Pergamon

Phytochemistry, Vol. 49, No. 5, pp. 1427–1430, 1998

Printed in Great Britain
0031–9422/98/$—see front matter

PII : S0031–9422(98)00081–8

IDENTIFICATION OF FLAVONOL AND ANTHOCYANIN METABOLITES IN LEAVES OF *PETUNIA* 'MITCHELL' AND ITS *LC* TRANSGENIC

STEPHEN J. BLOOR,* J. MARIE BRADLEY,† DAVID H. LEWIS† and KEVIN M. DAVIES†

Industrial Research Ltd, PO Box 31310, Lower Hutt, New Zealand; † New Zealand Institute for Crop and Food Research, Private Bag 4005, Levin, New Zealand

(*Received in revised form* 5 *January* 1998)

Key Word Index—*Petunia*; Solanaceae; leaves; petals; acylated flavonol glycosides; kaempferol sophoroside; quercetin sophoroside.

Abstract—The leaves of *Petunia* 'Mitchell', a model species employed for transgenic manipulation, contain a wider array of acylated flavonol glycosides than occur in the petals. Three new acylated flavonol glycosides are described, kaempferol-3-*O*-(2-*O*-feruloyl-β-D-glucosyl(1→2)6-*O*-malonylglucoside), quercetin-3-*O*-(2-*O*-caffeoyl-β-D-glucosyl(1→2)6-*O*-malonylglucoside), and quercetin-3-*O*-(2-*O*-feruloyl-β-D-glucosyl(1→2)glucoside). A transgenic Mitchell line expressing the maize *'Leaf colour'* (*Lc*) cDNA had enhanced levels of anthocyanins, particularly in their leaves. These anthocyanins were determined to be the same acylated petunidin glycosides as those which produce a slight red colouration in the tube of the flowers of *Petunia* 'Mitchell'.

INTRODUCTION

In studies focusing on the manipulation of the flavonoid biosynthetic pathway *Petunia* 'Mitchell', a white flowing cultivar, has been used as a model species to test genetic modification strategies aimed at colour modification. Two examples of such work are where the maize *Leaf colour* gene (*Lc*) [1] and chalcone reductase gene (CHR) [2] have been successfully introduced into 'Mitchell', leading to enhanced levels of pigmentation (*Lc*) or the introduction of yellow flower colour (CHR). *Petunia* 'Mitchell' and derived transgenics have also been employed in studies relating to the UV-B effects on plants [3]. A prerequisite of this work was the need for a detailed understanding of the existing flavonoid chemistry in this particular cultivar. Of particular interest was the relationship between the flavonols in the leaf and petal tissue.

The chemistry of the petal tissue of *Petunia* has been well studied. In particular, Griesbach's group have identified a range of kaempferol and quercetin glycosides [4, 5] and a number of anthocyanidin glycosides [6] in flowers of a variety of petunia cultivars. The flavonol glycosides previously reported are the 3-glucosides, 7-glucosides, 3,7-diglucosides, 3-caffeoylsophorosides and 3-caffeoyl-sophoroside-7-glucosides of kaempferol and quercetin. Petunoside, a ferulylated kaempferol 3-sophoroside has also been identified [7]. The anthocyanins are mainly the 3-[*p*-coumaroyl (or caffeoyl) rutinoside]-5-glucosides of malvidin, petunidin and peonidin or unacylated 3-rutinosides and 3-glycosides of delphinidin, cyanidin or pelargonidin.

In this work we have determined the structures of the major flavonol glycosides present in the leaves of *Petunia* 'Mitchell'. The anthocyanin structures for four of the major anthocyanins in the pigmented leaves of the transgenic *Lc* Mitchell petunia, have also been determined, but positions of acylation not specified.

RESULTS AND DISCUSSION

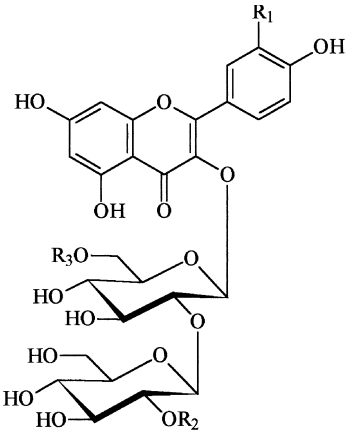
There are at least 13 discernible flavonol glycoside peaks in the HPLC diagram of extracts of Mitchell leaf. However, there are only two basic structural types, as base hydrolysis of the extract yields kaempferol- and quercetin-3-*O*-sophorosides, i.e. all of the other flavonols are acylated versions of these two glycosides. In contrast, the major petal part, the limb, contained only one dominant glycoside, quercetin 3-*O*-sophoroside.

The structures of eight major compounds **1**–**8**, were defined after isolation by polyamide CC and preparative HPLC. Compounds **1** and **4** co-eluted from a polyamide column and were readily identified, without further separation, as the sophorosides of

*Author to whom correspondence should be addressed.

Table 1. ^{1}H NMR data for acylated flavonol glycosides

	6	**7**	**9**	**10**	**11**	**12**
H-2′	**7.98** *d* (2H) 8.8	7.96 *d* (2H) 8.9	7.56 *dd* 8.5, 2.1	7.54 *dd* 8.5, 2.1	7.90 *d* (2-H) 8.8	7.47 *d* 8.8
H-6′			7.48 *d* 2.1	7.46 *d* 2.1		7.41*s*
caff/fer H-*β*	7.46 *d* 16	7.45 *d* 15.9	7.41 *d* 16	7.45 *d* 15.8	7.46 *d* 16	7.38 *d* 16
caff/fer H-2	6.98 *d* 2	7.14 *br s*	6.97 *d* 1.8	7.13 *br s*	7.15 *br s*	6.95 *s*
caff/fer H-6	6.87 *dd* 8.8,2	6.94 *d* 10.2	6.87 *m*	6.94 *d* 8.5	6.97 *br d* 8	6.83 *d* 8
H-3′	6.91 *d* (2H) 8.9	6.89 *d* (2H) 8.1	—	—	6.89 *d* (2H) 8.9	—
H-5′			6.87 *m*	6.86 *d* 8.5		6.85 *d* 8.3
caff/fer H-5	6.70 *d* 8.2	6.71 *d* 8.1	6.69 *d* 8.1	6.69 *d* 8.2	6.71 *d* 8.2	6.66 *d* 8
H-8	6.38 *d* 2	6.36 *d* 2	6.36 *d* 2	6.32 *d* 2	6.36 *d* 2	6.33 *s*
caff/fer H-*α*	6.23 *d* 16	6.35 *d* 15.9	6.22 *d* 16	6.34 *d* 15.8	6.35 *d* 16	6.21 *d* 16
H-6	6.18 *d* 2	6.18 *d* 1.9	6.19 *d* 2	6.18 *d* 2	6.18 *d* 2	6.16 *s*
1″	5.69 *d* 7.5	5.73 *d* 7.5	5.70 *d* 7.5	5.74 *d* 7.5	5.63 *d* 7.3	5.58 *d* 7.3
1‴	5.07 *d* 8	5.08 *d* 8.0	5.05 *d* 8	5.07 *d* 8	5.07 *d* 8	5.01 *d* 8.3
2‴	4.66 *t* 9	4.68 *t* 9	4.66 *t* 9	4.68 *t* 8.4	4.66 *t* 8.4	4.64 *t* 8.3
6″a					4.12 *d* 10.3	4.12 *d* 11.7
6″b						3.88 *m*
fer OMe		3.77 (3H) *s*		3.77 (3H) *s*	3.76 *s* (3H)	
mal CH_2					2.98 (2H) *m*	2.94 (2H) *m*



	R_1	R_2	R_3
1	H	H	H
2	H	caffeoyl	H
3	H	feruloyl	H
4	OH	H	H
5	OH	caffeoyl	H
6	OH	feruloyl	H
7	H	feruloyl	malonyl
8	OH	caffeoyl	malonyl

kaempferol and quercetin respectively (by co-chromatography on HPLC with authentic standards). Compounds **2** and **5** were the mono-caffeates of **1** and **4** respectively (by ^{1}H NMR, see Table 1). The position of acylation was determined as the C-2 of the distal glucose unit since a one proton triplet was clearly seen at about 4.6 ppm in the ^{1}H NMR spectrum. This proton was coupled to one of the anomeric protons which, since the C-2 of the proximal glucose is blocked by the second glucose, must be the H-2 proton of the distal glucose. A comparison of ^{13}C NMR data for **5** with that of quercetin-3-*O*-(2-*O*-caffeoyl-*β*-D-glucosyl(1→2)-glucoside)7-*O*-glucoside [8] support this structure assignment (Table 2).

The ^{1}H NMR spectra of **3** and **6** (Table 1) were similar to those of **2** and **5** apart from the appearance of a methoxyl signal showing these were the 2‴-*O*-ferulyl-sophorosides of kaempferol and quercetin respectively.

Compound **8** was similar to **2**; however an additional non-aromatic acyl group was present. The ^{1}H and ^{13}C NMR spectra of **8** (see Tables 1 and 2) showed the acyl group to be a malonyl group and its location to be at the C-6 of one of the sugars (from the downfield position of signals for one of the CH_2 groups. A TOCSY NMR experiment linked the acylated CH_2 protons to the anomeric proton of the proximal glucose. Hence **8** is quercetin-3-*O*-(2-*O*-caffeoyl-*β*-D-glucosyl(1→2)6-*O*-malonylglucoside). FAB(−) MS confirmed the molecular formula ($[M-H]^-$ at 873.17463, $C_{39}H_{37}O_{23}$ requires 873.17256).

Compound **7** was also a malonylated compound (from ^{1}H NMR) and by analogy was assigned the structure kaempferol-3-*O*-(2-*O*-feruloyl-*β*-D-glucosyl(1→2)6-*O*-malonylglucoside). FAB(+) MS $[MH]^+$ at 873.20786, $C_{40}H_{41}O_{22}$ requires 873.20895.

The structures of two of the other minor flavonol glycosides were tentatively assigned as the mono-malonylated derivatives of the two flavonol sophorosides, based on the on-line spectral data and retention times. There was no evidence for the presence of flavonols with 7-*O*- glycosylation in the leaf tissue of Mitchell

Table 2. ^{13}C NMR data for flavonol glycosides **5** and **8**

	Q-3caff-soph-7-glc[#]	**5***	**8***
Aglycone	156.4	156.2	156.2
	133.1	132.9	132.8
	177.4	177.3	177.3
	160.8	161.2	161.2
	99.2	98.5	98.6
	162.6	164.0	163.1
	94.1	93.4	93.5
	155.8	155.7	156.2
	105.5	103.9	103.9
	122.0	121.9	121.8
	115.3	115.4	115.7
	144.8	144.8	144.5
	148.9	148.4	148.5
	115.8	115.8	115.8
	120.8	121.2	121.1
3-*O*-glc	97.3	97.4	97.5
	79.5	79.6	79.7
	76.7	76.7	76.1
	70.2	70.2	70.1
	77.2	77.2	73.7
	60.5	60.7	63.5
2^{G}-*O*-glc	99.5	99.5	99.6
	73.7	73.7	73.7
	74.4	74.5	74.5
	70.0	70.0	69.9
	76.3	76.4	76.8
	60.5	60.7	60.9
7-*O*-glc	99.7		
	73.0		
	76.3		
	69.5		
	77.0		
	60.5		
Caffeoyl	165.7	165.77	165.8
	114.6	114.7	114.7
	144.5	144.5	144.6
	125.5	125.7	125.7
	114.6	114.8	114.7
	148.2	148.1	148.1
	145.5	145.4	145.5
	115.6	115.6	115.4
	120.8	120.7	120.8
Malonyl		166.4	
		41.0	
		166.4	

[#]Taken from Ref 8.

*Similar shifts within a column may be interchanged.

or the *Lc* transgenics. These comprise up to 20% of the glycosides reported previously from *Petunia* 'Mitchell' petal tissue [9].

Of the eight structures determined, the sophorosides (**1** and **4**), caffeoyl sophorosides (**2** and **5**) and petunoside (**3**) are part of the group described previously in petal tissue while the malonylated and/or ferulylated sophorosides (**6**, **7** and **8**) are new compounds. The location of the caffeoyl group in two of the known compounds, which was not specified previously, has also been determined. In most of the extracts of *Petunia* 'Mitchell' and *Lc* leaves, a non-flavonoid phenolic compound, was present in significant amounts, and in many cases it is the dominant peak in the HPLC diagram at 352 nm. This compound was also isolated and determined to be rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. Small amounts of caffeic and ferulic acid were also present.

Although the levels and types of flavonol glycosides remained relatively unchanged in the *Lc* transgenics, the introduction of the *Lc* transgene enhanced the production of anthocyanins throughout the plant. Anthocyanin accumulation occurred most dramatically in the leaves but was also enhanced in stems, sepals, tube and limb of the flower [1]. Although the limb of *Petunia* 'Mitchell' flowers generally appear white, the tubes of Mitchell flowers do show some pigmentation due to the presence of anthocyanins. The anthocyanin HPLC profile of the extract from the *Lc* leaves was identical to that from the Mitchell tubes. These extracts showed three major anthocyanins, **9**–**11**, along with several minor anthocyanins. Acid hydrolysis gave petunidin and delphinidin (approx. 3 : 1) and the relatively long retention times suggested they were acylated glycosides. Base hydrolysis resulted in the removal of these acyl groups and yielded one major glycoside, **12**, together with a minor glycoside (ratio ca 3 : 1) which had HLPC retention times indicative of tri- or higher glycosylation.

Previous work on petunia petals has shown the major petunidin glycoside to be petunidin-3-(*p*-coumaroylrutinoside) 5-glucoside [6], a compound also present in berries of *Solanum* species [10]. The major *Lc* leaf anthocyanin, **11**, was shown to be the same compound by cochromatography (HPLC) and on-line spectral comparison with an extract from *Solanum* berries. Further, the *Solanum* base hydrolysis product co-chromatographed with **12** i.e. **12** is petunidin-3-rutinoside-5-glucoside. The on-line UV spectrum of **10**, and its relative retention time, indicated it to be a petunidin 3-rutinoside 5-glucoside acylated with a caffeic acid group. The third anthocyanin, **9**, was more unusual. Base hydrolysis of a small amount of purified **9** yielded a mixture of the anthocyanidin glycoside, **12**, and a more polar compound sharing the UV spectral characteristics of the acyl group of **9**. Subsequent acid hydrolysis of this mixture yielded *p*-coumaric acid. From this evidence the most likely acyl group in **9** is a glycosylated p-coumaric acid i.e. **9** is a glycosylated derivative of **11** with the additional sugar attached to the *p*-coumaric moiety. This type of acyl group has been reported in other anthocyanins such as the ternatins [11]. The structures of the delphinidin glycosides were not determined.

While the glycosylation pattern of the flavonols of *Petunia* 'Mitchell' occurs commonly and is consistent with previous work on *Petunia* petals, the presence of both kaempferol and quercetin glycosides and the high degree of acylation of the leaf flavonols implicate the involvement of a larger range of biosynthetic genes in the leaves. The level of flavonols in leaf tissue is only ca 2% by dry weight compared with ca 10% for the petals. So, although the flavonoid biosynthesis is downregulated in the leaves, the biosynthetic complexity is enhanced in comparison to petal tissue.

EXPERIMENTAL

General

The Mitchell petunia (*Petunia axillaris* × (*P. axillaris* × *P. hybrida*)) line was obtained from the University of Auckland, N.Z. Plants were grown under standard greenhouse conditions. NMR spectra were recorded at ambient temperature in DMSO-d_6. FAB mass spectra were recorded on a VG70-250S instrument. Analytical HPLC was performed using a Waters 600 solvent delivery system with a Water 996 diode array detector. Column; Merck Lichrospher™ 100 RP-18 endcapped (5 μm, 4$\varnothing$ × 119 mm. Elution (0.8 ml/min, 30°C) performed using a solvent system comprising solvent A [HOAc : CH_3CN : H_3PO_4 : H_2O (20 : 24 : 1.5 : 54.5] and 1.5% H_3PO_4 mixed using a linear gradient starting with 20% A, increasing to 67% A at 30 min, 90% A at 33 min and 100% A at 39.3 min. Flavonols and rosmarinic acid were detected at 352 nm and anthocyanins at 530 nm. Flavonol standards were from a reference collection held in the laboratory. *Solanum* berries were collected from wide *Solanum nigrum* plants in the laboratory grounds. Flavonol levels were calculated as rutin equivalents from HPLC peak areas at 352 nm using a standard curve prepared from recrystallised rutin.

Isolation of Flavonols

Approximately 200 g of Mitchell petunia leaves were extracted by soaking in methanol and the concentrated extract was chromatographed on a polyamide column (MN SC-6). The flavonols were eluted by increasing the amount of methanol in 0.1% TFA. Individual fractions were analysed by HPLC. The major acylated flavonol glycosides were purified by preparative HPLC using an isocratic solvent system (methanol : water : acetic acid, 46 : 54 : 1, 1.5 ml/min), a semipreparative column (Phenomenex 5μm, 10$\varnothing$ × 250 mm) and detection at 350 nm.

Purification of **9**

Anthocyanins were extracted from *Lc* transgenic leaf tissue (3 g) by soaking in 0.2% aq. TFA for 4 h. The concentrated extract was chromatographed on polyamide eluting initially with 0.1% aq. TFA followed by a gradually increasing proportion of methanol in the same solvent. **9** was eluted as the first major coloured band and was sufficiently pure for degradation analysis.

Acknowledgements—This work was supported by the N.Z. Foundation for Research, Science and Technology (Contracts CO8504, CO2405).

REFERENCES

1. Bradley, J. M., Davies, K. M., Deroles, S. C., Bloor, S. J. and Lewis, D. H., *The Plant Journal*, 1998, **13**, 381.
2. Davies, K. M., Bloor, S. J., Spiller, G. B. and Deroles, S. C., *The Plant Journal*, 1998, **13**, 259.
3. Ryan, K. G., Markham, K. R., Bloor, S. J., Bradley, J. M., Mitchell, K. A. and Jordan, B. R., *Photochemistry and Photobiology*, submitted.
4. Griesbach, R. J., *HortScience*, 1993, **28**, 659.
5. Griesbach, R. J. and Asen, S., *Plant Science*, 1990, **70**, 49.
6. Griesbach, R. J., Asen, S. and Leonnarat, B. A., *Phytochemistry*, 1991, **30**, 1729.
7. Birkhofer, L. and Kaiser, C., *Z. Naturforsch*, 1962, **17b**, 279.
8. Nielsen, J. K., Olsen, C. E. and Perersen, M. K., *Phytochemistry*, 1993, **34**, 539.
9. Griesbach, R. J. and Kamo, K. K., *Phytochemistry*, 1996, **42**, 361.
10. Price, C. L. and Wrolstad, R. E., *J. Food Sci.*, 1995, **60**, 369.
11. Terahara, N., Saito, N., Honda, T., Toki, K. and Osajima, Y., *Phytochemistry*, 1990, **29**, 949.