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NOVEL ANTHOCYANINS PRODUCED IN PETALS OF GENETICALLY TRANSFORMED LISIANTHUS

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Key Word Index—*Eustoma grandiflorum*; Gentianaceae; lisianthus; anthocyanins; transgenic; UFGT transgene; delphinidin-3-*O*-β-D-[6-*O*-α-L-rhamnopyranosylglucopyranoside]-5-*O*-β-D-[6-*E* (and *Z*)-*p*-coumaroylglucopyranoside]; NMR; TOCSY.

Abstract—The structures of the pigments in the deep purple flowers of a lisianthus line, transformed with a UDP-glucose:flavonoid-3-*O*-glucosyltransferase cDNA from *Antirrhinum majus*, have been studied using paper chromatography, HPLC and ^{1}H and ^{13}C NMR spectroscopy involving the use of ^{1}H–^{1}H COSY and TOCSY techniques. The transgenic line is shown to have produced the new anthocyanins delphinidin-3-*O*-β-D-(6-*O*-α-L-rhamnopyranosylglucopyranoside)-5-*O*-β-D-[6-*E* (and *Z*)-*p*-coumaroylglucopyranoside] and delphinidin-3-*O*-β-D-glucopyranoside-5-*O*-β-D-(6-*O*-*E*-*p*-coumaroylglucopyranoside) in addition to those found in the untransformed control plants.

INTRODUCTION

Lisianthus (*Eustoma grandiflorum* Grise.) is becoming increasingly popular as a cut flower. The range of flower colours available in commercial lines is, however, limited to white and shades of cream, pink, mauve and purple. The pigment and co-pigment chemistry of lisianthus flowers have been reported in recent publications [1, 2] together with an extensive study of the factors influencing their expression in flower colour [2]. One notable difference between lisianthus pigments and those of many ornamentals is the predominance of galactose residues at the 3-position of the anthocyanins and co-pigments, rather than the more common glucose. As part of a programme aimed at generating new flower colours in lisianthus, transgenic lisianthus containing a cDNA encoding the UDP-glucose: flavonoid-3-*O*-glucosyltransferase (UFGT) from *Antirrhinum majus* have recently been produced. One line of these plants, whose production and general analysis is detailed elsewhere [3], produces a range of anthocyanins novel to lisianthus. In this paper the detailed analysis and identification of the anthocyanins present in petals of transgenic line TL851 is presented.

RESULTS AND DISCUSSION

The pigment and co-pigment contents of purple petals from lisianthus, transformed with UFGT cDNA from *A. majus* (line TL851), were compared directly with those from non-transformed plants by two-dimensional paper chromatography (2D-PC), HPLC and a variety of NMR techniques. Whilst no qualitative differences were seen in the flavonol glycoside profiles [3], significant changes were evident in the anthocyanin composition of the transformed plant.

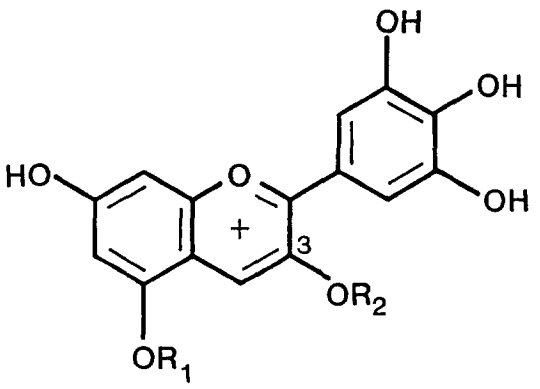
2D-PC flavonoid patterns of the transformed lisianthus petals revealed some doubling of the anthocyanin spots, and HPLC confirmed the presence of several major new anthocyanins. All anthocyanins in the petals of the non-transgenic control and UFGT-transgenic plants were present in their acylated forms as evidenced by their intense absorption in the 320 nm region. A quantitative HPLC comparison (λ 530 nm) revealed, through peak integration, that the new anthocyanins accounted for *ca* 33% of the total anthocyanins in the transformed petals [3].

Isocratic preparative HPLC was used to isolate individual anthocyanins from transformed plant material following part-purification by 1D-PC. Four fractions (2–5) containing workable amounts of anthocyanins were obtained. 1D-PC analysis in 15% acetic acid revealed that all but fraction 4 consisted of mixtures of major compounds with R_fs close to those reported for the *trans*/*cis* (*E*/*Z*) isomer mixes **22**/**23** and **21**/**22** (its *Z*-isomer) from untransformed plants [2]. Indeed, **22** and **23** (delphinidin-3-*O*-β-D-[6-*O*-α-L-rhamnopyranosylgalactopyranoside]5-*O*-β-D-[6-*E* (and *Z*)-*p*-coumaroylglucopyranoside] proved to be the predominant anthocyanins in the largest HPLC fraction (3). These compounds were identified by co-chromatography and from their ^{13}C and ^{1}H NMR spectra (Tables 1 and 2), which were identical with those previously recorded [2], but which are now fully assigned in this study. Compound **22**, confirmed as the more stable *E*-isomer by ^{1}H NMR spectroscopy (Table 2), was also

Table 1. ^{13}C NMR spectra (saccharide region) of the major anthocyanins in petals of the lisianthus transgenic line TL851*

Sugar carbon		Compound (fraction no.)			
		22/23 (3)	**1** (4)	**2** (2)	**3** (3)
3-*O*-gal	C-1	104.1			
	C-2	72.0			
	C-3	76.2			
	C-4	70.3			
	C-5	74.9			
	C-6	67.3			
3-*O*-glc	C-1		102.6	102.7	102.7
	C-2		74.6	74.6	74.9
	C-3		78.1†	78.1	78.1
	C-4		71.2	71.2	71.8
	C-5		77.6	77.6	76.2
	C-6		67.5	67.4	62.7
6″-*O*-Rha	C-1	102.0	102.1	102.1	
	C-2	72.4	72.4	72.4	
	C-3	71.7	71.7	71.7	
	C-4	74.0	74.0	74.0	
	C-5	70.0	69.9	69.9	
	C-6	18.1	18.0	18.2	
5-*O*-Glc	C-1	102.9	103.0	103.0	102.9
	C-2	74.8	74.9	74.9	74.8
	C-3	78.0	78.2†	78.1	78.0
	C-4	72.1	72.1	72.1	72.1
	C-5	76.4	76.4	76.4	76.4
	C-6	64.7	64.7	64.7	64.7

*Solvents as in Experimental.
†Assignments may be interchanged.



1 R_1 = glc^6—*E*-*p*-coum; R_2 = glc^6—rha
(**22** R_1 = glc^6—*E*-*p*-coum; R_2 = gal^6—rha)
2 R_1 = glc^6—*E*-*p*-coum; R_2 = glc^6—rha
(**23** R_1 = glc^6—*E*-*p*-coum; R_2 = gal^6—rha)
3 R_1 = glc^6—*E*-*p*-coum; R_2 = glc
(**21** R_1 = glc^6—*E*-*p*-coum; R_2 = gal)

found as the major component of fraction 5. The *Z*-isomer was lacking from this fraction indicating that the *E* to *Z* isomerization is much less favoured than the reverse, and thus that fraction 3 probably contains the *Z*-isomer, which had in part isomerized.

Fractions 2 and 4, which represent the major new anthocyanins in transformed lisianthus TL851, mirrored fractions 3 and 5 in that 2 contained a mixture of *E*- and *Z*-isomers and 4 a single *E*-isomer. This was evident from the coupling constants of the α- and β-protons of the *p*-coumaroyl groups (Table 2). The ^1H NMR spectra of both fractions also confirmed a 1:1 ratio between the *p*-coumaroyl and delphinidin moieties and showed the presence of three sugar H-1 signals. In fraction 4, two of these H-1 signals were assignable to 5-*O*-β-D-glucosyl and terminal-*O*-α-L-rhamnosyl functions (Table 2). The third H-1 signal at δ 5.38, while superficially analogous to that of the 3-linked β-D-galactose in **22** (at *ca* δ 5.4), was shown by ^1H–^1H COSY to be coupled with a proton resonating at *ca* δ 3.7 whereas the H-2 in the 3-*O*-β-D-galactoside resonates at δ 4.05. The nature of this 3-linked sugar was clearly defined as glucose in the ^{13}C NMR spectrum, which also confirmed the identity of the 5-*O*-glucoside and the terminal rhamnose.

From the above, the two major new anthocyanins produced in the petals of the UFGT-transgenic plant, TL851, are the mono-*E*- and *Z*-*p*-coumaroyl derivatives of terminally rhamnosylated 3,5-diglucosyldelphinidin. Both the ^{13}C and ^1H NMR spectra (Tables 1 and 2) show that one glucose is rhamnosylated at the 6-hydroxyl (C-6, 67.5 ppm; H-6, δ 3.7/4.0) whilst the other is acylated at the 6-hydroxyl (C-6, 64.7 ppm; H-6, δ 4.4/4.5). However, which of the glucoses is acylated and which rhamnosylated is not defined. 1D- and 2D-TOCSY measurements were used to resolve this problem. Thus, with fraction 4, H–H couplings were tracked along the backbone of each glucose starting from the H-1 signal. The H-2 to H-6 signals were identified sequentially via irradiation of each newly identified signal in turn, using a series of mixing times from 40–130 msec. In this way also, assignments for all protons in both 3- and 5-linked glucoses were made (see Table 2). Thus, the 3-linked glucose H-1 with its signal at δ 5.38 was shown to reside in the same glucose unit as the H-6A/6B protons represented by the signals at δ 3.62/3.92. H-6 signals in this range indicated 6-*O*-glycosylation rather than acylation [4]. Cross-correlation of these signals in an ^1H–^{13}C COSY spectrum with the carbon at 67.4 ppm confirms this [5]. In a like manner, the 5-linked glucose H-1 (δ 5.17) was related to the 6-*O*-acylated glucose with H-6A/6B at δ 4.53/4.36 and C-6 at 64.7 ppm. The same relationships were established for fraction 2, thus defining the structures of the new anthocyanins as: delphinidin-3-*O*-β-D-(6-*O*-α-L-rhamnopyranosylglucopyranoside)5-*O*-β-D-[6-*E* (and *Z*)-*p*-coumaroylglucopyranoside] (**1** and **2**).

Minor lower R_f components in fractions 3 and 5 are thought to be the analogous de-rhamnosyl derivatives of the above, although evidence in support of this is based only on their relative R_f values and on chemical shifts of lower signals in the NMR spectra. For example, fraction 3, which contains the *E*-/*Z*-isomer

Table 2. ^{1}H NMR spectra of the major anthocyanins in petals of the lisianthus transgenic line TL851*

Proton		Compound (fraction no.)			
		23 (3)	**22** (5)	**1** (4)	**2** (2)
Delphinidin	H-4	8.86*s*	8.87*s*	8.86*s*	8.92*s*
	H-6	6.93 (2)	6.95 (1.5)	6.95	7.00 (2)
	H-8	6.97 (2)	6.98 (2.0)	6.98	7.01 (2)
	H-2′6′	7.72*s*	7.72*s*	7.73*s*	7.73*s*
p-Coumaroyl	H-α	5.78 (13)	6.35 (16.0)	6.29 (15.8)	5.83 (13.0)
	H-β	6.77 (13)	7.56 (15.5)	7.51 (15.8)	6.83 (13.0)
	H-2,6	7.43 (9.0)	7.44 (8.0)	7.36 (8.5)	7.49 (8.5)
	H-3,5	6.48 (9.0)	6.80 (8.5)	6.73 (8.5)	6.54 (8.5)
3-*O*-Gal	H-1	5.37 (7.5)	5.46 (8.0)		
	H-2	4.0	4.08		
	H-3	3.3–3.9	3.3–4.0		
	H-4	3.3–3.9	3.3–4.0		
	H-5	3.3–3.9	3.3–4.0		
	H-6A	3.90	3.91		
	H-6B	3.68	3.72		
3-*O*-Glc	H-1			5.38 (7.5)	5.44 (8.0)
	H-2			3.68	3.77
	H-3			3.52	3.4–4.1
	H-4			3.41	3.4–4.1
	H-5			3.7	3.4–4.1
	H-6A			3.95 (12, 1.5)	3.4–4.1
	H-6B			3.62 (12, 6)	3.4–4.1
6″-*O*-Rha	H-1	4.64	4.69 (1)	4.65	4.67
	H-2	3.3–3.9	3.74	3.4–3.9	3.4–4.1
	H-3	3.3–3.9	3.3–4.0	3.4–3.9	3.4–4.1
	H-4	3.3–3.9	3.3–4.0	3.4–3.9	3.4–4.1
	H-5	3.5	3.6	3.52 (9.5, 6.5)	3.6
	H-6	1.19 (6.1)	1.24	1.04 (6.4)	1.11 (6.1)
5-*O*-Glc	H-1	5.21 (7.5)	5.26 (7.0)	5.17 (8.0)	5.25 (7.5)
	H-2	3.7	3.77	3.67	3.75
	H-3	3.3–3.9	3.3–4.0	3.53	3.4–4.1
	H-4	3.64	3.3–4.0	3.52	3.4–4.1
	H-5	3.82	3.3–4.0	3.81	3.4–4.1
	H-6A	4.57 (12.5, 2.5)	4.61 (12, 1.5)	4.53 (12, 1.5)	4.60
	H-6B	4.41 (12.0, 8.0)	4.45 (12.0, 8.0)	4.36 (12, 7.5)	4.44

*Solvents as in Experimental; assignments determined by COSY experiments, and for **1** additionally by TOCSY; multiplicities (in Hz) appear in parentheses, and where not quoted were obscured by other signals.

mix, **22/23**, as major components, also contains a lower R_f component which exhibits in the ^{13}C NMR spectrum a number of lower intensity sugar carbon signals assignable to an unsubstituted 3-*O*-glucoside. In particular, the distinctive signal at 62.7 ppm is representative (in CD_3OD–trifluoracetic acid) of an unsubstituted C-6 carbon [6]. As expected, this cross-correlates with signals in the ^{1}H NMR spectrum at δ 3.88 and 3.71, which occur in the expected range for unsubstituted H-6A and 6B protons [4]. Structure **3** is therefore assigned to this compound.

The galactose containing equivalent of this compound, delphinidin 3-*O*-β-D-galactopyranoside-5-*O*-β-D-(6-*O*-*E*-*p*-coumaroylglucopyranoside), **21**, is seen as a minor constituent in the 1D-PC of fraction 5. It is also evidenced in the ^{13}C NMR spectrum by a signal at 62.7 ppm (representing an unsubstituted C-6 carbon) which cross-correlates with H-6A/6B signals at δ 3.9/3.8 in the ^{1}H NMR spectrum. In addition, a clear 8 Hz doublet at δ 5.34 represents the H-1 signal, which is cross-coupled with a C-1 signal at 104.6 ppm. This H-1 signal is confirmed as representing a 3-linked galactose by ^{1}H–^{1}H COSY in which H-2 was identified at δ 4.06 rather than the *ca* δ 3.7 expected for glucose. The H-1 of the 5-linked glucose in this compound appears at δ 5.23 and, as expected, cross-links to an H-2 signal at δ 3.75. In neither the galactose nor glucose forms of **21** are the *E*- and *Z*-isomers distinguishable by NMR, due to the dominance of signals from the major components. However, the 1D-PC R_f values prove that *E*-isomers are present in both fractions 3 and 5.

On the basis of the above it is concluded that the transgenic lisianthus line TL851, produced by insertion of an *A. majus* cDNA coding for UDP-glucosyl:flavonoid-3-*O*-glucosyltransferase [3], contains in its petals significant levels of anthocyanins in which glucose has replaced the 3-*O*-linked galactose present in the original anthocyanins. The anthocyanins are new to lisianthus and have not previously been found elsewhere [7, 8].

EXPERIMENTAL

Plant material. Petal tissue from untransformed line 54 control plants [9], and the transgenic line TL851 into which the *A. majus* cDNA coding for UFGT had been inserted [3], was supplied by the Plant Pigment Group, Crop & Food Research, Levin, NZ. The TL851 petal tissue sample was supplemented with like tissue from its clonal line TL846.

Sample extraction and work-up. A total of 28 g (wet wt) of fresh petals from the transformed plant (TL851) was ground and extracted with MeOH–H_2O–HOAc (11:5:1) (500 ml). Most of the vacuum-reduced extract was applied to 30 1D-PCs and chromatographed in *t*-BuOH–HOAc–H_2O (3:1:1) to permit sepn of anthocyanins (low mobility) from the flavonol glycoides (higher mobility) [2].

Flavonol glycosides. 2D-PC and HPLC (conditions as for quantitative HPLC, see below) analyses of the total extract revealed only quantitative differences between untransformed reference material and the two transgenics. For details see ref. [3].

Anthocyanins. The flavonol-free anthocyanin fr. isolated by 1D-PC from crude extract (above) was cleaned-up on a small RP-8 column using 7% aq. HOAc and 7% methanolic HOAc. The eluted anthocyanins were concd prior to analysis and sepn by isocratic analyt. and then prep., HPLC, respectively. Conditions (analyt.): column—Econosphere C18, 25 cm, 5 μm; solvent—a 1:1 mix of A (1.5% H_3PO_4 in H_2O) and B (H_2O–MeCN–HOAc–H_3PO_4, 107:50:40:3), flow rate 0.8 ml min^{-1}; detection at 352 and 530 nm. Conditions (prep.): column—Whatman Partisil 10, ODS-2, 25 cm; solvents as above, but 43% A and 57% B, flow rate 2 ml min^{-1}; detection at 440 nm. The collected frs were analysed by 1D-PC in 15% HOAc to reveal the following: fr. 1 (low level)—non-acylated anthocyanin? R_f 0.75; fr. 2—compound **2**, R_f 0.54, and compound **1**, R_f 0.38; fr. 3—compound **23**, R_f 0.50, compound **22**, R_f 0.32, and compound **3**, R_f 0.1; fr. 4—compound **1**, R_f 0.39; fr. 5—compound **22**, R_f 0.31, and two minor compounds, R_f 0.14, 0.05; fr. 6 (low level)—compound **22**, R_f 0.31, and two minor compounds, R_f 0.10, 0.05. Frs 2–5 were concd and each applied to an RP-8 column after dilution with 8% aq. HOAc. H_3PO_4 was removed by washing with 8% aq. HOAc, and the anthocyanins were eluted with 8% methanolic HOAc. Each purified fr. was evapd to dryness and then freeze-dried prior to dissolution in MeOH-d_4 for NMR studies. Routinely, ^1H NMR (500 HMz), ^{13}C NMR (75 MHz) and ^1H–^1H and ^{13}C–^1H COSY measurements were made on each fr. Additionally, 1D- and 2D-TOCSY experiments were carried out on fr. 4. Data derived are presented in Tables 1 and 2.

Quantitative HPLC analyses. Analyses of anthocyanins (and flavonol glycosides) were carried out using an RP-18, 5 μm, Econosphere column, a flow rate of 0.8 ml min^{-1} and the solvents A and B as a gradient changing linearly from 3:2 to 1:4 over 25 min and held at 1:4 for a further 10 min. Plots were recorded routinely at 352 and 530 nm.

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