm excitation filter, a 488 nm dichroic mirror and a fluorescein isothiocyanate band pass suppression filter.

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## References

Asen, S., Griesbach, R.J., Norris, K.H., Leonhardt, B.A., 1986. Flavonoids from *Eustoma grandiflorum* flower petals. Phytochemistry 25, 2509.

Binding, H., Nehls, R., 1977. Regeneration of isolated protoplasts to plants in *Solanum dulcamara* L. Z. Pflanzenphysiol 85, 279.

Bloor, S.J., 1997. Blue flower colour derived from flavonol–anthocyanin copigmentation in *Ceonothus papillosus*. Phytochemistry 45, 1399–1405.

Bloor, S.J., 1999. Novel pigments and copigmentation in the blue marguerite daisy. Phytochemistry 50, 1395–1399.

Deroles, S.C., Bradley, M.J., Schwinn, K.E., Markham, K.R., Bloor, S.J., Manson, D.G., Davies, K.M., 1998. An antisense chalcone synthase cDNA leads to novel colour patterns in lisianthus (*Eustoma grandiflorum*) flowers. Molecular Breeding 4, 59.

Gadek, P.A., Quinn, C.J., Ashford, A.E., 1984. Localisation of the biflavonoid fraction in plant leaves with special reference to *Agathis robusta*. Aust. J. Chemistry 32, 15–31.

Grandmaison, J., Ibrahim, R.K., 1996. Evidence for nuclear protein binding of flavonol sulphate esters in *Flaveria chloraefolia*. J. Plant Physiol. 147, 653–660.

Harborne, J.B., 1994. The Flavonoids — Advances in Research Since 1986. Chapman and Hall, London (and earlier volumes referenced therein).

Hopp, W., Seitz, H.U., 1987. The uptake of acylated anthocyanin into isolated vacuoles from a cell suspension culture of *Daucus carota*. Planta 170, 74–85.

Hrazdina, G., 1992. Compartmentation in aromatic metabolism. In: Stafford, H.A., Ibrahim, R.D. (Eds.), Phenolic Metabolism in Plants. Plenum Press, New York, pp. 1–23.

Hutzler, P., Fischbach, R., Heller, W., Jungblut, T.P., Reuber, S., Schmitz, R., Veit, M., Weissenbock, G., Schnitzler, J.-P., 1998. Tissue location of phenolic compounds in plants by confocal laser scanning microscopy. J. Exp. Botany 49, 953–965.

Ibrahim, R.K., De Luca, V., Khouri, H., Latchinian, L., Brisson, L., Charles, P.M., 1987. Enzymology and compartmentation of polymethylated flavonol glucosides in *Chrysoplenium americanum*. Phytochemistry 26, 1237–1245.

Ibrahim, R.K., 1992. Immunolocalization of flavonoid conjugates and their enzymes. In: Stafford, H.A., Ibrahim, R.K. (Eds.), Phenolic Metabolism in Plants. Plenum Press, New York, pp. 25–61.

Lozovaya, V.V., Gorshkova, T.A., Yablokova, E.V., Rumyantseva,

N.I., Valieva, A., Ulanov, A., Widholm, J.M., 1999. Cold alkali can extract phenolic acids that are ether linked to cell wall components in dicotyledonous plant (buckwheat, soybean and flax). Phytochemistry 50, 395–400.

Markham, K.R., Ofman, D.J., 1993. Lisianthus flavonoid pigments and factors influencing their expression in flower colour. Phytochemistry 34, 679–685.

Markham, K.R., 1972. A novel flavone polysaccharide compound from *Monoclea forsteri*. Phytochemistry 11, 2047–2053.

Markham, K.R., 1982. Techniques of Flavonoid Identification. Academic Press, London, pp. 49–51.

Matern, U., Reichenbach, C., Heller, W., 1986. Efficient uptake of flavonoids into parsley (*Petroselinum hortense*) vacuoles requires acylated glycosides. Planta 167, 183–189.

McClure, J.W., 1975. Physiology and functions of flavonoids. In: Harborne, J.B., Mabry, T.J., Mabry, H. (Eds.), The Flavonoids. Chapman and Hall, London, pp. 970–1055.

Stafford, H.A., 1990. Flavonoid Metabolism. CRC Press, Boca Raton, FL, pp. 202–208.

Strack, D., Heilemann, J., Klinkott, E.S., 1988. Cell wall bound phenolics from Norway spruce (*Picea abies*) needles. Z. Naturforschung 43c, 37–41.