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Modifying the acylation of flavonols in *Petunia hybrida*

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ABSTRACT

The potential for chemically-regulating the acylation of natural products in whole plants has been determined by treating petunia leaves with phenylpropanoid acyl donors supplied as the respective methyl esters. Treatment with derivatives of the naturally-occurring acylating species caffeic acid resulted in a general increase in flavonol derivatives, notably caffeoylated quercetin-3-*O*-diglucoside (QDG) and kaempferol-3-*O*-diglucoside (KDG). Similarly, methyl ferulate increased the content of feruloylated KDG 40-fold. Treatment with methyl coumarate resulted in the appearance of a coumaroylated derivative of quercetin-3-*O*-glucuronyl-glucoside (QGGA). When the feeding studies were repeated with the equivalent phenylpropanoid isosubstituted with fluorine groups a semi-synthetic 4-fluorocinnamoyl ester of QGGA was observed. Our results demonstrate the potential to regulate the acylation of flavonols and potentially other natural products by treating whole plants with methyl esters of natural and unnatural acyl donors.

1. Introduction

Acylation, the esterification of a hydroxyl acceptor by an acid donor, is a commonly observed modification of plant secondary metabolites leading to products with altered physical and biological properties. Acylating moieties include acetyl, malonyl, benzoyl, and phenylpropanoyl groups, with the acceptors varying from simple sugars to complex alkaloids (Ghangas, 1999; Walker and Croteau, 2000). As a well studied example, many flavonoids undergo acylation, most commonly with malonic acid or the phenylpropanoids coumaric, caffeic or ferulic acid (Bloor and Abrahams, 2002; Fujiwara et al., 1998). Flavonoid acceptors include anthocyanins, isoflavones, flavones and flavonols (Nielsen et al., 1993; Stochmal et al., 2001; Suzuki et al., 2001). While the exact roles of acylation are not always known, malonylation is known to direct flavonoid glycoside metabolites for storage in the vacuole (Boller and Wiemken, 1986), while esterification of anthocyanins with phenylpropanoids promotes intramolecular aggregation or stacking, which protects the oxonium ion from decomposition (Cevallos-Casals and Cisneros-Zevallos, 2004). With respect to novel biological activities, acylation of flavonoids can result in changes in pigmentation (Bloor, 2001), insect antifeedant activity (Harborne and Williams, 1998) and antioxidant properties (Alluis and Dangles, 1999). In addition, the pharmacological activities of several plant secondary metabolites are due to specific acylations by aromatic donors (Ma et al., 2005). As such the acyltransferases responsible for these modifications have recently been the subject of considerable research effort (reviewed by D'Auria, 2006). Typically, the acylation of complex natural products proceeds via a two step pathway in which the donor acid is activated through the formation of the respective CoA thioester through the action of an ATP-dependent CoA ligase, followed by acyl-transfer to the acceptor through the action of a BAHD acyltransferase (D'Auria, 2006). Other acyltransferases operating in plant secondary metabolism include acyl-glucose dependent enzymes, which belong to the SCPL enzyme family (Fraser et al., 2007).

With our background in foreign compound metabolism in plants, we have become interested in the possibility that xenobiotic acids can be mistaken for natural products and enter plant secondary metabolism as acyl donors. Such semi-synthetic acylated products have the potential to have novel biological activities, as demonstrated with fluoroacylated derivatives of the anticancer taxane drugs (Ojima, 2004). Fluorine-substitution is particularly effective in regulating the properties of bioactive molecules, due to the combination of the element's unique electronic properties and non-intrusive size (Smart, 2001). While such fluoroacylated derivatives can be prepared synthetically, there are potentially many advantages in using biotransformation reactions to achieve regio- and stereo-selective incorporation into natural product acceptors. Significantly, halogenated xenobiotic acids are known to enter acylation pathways in plant primary metabolism, with fatty acid derivatives of 2,4-dichlorophenoxyacetic acid accumulating as triglycerides in coconut (Lopez-Villalobos et al., 2004). With this in mind, we wanted to see if the acyl-incorporation of halogenated acids could be demonstrated in secondary metabo-

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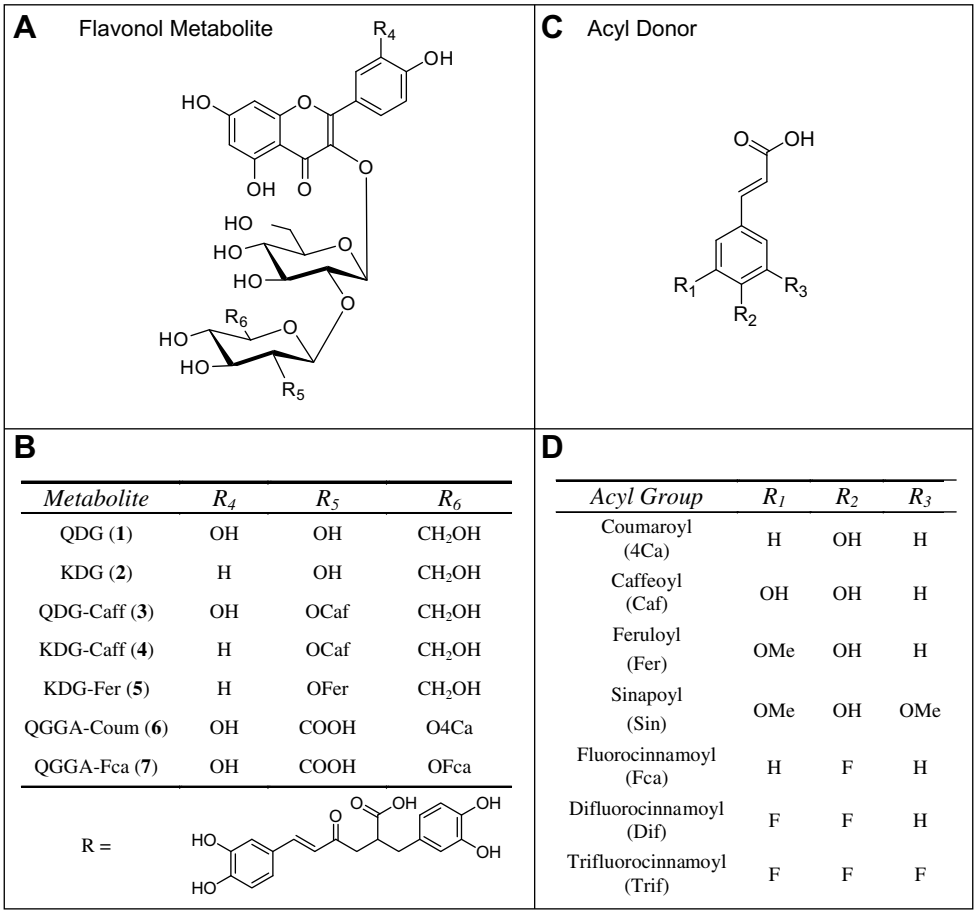
lism using commonly encountered acceptor species such as flavonoids. The acylation of flavonols in the leaves of petunia (*Petunia hybrida* L.) plants was selected as a suitable test system, as the respective natural products are well characterized and include a range of aromatic and aliphatic esters of glycosides of quercetin and kaempferol (Bloor et al., 1998). The experimental strategy has been to treat plants with caffeic and ferulic acid which are known to serve as acyl donors and then test the system with other naturally-occurring phenylpropanoids which are not normally incorporated. Having established the tractability of the acylating pathway, the plants were then fed with synthetic phenylpropanoids, where ring substituents have been replaced with fluorine atoms and the incorporation into the acylated flavonol pool determined.

2. Results

2.1. Acylated flavonols in petunia leaves

To facilitate the active accumulation of flavonoids, petunia plants were exposed to high level illumination (160 μEinstein m^{-2} s^{-1}) for 24 h prior to harvest. High level irradiance with the attendant exposure to UV light is known to provoke a major increase in the content of phenolic metabolites in a range of plant species (Himer et al., 2001; Li et al., 1993). The plants were either otherwise untreated, or sprayed with 0.1% (v/v) Triton X-100 to simulate the feeding conditions to be used later in the project with the potential acyl donors. As determined by HPLC, leaf extracts accumulated a range of UV-absorbing metabolites with the profiles obtained being essentially identical in individual plants. The individual UV-absorbing metabolites were identified with reference to published data (Cuyckens et al., 2003), using a combination of their UV–Vis absorbance spectra determined using photodiode array (PDA) detection and MS analysis of parent and fragment ions. The structures of the compounds identified are shown (Fig. 1) and are numbered in bold with reference to the respective peaks resolved by HPLC (Fig. 2A). The individual compounds were then quantified based on their absorbance at 330 nm, using a standard curve prepared from quercetin-3-*O*-diglucoside. By quantifying at 330 nm the concentration of the parent flavonoids was determined, with minimal interference from the acylating groups. The concentrations of the metabolites present were then compared in the plants treated with high level illumination (± detergent treatment) with plants grown under a conventional (80 μEinstein m^{-2} s^{-1}) lighting regime (Table 1). The major UV-absorbing metabolites identified in the light-stressed leaves were derivatives of the flavonols quercetin-3-*O*-diglucoside (QDG) and kaempferol-3-*O*-diglucoside (KDG), respectively. The relative abundances of the individual compounds were similar in all extracts, though their overall contents were approximately tripled by the high light treatment (Table 1). Spraying with detergent had no significant effect on the accumulation of the metabolites, as compared with the unsprayed controls (data not shown), so the results shown in Table 1 are derived from the plants sprayed with formulation. QDG accumulated as a major metabolite in its unmodified form (**1**), as well as being acylated with caffeic acid (QDG-Caf; **3**). In contrast, non-



Metabolite	R_4	R_5	R_6
QDG (**1**)	OH	OH	CH_2OH
KDG (**2**)	H	OH	CH_2OH
QDG-Caff (**3**)	OH	OCaf	CH_2OH
KDG-Caff (**4**)	H	OCaf	CH_2OH
KDG-Fer (**5**)	H	OFer	CH_2OH
QGGA-Coum (**6**)	OH	COOH	O4Ca
QGGA-Fca (**7**)	OH	COOH	OFca

R =

Acyl Group	R_1	R_2	R_3
Coumaroyl (4Ca)	H	OH	H
Caffeoyl (Caf)	OH	OH	H
Feruloyl (Fer)	OMe	OH	H
Sinapoyl (Sin)	OMe	OH	OMe
Fluorocinnamoyl (Fca)	H	F	H
Difluorocinnamoyl (Dif)	F	F	H
Trifluorocinnamoyl (Trif)	F	F	F

Fig. 1. The UV absorbing metabolites identified and acyl donors used in the studies with petunia. The templates of the flavonol acceptors are shown (A), along with the respective acylated metabolites (B), which are numbered in bold and refer to the peaks shown in Fig. 2. The phenylpropanoid metabolite rosmarinic acid (R) is also shown. The template of the acyl donors used is shown (C) and their specific substituents (D). K = kaempferol, Q = quercetin, DG = diglucoside, GGA = glucosyl-glucuronide.

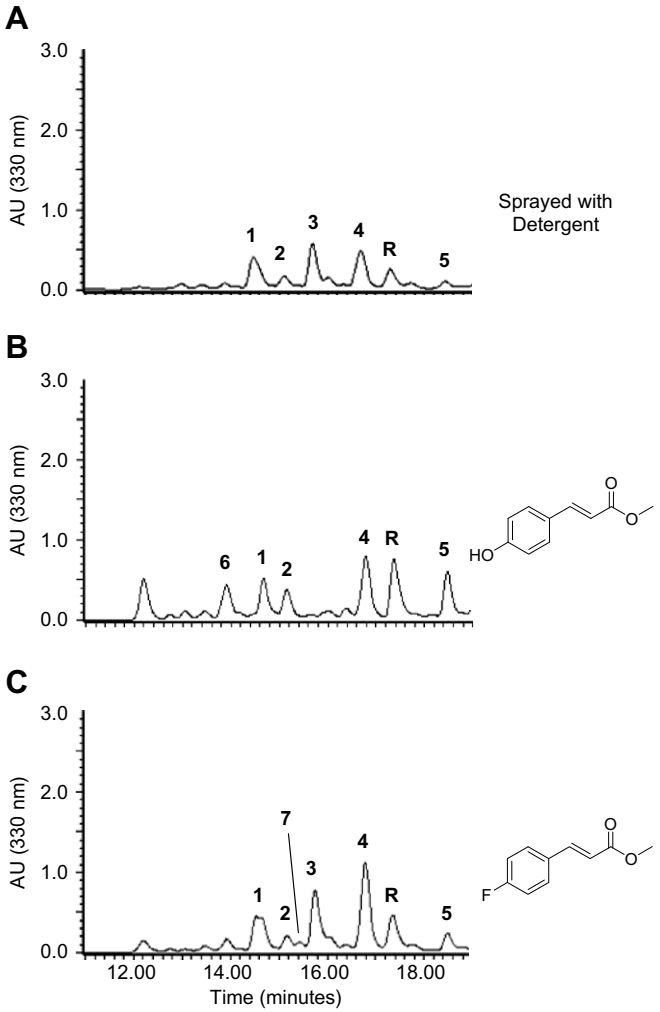


Fig. 2. UV-absorbing metabolites extracted from Petunia leaves exposed to high level illumination for 24 h in the presence of (A) formulation alone; (B) 1 mM methyl coumarate acid; and (C) 1 mM methyl 4-fluorocinnamate. The numbered peaks refer to the compounds identified in Fig. 2.

acylated KDG (**2**) was a relatively minor component of the total kaempferol pool, with the respective caffeoyl-ester (KDG-Caf; **4**) dominating. In addition, KDG also accumulated to a lesser extent as the feruloyl ester (KDG-Fer; **5**). In addition to these flavonol derivatives, the caffeic acid derivative rosmaric acid (Fig 1B; **R**) was also identified as a phenolic metabolite in petunia foliage, as described in earlier studies (Bloor et al., 1998). However, in contrast to these studies, we were unable to detect any 6″-*O*-malonylated derivatives of QDG. Based on previously published data (Bloor et al., 1998) the substitutions with the acyl groups most likely occurred at the 2-*O*-position of the terminal glucose substituent (Fig. 1A and B).

2.2. Feeding studies with phenylpropanoids

The petunia leaves were first sprayed with caffeic acid, as this was the most abundantly incorporated acyl group in the flavonol pool (Table 1). The caffeoyl compound was applied either as its free acid or as the corresponding methyl caffeate ester. The application of the methylated derivative led to over a 10-fold increase in the pool size of QDG-Caf and a 3-fold enhancement in KDG-Caf (Table 1). In addition, feeding with methyl caffeate also increased the pool sizes of all the other UV-absorbing metabolites except for QDG. In addition to the originally identified compounds, feeding with methyl caffeate led to the appearance of a series of unidentified polar UV-absorbing metabolites (Fig. 2B). Applications of the methyl ester led to an overall 61% enhancement in incorporation into QDG-Caf as compared with feeding with free caffeic acid. All future feeding studies with the phenylpropanoids therefore used the respective methyl esters.

The plants were then sprayed with the methyl esters of coumaric, ferulic and sinapic acids. Concentrating on the major effects determined on the HPLC profiles of the flavonols, treatment with the other natural acyl donor, ferulic acid, resulted in a selective 40-fold enhancement in KDG-Fer (Table 1). Feeding with methyl sinapate had no effect on the flavonol content (data not shown), while feeding with methyl coumarate (Fig 2B; Table 2) resulted in a large enhancement in the levels of KDG and its feruloyl ester. In addition a new metabolite, compound **6**, was determined (Fig. 2B). Based on MS fragmentation analysis (Fig. 3), this compound was tentatively identified as quercetin-3-*O*-(5‴-*O*-coumaroyl-glucuronyl(1 → 2)glucoside) (QGGA-Coum; **6**). This

Table 2
Determination of parent mass ions and yield of synthesized methyl esters of phenylpropanoates

Methyl ester	Expected $[M+H]^+$ (Da)	Obtained (ES^+) (Da)	Yield (%)
Coumarate	179.1	179.2	93
Ferulate	209.07	209.1	89
Caffeate	195.06	195.1	92
Sinapate	239.08	239.2	91
Fluorocinnamate	181.06	181.2	86
Difluorocinnamate	199.05	199.1	95
Trifluorocinnamate	217.04	217.1	86

Table 1
The effect of exogenously-fed methyl esters of phenylpropanoids upon the accumulation of acylated metabolites in the foliage of *Petunia hybrida*

Compound	Control LL	Control HL	Phenylpropanoid feeding treatment				
			4Ca	Fca	Caf	Fer	Dif
Concentration (nmol g^{-1} FW)							
QDG (1)	72 ± 16.8	200 ± 21.0	191 ± 20.7	257 ± 34.3	**127*** ± 12.5	148 ± 9.4	263 ± 14..8
KDG (2)	25 ± 5.7	62 ± 8.4	**124*** ± 12.1	59 ± 10.3	**118*** ± 10.3	**110*** ± 6.9	64 ± 4.8
QDG-Caff (3)	49 ± 10.9	235 ± 19.9	**46*** ± 11.9	326 ± 39.4	**2891*** ± 143.2	**16*** ± 1.2	**140*** ± 7.2
KDG-Caff (4)	96 ± 5.0	338 ± 64.0	366 ± 11.6	511 ± 29.0	**1018*** ± 24.2	469 ± 32.2	510 ± 13.9
KDG-Fer (5)	22 ± 6.8	37 ± 1.4	**239*** ± 9.7	81 ± 18.7	**121*** ± 15.6	**1509*** ± 48.7	**0*** ± 0.0
QGGA-Coum (6)	0 ± 0.0	0 ± 0.0	**220*** ± 18.8	**59*** ± 7.7	**144*** ± 10.2	0 ± 0.0	0 ± 0.0
QGGA-Fca (7)	0 ± 0.0	0 ± 0.0	0 ± 0.0	**15*** ± 0.2	0 ± 0.0	0 ± 0.0	0 ± 0.0
R	79 ± 10.8	140 ± 28.6	**368*** ± 9.6	217 ± 2.8	**328*** ± 23.1	**53*** ± 6.2	**15*** ± 4.5

Untreated plants were grown under ambient lighting (= Con LL) or were sprayed with formulated treatments containing 1 mM of the methyl esters then exposed to high level lighting for 24 h. Con HL refers to plants treated under high light with formulant solution only. Values are the mean of two biological replicates ± standard deviation (* = values significantly greater than control ($P < 0.05$)).

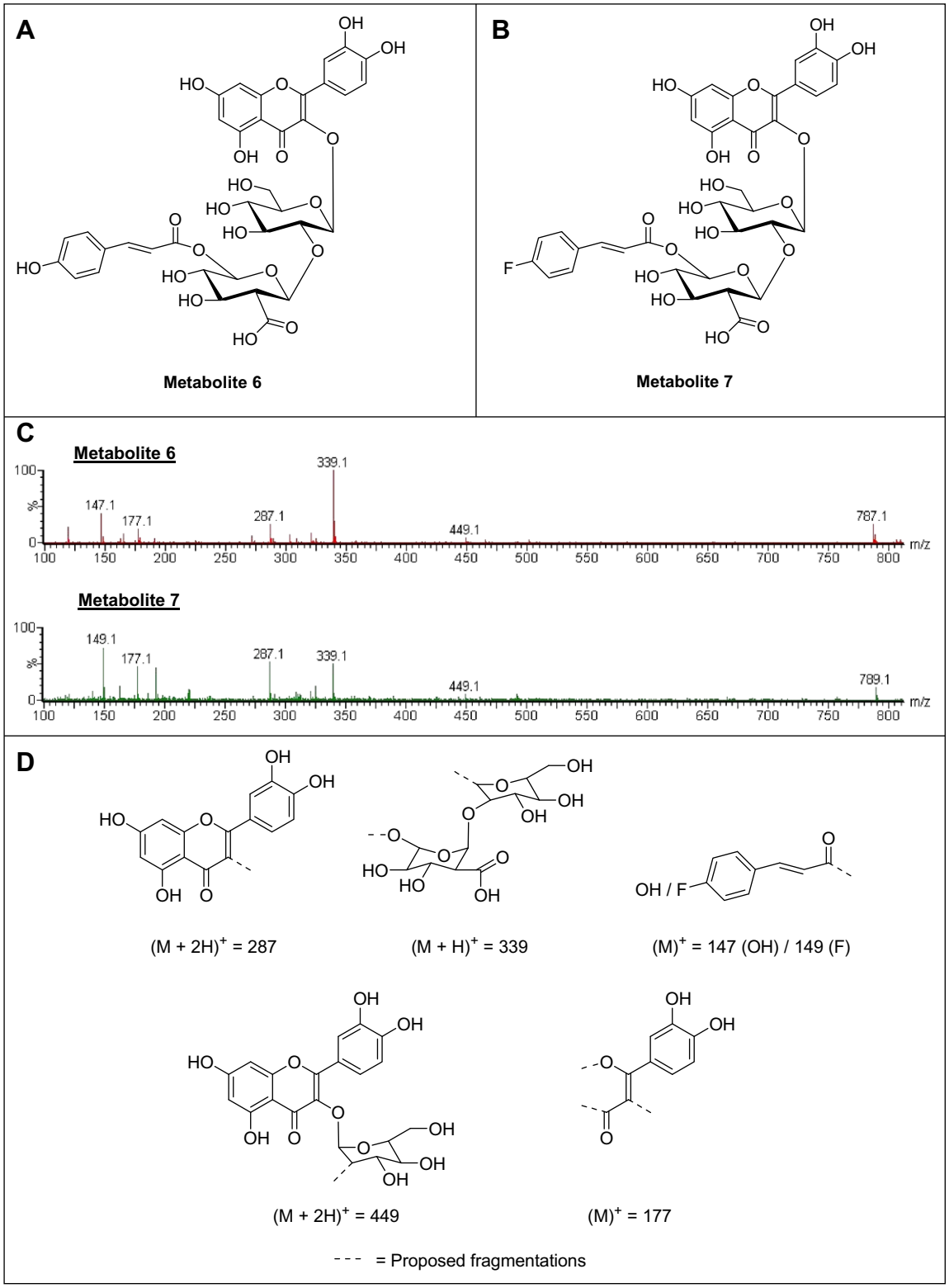


Fig. 3. The identification of two novel and analogous acylated derivatives of quercetin-3-*O*-glucuronyl-glucoside showing the incorporation of coumaric acid (A) and 4-fluorocinnamic acid (B) with characteristic fragmented mass ions determined by MS shown (C) along with the identities of the assigned mass ions (D).

identification was based on the parent mass ion and generation of fragments corresponding to quercetin (287 and 177 Da), quercetin glucosyl (449 Da) and coumaroyl (147 Da), as found in previous studies on flavonol glycoside fragmentation (Cuyckens et al., 2003). In addition, a fragment corresponding to glucuronyl-glucosyl (339 Da) was also identified. While glucosyl-glucuronic acid conjugates of flavonols, have not been previously described in petunia, similar diglycosides are known in other plants and the proposed structure of the metabolite, as derived from MS fragmentation experiments, is consistent with those described in the literature (Sawada et al., 2005). A careful examination of the plants treated in high light only, showed that this compound was in fact

a natural constituent of the flavonol pool in Petunia, albeit as a trace metabolite. A re-examination of the other feeding experiments showed that this coumaroylated metabolite also accumulated in plants fed with methyl caffeate (Table 2).

Having established the perturbation in flavonol acylation following feeding with naturally-occurring phenylpropanoids, the study was extended by spraying the plants with the methyl esters of 4-fluoro- (Fca), 3,4-difluoro- (Dif), and 3,4,5-trifluoro- (Trif) substituted cinnamoyl acid derivatives. The mono- and tri-fluorinated compounds did not significantly perturb the accumulation of the endogenous metabolites, while feeding with 3,4-difluorocinnamic acid led to a selective decline in QDG-Caf, KDG-Fer and rosmarinic acid. To look for incorporation of the fluoroacyl donors into novel flavonols, for each feeding study, the eluting metabolites were fragmented by MS and monitored for evidence of the fluorcinnamoyl species. This is effectively a sensitive means of looking for fluoroacylation due to the predictable cleavage of the phenylpropanoid ester during ionization of the parent acylated flavonol (Fig. 3). Using this approach we were unable to find any evidence for incorporation of 3,4-difluorocinnamic acid (167 Da) or 3,4,5,-trifluorocinnamic acid (185 Da). However, a compound fragmenting to release a 149 Da fragment characteristic of 4-fluorocinnamoylated compounds was identified in a minor peak eluting just after KDG (Fig. 2C). MS analysis of this compound (**7**) (Fig 3), subsequently identified it as quercetin-3-*O*-(5‴-*O*-4-fluorocinnamoylglucuronyl(1 → 2)glucoside) (QGGA-Fca; **7**), with the compound accumulating to a final concentration of 15 nmol g^{-1} FW (Table 2).

With respect to the levels of incorporation and recovery of the applied methylated-acyl donors; in the absence of isotopic labeling these could not be calculated with any confidence for the naturally-occurring phenylpropanoids. For example, whereas only approximately 200 nmol of methyl caffeate was sprayed onto the plants, the large increase in the levels of caffeoylated flavonols could account for over four times the applied dose. The one exception was with 4-fluorocinnamate, where a final level of incorporation of 7.5% into the acylated flavonol pool could be calculated (Table 1). Intriguingly, in all cases methanolic washes of the leaf surface 24 h after application failed to recover any of the methyl esters. This, together with the absence of further novel absorbing peaks in the HPLC profiles of the leaf extracts (Fig. 2), suggested that the applied phenylpropanoids had either been metabolised to non-UV-absorbing soluble compounds or incorporated into insoluble matrices.

3. Discussion

Our results demonstrate that the accumulation of acylated flavonols in the foliage of petunia can be selectively regulated by simply spraying the plants with the acyl donor species supplied as the respective methyl ester. This was most dramatically demonstrated in the major increases in the content of endogenous feruloylated and caffeoylated QDG and KDG achieved by feeding with methyl ferulate and methyl caffeate respectively. In fact feeding with methyl caffeate caused an accumulation of all of the acylated flavonols. While methyl sinapate was not incorporated, treatment with the coumarate ester led to the accumulation of the novel acylated flavonol derivative QGGA-Coum. The acylating pathway in petunia also had a limited capacity to accept non-natural phenylpropanoids. Thus, when the plants were fed with methyl 4-fluorocinnamate, the respective phenylpropanoid entered the flavonol acylation pathway to give rise to relatively small amounts of the semi-synthetic ester QGGA-Fca.

These simple observations may be of great utility in selectively enhancing the content of valuable acylated natural products in medicinal plants. Our results suggest that (a) levels of endogenous acylated metabolites can be usefully enhanced by spraying whole plants with methylated donor acids and (b) that these feeding studies can usefully incorporate unnatural fluorinated acids to derive novel semi-synthetic esters. Previous studies in suspension-cultured cells of wild carrot (*Daucus carota* spp. *carota*) have shown that it is possible to manipulate the acylation of anthocyanin glycosides by feeding with naturally-occurring benzoic and phenylpropanoid acids to form novel ester species (Baker et al., 1994; Dougall et al., 1998). Additionally, in the later studies incorporation of halogenated cinnamic acids into the anthocyanin pool was also observed (Dougall et al., 1998). From an applied perspective, studies in Taxus cultures have shown that feeding with benzoic acid acyl donors can enhance the content of the bioactive acylated taxanes (Fett-Neto et al., 1994). Potential application also exists in other medicinal plant species which are known to accumulate acylated bioactive compounds, such as *Rauvolfia* (Bayer et al., 2004) or *Catharanthus* (St-Pierre et al., 1998). Our results show that it is also possible to achieve useful enhancement in yields (mM) of acylated flavonoids in whole plants without the need to generate and maintain suspension cultures. In addition to boosting the content of useful natural acylated secondary metabolites, the simplicity of our treatment suggests that it would be relatively straight-forward to screen large numbers of plant species for their ability to incorporate fluoroacyl donors into their natural products pools, thereby extending chemical diversity and potential biological activity. The potential for the incorporation of fluoroacyl groups is particularly attractive, based on the associated novel biological properties associated with such semi-synthetic plant secondary products (Ojima, 2004).

The enhancement of the acylated flavonol pool following feeding with phenylpropanoid donors is also of interest in understanding how these pathways are regulated. The scale of the enhancement in the acylated flavonol pool on feeding caffeoyl phenylpropanoids is strongly suggestive of metabolic regulation by positive feed-back from the end products of the pathway. This conclusion can be made as the scale of the accumulation of the acylated flavonol glycosides greatly exceeded the depletion in the levels of the KDG and QDG intermediates, implying that the pool was being replenished by *de novo* flavonol synthesis. Drawing analogies with what is known of the detoxification of other exogenously supplied compounds in plants, a short-term response to the influx of xenobiotic acids is to biotransform them to the respective esters, classically using sugar acceptors (Cole and Edwards, 2000). Our results with caffeic acid and ferulic acid in Petunia suggest that these compounds are recognized by the endogenous CoA ligases and acyltransferases and removed from the cell by incorporation into the flavonol glycosides. In this respect the QDG and KDG are serving the functions as glycoside acceptors which can then be recycled and re-used as required following deacylation. This model for detoxifying exogenously-supplied acids may suggest that xenobiotics could become incorporated into a diverse range of plant esters. The range of plants which can incorporate xenobiotic acids into their acylated natural products, the regulation of the respective pathways and the in vitro biosynthesis of xenobiotic natural compounds is currently under further investigation.

4. Experimental

4.1. Synthesis of methyl phenylpropanoate esters

All chemicals were analytical grade and obtained from Sigma–Aldrich (Poole, Dorset, UK). The cinnamic acid derivatives (0.91 mmol) was dissolved in dry dichloromethane (5 ml) and dimethylformamide (4 μl) added. Oxalyl chloride (1.82 mmol) was subsequently added drop-wise, followed by methanol

(2.73 mmol) and the solution stirred for 30 min. The reaction mixture was washed with water (3 × 10 ml) and dried over magnesium sulphate. Concentration *in vacuo* afforded the corresponding cinnamate derivative as a pale yellow–brown oil. The yield and identity of the corresponding methyl esters was determined by HPLC-MS (Table 2).

4.2. Plant experiments

P. hybrida ('Grandiflora Selection Mixed' F1) seeds were purchased from Mr Fothergill's Seeds Ltd. (Kentford, Suffolk, UK) and sown onto multipurpose potting compost. Plants were maintained in a growth room for 28 days under a regime of 16 h light (80 μEinstein m^{-2} s^{-2}, 24 °C), 8 h dark (22 °C). Plants were sprayed ± formulation consisting of an aqueous solution of Triton-X 100 detergent (0.1% v/v) containing ± the phenylpropanoid derivatives at a final concentration of 1 mM. The solution was applied as a fine spray at a rate of 25 ml per seed tray (800 cm^2), with each tray containing 24 plants. After spraying, the plants were immediately transferred into a Sanyo versatile environmental test chamber set to 24 h lighting (160 μEinstein m^{-2} s^{-2}, at 17 °C). After a 24 h treatment, their leaf tissue was weighed and frozen in liquid nitrogen prior to storage at −80 °C.

4.3. Extraction and analysis of metabolites

Plant samples (1 g) were extracted in ice-cold methanol (3 ml) using a pestle and mortar and acid-washed sand as an abrasive. After centrifugation (16,000*g*, 5 min), the extract was concentrated to 1 ml under reduced pressure. Chloroform (1 ml) and distilled water (200 μl) were added and the resulting emulsion re-centrifuged for 1 min. The upper methanolic layer was removed and 10 μl injected onto an UPLC™ BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters Acuity). The column was then eluted at 0.2 ml min^{-1} with a mixture of 0.5% formic acid (solvent A) and acetonitrile containing 0.5% formic acid (solvent B) using a linear gradient of 5–95% solvent B over 9 min, followed by isocratic elution with 95% B for a further 2 min. The eluant was monitored by diode array detection for UV–Vis-absorbing metabolites, with the absorbance at 330 nm used for quantification after calibrating the system with a flavonoid standard (quercetin 3-*O*-diglucoside). The eluant was then analysed by MS after electrospray ionization using a Micromass QTof spectrometer operating in positive ion mode. Settings were sample cone voltage = 41 kV, capillary voltage = 2.55 kV, extraction cone voltage = 5 kV, source temperature = 100 °C and desolvation temperature = 180 °C. Metabolites were identified from parent and fragment mass ions with reference to published spectra (Cuyckens et al., 2003).

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