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Characterization and Content of Flavonoid Glycosides in Genetically Modified Tomato (*Lycopersicon esculentum*) Fruits

GWÉNAËLLE LE GALL,† M. SUSAN DUPONT,† FRED A. MELLON,† ADRIENNE L. DAVIS,‡ GEOFF J. COLLINS,‡ MARTINE E. VERHOEYEN,‡ AND IAN J. COLQUHOUN*,†

Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom, and Unilever Research, Colworth House, Sharnbrook, Bedfordshire MK44 1LQ, United Kingdom

There is a growing interest in producing food plants with increased amounts of flavonoids because of their potential health benefits. Tomatoes contain small amounts of flavonoids, most of which are located in the peel of the fruit. It has been shown that flavonoid accumulation in tomato flesh, and hence an overall increase in flavonoid levels in tomato fruit, can be achieved by means of simultaneous overexpression of the maize transcription factors *LC* and *C1*. Fruit from progeny of two modified lines (2027 and 2059) was selected for a detailed analysis and individual identification of flavonoids, at different stages of maturity. Nine major flavonoids were detected in the flesh of transgenic ripe tomatoes. LC/NMR, LC/MS, and LC/MS/MS enabled us to identify these as kaempferol-3,7-di-*O*-glucoside (**1**), kaempferol-3-*O*-rutinoside-7-*O*-glucoside (**2**), two dihydrokaempferol-*O*-hexosides (**3** and **4**), rutin (**5**), kaempferol-3-*O*-rutinoside (**6**), kaempferol-3-*O*-glucoside (**7**), naringenin-7-*O*-glucoside (**8**) and naringenin chalcone (**9**), which were quantified by HPLC/DAD. All but **5**, **6**, and **9** were detected in tomato for the first time. The total flavonoid glycoside content of ripe transgenic tomatoes of line 2059 was about 10-fold higher than that of the controls, and kaempferol glycosides accounted for 60% of this. Kaempferol glycosides comprised around 5% of the flavonoid glycoside content of ripe control tomatoes (the rest was rutin and naringenin chalcone). The rutin concentration in both transgenic and control fruits was similar.

KEYWORDS: Metabolomics; tomato; ripening; transgenic; GMO; flavonoids; kaempferol glycosides; naringenin-7-*O*-glucoside; dihydrokaempferol glycosides; transcription factors; *LC*; *C1*; *Lycopersicon esculentum*

INTRODUCTION

Flavonoids are a class of secondary metabolites that are produced ubiquitously in fruits and vegetables. These polyphenol compounds have been studied extensively because of their potential health benefits. Their antioxidant activity is thought to slow the aging of cells and to protect against lipid peroxidation, a reaction that could be an important step in the development of illnesses such as cardiovascular or coronary heart disease and chronic inflammation (*1*−*5*). Their chemical structure (a $C_6-C_3-C_6$ configuration consisting of two aromatic rings joined by a three-carbon link) renders the flavonoids good hydrogen and electron donors. These characteristics make them effective scavengers of free radicals such as the products of lipid peroxidation, which are generated by oxidative chain reactions and lead to tissue damage over time (*6*). In vitro studies have also shown that flavonoids can inhibit, and sometimes induce, enzyme systems. They are thought to reduce the proliferative activity of certain types of tumor cells (*7, 8*) and to be involved in the apoptosis of HL-60 leukemia cells (*9*). Two recent in vivo studies have added further support to the potential health benefits of flavonoids. Duarte et al. (*10*) reported that supplementation with quercetin had a blood pressure lowering effect on spontaneously hypertensive rats. Chopra et al. (*11*) showed that alcohol-free red wine extract and one of its components, quercetin, can inhibit LDL oxidation in humans after in vivo supplementation.

The most common classes of polyphenol compounds occurring in foods of plant origin are the flavones (celery), isoflavones (soya), flavanones (citrus fruits), flavanols (tea, apple, wine), flavonols (onion, tea, wine), and anthocyanins (wine, blueberry).

Tomato is a major food crop worldwide, and its fruit contains several flavonoids of which naringenin chalcone and rutin (quercetin-3-*O*-rutinoside) are predominant. However, these compounds are found at low levels and are restricted to the peel. Only traces of rutin are found in the flesh, which constitutes 95% of the fruit. Genetic modification can be used to up-regulate

* Corresponding author. Tel.: +44 1603 255353. Fax: +44 1603 507723. E-mail: ian.colquhoun@bbsrc.ac.uk.
† Institute of Food Research.
‡ Unilever Research.

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flavonoid biosynthesis in this fruit (*12*, *13*). The transgenic tomatoes used in this study were generated (*12*) by simultaneous overexpression of two maize regulatory genes, *leaf color* (*LC*) and *colorless-1* (*C1*). The flesh of the *LC/C1* ripe tomatoes was found to contain a number of kaempferol and naringenin glycosides, which were not present in wild-type red or transgenic green fruits. Whole red fruits from numerous *LC/C1* lines were analyzed for flavonoid content after hydrolysis of the glycosides. They showed higher levels of kaempferol and, to a lesser extent, naringenin compared with their controls (*12*).

The flavonoids in *LC*/*C1* tomatoes are present as conjugates, as in many plants. Recent studies suggest that the degree of glycosylation may have an impact on the ability of these compounds to be absorbed (*14*, *15*); hence, the structures of the flavonoid glycosides introduced into the *LC*/*C1* tomatoes are of considerable interest. This work reports on the further characterization, individual identification, and quantification of flavonoid glycosides found in two of the lines of transgenic and control tomatoes produced. The effect of the fruit maturity is also reported. This work is complemented by metabolite profiling, described in the accompanying paper (*16*), which was aimed at evaluating possible effects of the modification on compounds other than flavonoids.

MATERIALS AND METHODS

Materials. Details of the procedure used to produce transgenic tomatoes and their controls have been given by Bovy et al. (*12*). Briefly, the binary vector pBBC3 was used to create plasmid pBBC200. This plasmid was transferred to *Agrobacterium tumefaciens* strain LBA4404, which was then used to transform tomato variety FM6203 (Unilever commercial variety). Plants transformed with pBBC200 were numbered series 2000. The particular fruits studied in this work are from lines 2059 and 2027, both generated using the same gene construct [transgenic (+) and control (−)]. The fruit samples analyzed here are progeny of transgenic parents of lines 2059 (+) and 2027 (+), the control plants are azygous, where the azygous line, e.g. 2059(−), has lost the transgene through segregation. Azygous plants are considered to be the ideal matched controls (*12*).

Samples of line 2059 were obtained from five pairs of plants (transgenic and control) grown hydroponically in a glasshouse under identical conditions. Eight fruits were taken from each plant (six red plus one from each plant at both the mature green and turning stages. Harvest date was determined by visual assessment using a reference color chart specific for the variety. Individual fruits were date tagged at the breaker stage, with harvest of turning fruit typically 2−4 days postbreaker and red fruit 15−18 days postbreaker). Thus, eighty samples were prepared in total (*16*). Only six pooled samples were used for this work: two red, two turning, and two green, with transgenic and control in each case. Each pooled sample consisted of 2.50 g of freeze-dried powder (0.5 g per fruit) coming from five individual fruits, each from a different plant (each red fruit was randomly chosen out of the six samples that comprised a batch). Plants of line 2027 were grown under the same hydroponic conditions but only fully red ripe fruit were harvested.

All solvents were of HPLC grade and the water was purified via a Millex Q-plus system (Millipore Ltd., Watford, UK). MN polyamide SC6 was purchased from Macherey-Nagel GmbH & Co.

Quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-β-D-glucoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-β-D-glucoside, naringenin-7-*O*-β-D-glucoside, and eriodictyol-7-*O*-β-D-glucoside were purchased from Extrasynthese, Genay, France. Quercetin, kaempferol, and naringenin were from Sigma-Aldrich (Gillingham, UK), and naringenin chalcone was from Apin Chemicals (Abingdon, UK). The sample of dihydrokaempferol was kindly donated by Prof. J. B. Harborne, School of Plant Sciences, University of Reading.

Methods. *Extraction*. This extraction method was adapted from the one described by Price et al. (*17*). For line 2059, the whole fruit (flesh, peel, seeds) was analyzed. Each tomato fruit was freeze-dried for subsequent quantitative extraction and analysis. Each sample was ground to a fine powder in a domestic food processor and extracted as follows: duplicate samples of the dried powder (1 g) were homogenized in 50 mL of 100% methanol at 1200 rpm for 2 min (Ultra Turrax, Janke & Kundel). The extract was filtered under reduced pressure through filter paper (Whatman No. 541). The residue was re-extracted twice with 50 mL of 70% aqueous methanol. The combined fractions were evaporated in vacuo at 40 °C to ~10 mL and made up to 20 mL with water. An aliquot (10 mL) was added to a polyamide (1 g) column that had been preconditioned with methanol (20 mL) followed by water (60 mL). The column was washed with water (20 mL) and further eluted with methanol (50 mL) to elute the flavonoids. Methanol eluates were then evaporated to dryness in vacuo at 40 °C, redissolved in methanol (1 mL), and filtered prior to HPLC analysis (0.2 μm). For line 2027, only the flesh was analyzed: the fruits were quartered, the flesh (including columella) was separated from the peel and seeds, the flesh was immediately frozen in liquid nitrogen, ground to a fine powder, and freeze-dried. The extraction and concentration procedure was similar to that described above, except that 3 g of freeze-dried powder/80 mL of 100% methanol was used and the initial extract was clarified by centrifugation (12 000 rpm for 20 min) rather than filtration.

High-Pressure Liquid Chromatography. *Method I. Samples from Line 2059*. The method was adapted from that of DuPont et al. (*18*). A Hewlett-Packard 1100 system (Agilent, Stockport, UK) comprising an autosampler and a quaternary pump coupled to a diode array detector and controlled by Chemstation software was used. The solvent system was A (water/tetrahydrofuran/trifluoroacetic acid 98:2:0.1 v/v) and B (acetonitrile) used in the proportion of 10% B for 5 min increasing to 15% B after 15 min to 17% B at 20 min, 20% B at 25 min, 25% B at 30 min, 35% B at 35 min, 50% B at 40 min to 90% B at 45 min. A column cleanup stage was used holding at 90% B for a further 5 min followed by reequilibration for 15 min at 10% B. The column was a Prodigy 5 μm ODS3 reversed phase silica (250 mm × 4.6 mm i.d., with 30 mm × 4.6 mm i.d. guard, Phenomenex Ltd.), the temperature was 30 ° C, and the effluent (1 mL/min) was monitored by a diode array detector (signals measured at 270, 292, and 370 nm). An external standard (quercetin-3-*O*-glucoside) was injected after every three samples. Duplicate samples were injected at levels of 5 and 10 μL.

Individual flavonoid glycosides were quantified from the response at 370 nm for compounds **1**, **2**, **5**, **6**, **7**, and **9** and at 292 nm for **3**, **4**, and **8**, using calibration plots obtained for standard solutions of kaempferol-3-*O*-rutinoside (for **1**, **2**, **6** and **7**), rutin (for **5** itself), naringenin chalcone (for **9** itself), and naringenin (for **3**, **4** and **8**). The calibration for naringenin chalcone was done after correcting for a small amount of naringenin present in the standard. Calibration plots of peak area versus amount of standard injected were linear ($r^2 > 0.999$) across a range from 0.5 to 5 μg. Results were expressed in terms of the amount of aglycon present in each compound.

Method II. Line 2027+. A similar Hewlett-Packard 1100 system was used to separate flavonoid extracts. A Prodigy C_{18} (Phenomenex), 150 × 4.6 mm i.d., particle size 5 μm column was used at 30 °C at a flow rate of (1 mL/min). A solvent gradient of A (water/trifluoroacetic acid 99.9:0.1 v/v) and B (50% A and 50% acetonitrile (v/v)) was used in the proportion of 20% B, increasing to 35% B after 8 min and 50% B after 20 min for 22 min. A column cleanup stage was used at 100% B for a further 6 min and finally reequilibration for 5 min at 20% B.

Method III. Line 2027+, Preparation of Compounds for NMR. Compounds **1** and **2** were separated using the same solvent as in method II but with a solvent gradient of A (water/trifluoroacetic acid 99.9:0.1 v/v) and B (50% A and 50% acetonitrile (v/v)) used in the proportion of 18% B increasing to 100% B after 26 min until 31 min followed by a re-equilibration stage of 18% B for 6 min. Twelve 20−35 μL injections of the flavonol glycoside supernatant were made and fractions corresponding to kaempferol glycoside peaks at 21.5 min (**1**) and 22.8 min (**2**) were collected and combined. Peak fractions collected omitted the leading edge and tailing end of each peak to limit both cross contamination of the two kaempferol glycoside peaks and the inclusion of other minor contaminants eluting close by. The combined fractions were rotary evaporated at 50 °C and then freeze-dried. Approximately 1.9 mg (dry weight) of **1** and 4.1 mg of **2** were provided for subsequent analysis.

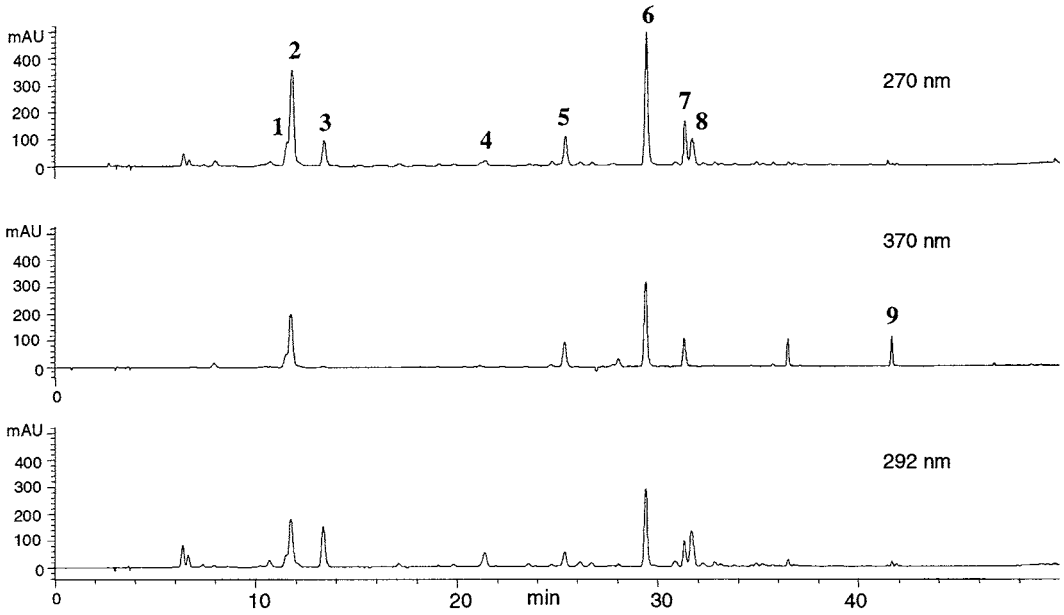


Figure 1. HPLC analysis of a transgenic red tomato extract of line 2059, whole fruit (5 μL injection): **1**, kaempferol-3,7-di-*O*-glucoside (11.52 min); **2**, kaempferol-3-*O*-rutinoside-7-*O*-glucoside (11.75 min); **3**, dihydrokaempferol-hexoside 1 (13.37 min); **4**, dihydrokaempferol-hexoside 2 (21.36 min); **5**, rutin (25.37 min); **6**, kaempferol-3-*O*-rutinoside (29.40 min); **7**, kaempferol-3-*O*-glucoside (31.32 min); **8**, naringenin-7-*O*-glucoside (31.68 min) and **9**, naringenin chalcone (41.63 min).

NMR spectra were measured in 99.95% MeOH-d_4 solvent on a Bruker AMX400 spectrometer equipped with either a dual ^{1}H/^{13}C probe or an inverse probe, operating at a temperature of 300 K. Standard parameter sets were used for 2D NMR. Spectra were referenced on internal TMS.

Liquid Chromatography/Nuclear Magnetic Resonance. *Line 2027+*. The material for LC/NMR analysis was prepared from *LC/C1* tomatoes using the methodology described in Extraction above. A Waters Novapak C_{18} 150 × 4.6 mm i.d., particle size 4 μm, column was used at a flow rate of 1 mL/min at ambient temperature with a linear solvent gradient of A (water/trifluoroacetic acid in D_2O 99.9:0.1 v/v) and B (50% A and 50% acetonitrile (v/v)) of 10 to 60% B in 40 min. Under these conditions, the kaempferol-3-*O*-rutinoside (**6**) eluted at ca. 25 min, while the kaempferol-3-*O*-rutinoside-7-*O*-glucoside (**2**) was the second (and larger) of the two peaks that eluted at ca. 15 min. The extract was dissolved in 250 μL of 50:50 deuterated water/deuterated acetonitrile, and injections were typically 60−100 μL. LC/NMR spectra were acquired on a Bruker DMX 500 spectrometer using a 4 mm inverse detection LC/NMR probe. Spectra were referenced on the solvent peak: acetonitrile = 2.065 ppm.

Liquid Chromatography/Mass Spectrometry. *Samples from line 2059*. Electrospray spectra were obtained using the following LC/MS system: JASCO-1585 (Jasco (UK) Ltd., Great Dunmow) ternary system, equipped with JASCO-1559 cooled autoinjector, 1575 programmable UV detector, and 1560 column heater/cooler connected to a Micromass Quattro II (Micromass UK Ltd, Manchester) mass spectrometer operated in the positive ionization mode. Typical tuning parameters were as follows: ion source, Z-spray; ionization mode, electrospray; capillary voltage, 3.5 kV; cone voltage, 28 V; source block temperature, 140 °C; desolvation temperature, 350 °C. The mass range scanned was 50−1500 Da, at a rate of 3 s/scan. The column and the conditions used to analyze the six tomato samples (red, turning, and green stages, both transgenic and control) were the same as used for HPLC/DAD analysis (method I).

Liquid Chromatography/Mass Spectrometry/Mass Spectrometry. The MS/MS conditions were optimized to maximize information that would assist in confirming the identity of the aglycon moiety of the flavonoid glycosides. Electrospray LC/MS/MS spectra were obtained using the following conditions (previously optimized using flavonoid standards): positive ion electrospray; cone voltage, (glycosides) 46 V or (aglycons) 28 V; collision energy, 17 eV; CID gas pressure, 1.1 × 10^{-3} bar. MS/MS product ion spectra of *m*/*z* 289 were scanned in continuum mode from *m*/*z* 75 to 300 at a rate of 1 s/scan with an interscan time of 0.2 s. Spectra were processed using MassLynx version 3.4 software (Micromass UK, Ltd., Manchester, UK).

RESULTS

Identification of Flavonoids. Separation of compounds **1**−**9** in transgenic red tomato, line 2059+, by HPLC method I is shown in **Figure 1**. The compounds were identified by a combination of comparison of the retention times of standards, matching of UV−visible spectral characteristics, LC/NMR, NMR (isolated compounds), LC/MS, and LC/MS/MS analysis.

Identification of 5−**9**. Rutin (**5**), kaempferol-3-*O*-rutinoside (**6**), kaempferol-3-*O*-glucoside (**7**), naringenin-7-*O*-glucoside (**8**), and naringenin chalcone (**9**) were unequivocally identified as the peaks eluting at 25.37, 29.40, 31.32, 31.68, and 41.63 min, respectively. Standards were used to identify peaks by retention times, and UV characteristics were compared. Major ions were 649 (8.8, MK^+), 633 (28.0, MNa^+), 611 (12.6, MH^+), 465 (33.5, $[MH - rha]^+$), and 303 (100, $[MH - rha - glc]^+$, aglycon) for **5**; 633 (4.7, MK^+), 617 (16.1, MNa^+), 595 (9.6, MH^+), 449 (34.4%, $[MH - rha]^+$), and 287 (100, $[MH - rha - glc]^+$, aglycon) for **6**; 487 (7.0, MK^+), 471 (24.3, MNa^+), 449 (3.1, MH^+), and 287 (100, $[MH - glc]^+$, aglycon) for **7**; and 457 (10.8, MNa^+), 435 (4.7, MH^+), and 273 (100, $[MH - glc]^+$, aglycon) for **8**.

Identification of 1 and 2. The two kaempferol glycosides at 11.52 and 11.75 min were identified as kaempferol-3,7-di-*O*-β-D-glucoside (**1**) and kaempferol-3-*O*-rutinoside-7-*O*-β-D-glucoside (**2**), respectively. The UV spectra reveal that both have a kaempferol structure. The MS analysis (**Figure 2**) shows that the major ions for **2** are 795 (16.2, MK^+), 779 (40.7, MNa^+), 757 (75.3, MH^+), 611 (72.6, $[MH - rha]^+$), 595 (23.6, $[MH - glc]^+$), 449 (100, $[MH - rha - glc]^+$), and 287 (12.4, $[MH - rha - glc - glc]^+$, aglycon). It is likely that the lower trace in **Figure 2** contains contributions from both **1** and **2**. However, the lower trace shows an ion of *m*/*z* 633 that could be attributed to the sodium adduct of **1** (25.9%, MNa^+).

The full structures of these two compounds were deduced by LC/NMR and detailed ^{1}H and ^{13}C NMR analyses performed

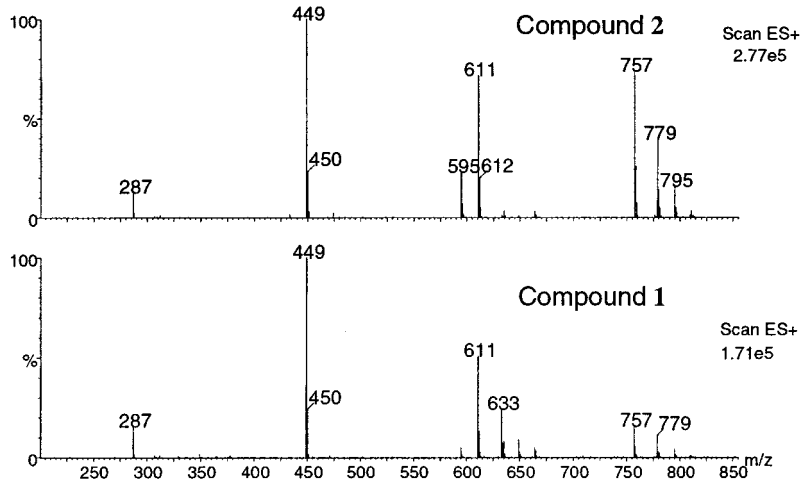


Figure 2. Positive-ion ESI mass spectra of **1** (kaempferol-3,7-di-*O*-glucoside) and **2** (kaempferol-3-*O*-rutinoside-7-*O*-glucoside) (sample from line 2059+).

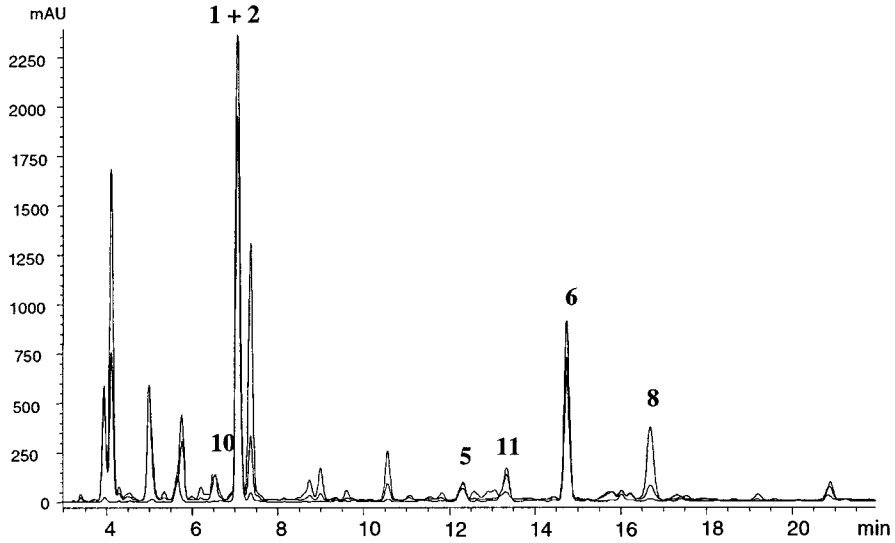


Figure 3. Chromatogram of *LC/C1* (2027+) tomato flesh showing kaempferol glycosides peaks (**1**, **2**, and **6**). Signal wavelengths of 280, 370, and 325 nm are overlaid. Peaks **5**, **8**, **10** (chlorogenic acid), and **11** (isoquercitrin) were identified by comparison with known standards. See **Figure 1** for other assignments.

on samples from line 2027. The HPLC/DAD chromatogram of a red transgenic tomato flesh sample from line 2027+ shown in **Figure 3** (HPLC method II) is comparable to that from line 2059+ (whole fruit) in **Figure 1**. The analysis procedure used to obtain the chromatogram in **Figure 3** was much quicker, and although separation is incomplete, the overall pattern is broadly similar to that given by 2059+. Compounds **1** and **2** are not separated in **Figure 3** nor is compound **7** assigned. For definitive identification, compounds **1** and **2** were separated using a modified chromatographic procedure (method III) and isolated for NMR analysis. Prior to the NMR analysis of the isolated compounds, LC/NMR was performed on an extract of line 2027+. This analysis confirmed that **6** was kaempferol-3-*O*-rutinoside and revealed that **2** and **6** shared many common features: the aglycon was identifiable as kaempferol and characteristic signals of the rutinoside group [R6 (rhamnose H-6) and G1 (rutinoside glucose H-1)] appeared in the spectra of both fractions. However, there were some additional signals in the spectrum of **2**, the most obvious being an additional sugar anomeric peak at ca. 5.25 ppm (J = 7.02 Hz). These data indicated that the unknown, **2**, was a kaempferol trisaccharide comprising 3-*O*-rutinoside plus an additional sugar unit.

Using NMR on the isolated compound **2** and comparing the ^{1}H and ^{13}C NMR data of **2** to those of rutin (**5**) (**Table 1**; data not shown for rutin) confirmed that **2** was a rutinoside. The remaining sugar resonances were identified by ^{13}C chemical shifts and ^{1}H−^{1}H coupling patterns as arising from a β-D-glucoside (see **Table 1**). The downfield shift of both the H-6 and H-8 protons relative to rutin and quercetin-3-*O*-glucoside (data not shown) strongly suggested that the additional glucose was *O*-linked to the 5- or 7-position. A ROESY experiment confirmed this conclusion and proved that the point of attachment was the 7-position: strong through-space correlations were observed from the glucose anomeric proton to both H-6 and H-8. No ROESY signals were observed from the rutinoside glucose anomeric proton to the kaempferol moiety, which suggested that the rutinoside was *O*-linked to the 3-position, and this was confirmed by the similarity of the ^{13}C chemical shifts of C-3 and C-4 in **2** and rutin. Therefore, **2** was identified as kaempferol-3-*O*-rutinoside-7-*O*-β-D-glucoside. The molecular weight of **2** deduced from the mass spectrum was 756, which is consistent with the proposed structure.

NMR data for **1**, isolated in the same experiment, are given in **Table 1**. The aromatic part of the ^{1}H NMR spectrum of **1** demonstrated the presence of the kaempferol moiety. In the aliphatic part of the spectrum, the observation of two anomeric protons suggested that **1** was a diglycoside: this was confirmed by integration of all the sugar signals. 2D TOCSY and HMQC

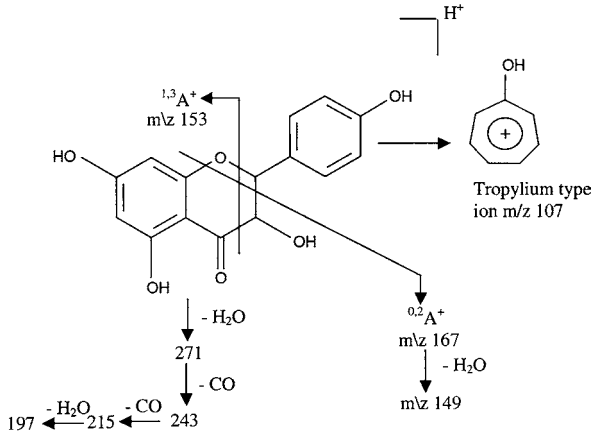
Table 1. ^{1}H and ^{13}C NMR Data for Kaempferol-3,7-di-*O*-β-D-glucoside (**1**) and Kaempferol-3-*O*-rutinoside-7-*O*-β-D-glucoside (**2**)[d]

	kaempferol-3,7-di-*O*-β-D-glucoside			kaempferol-3-*O*-rutinoside-7-*O*-β-D-glucoside		
	^{1}H		^{13}C	^{1}H		^{13}C
atom	δ (ppm)	*J* (Hz)	δ (ppm)	δ (ppm)	*J* (Hz)	δ (ppm)
2			nm			164.7
3			nm			135.6
4			nm			179.6
4a			nm			107.5
5			nm			162.5[c]
6	6.50[a] d	2.2	100.9[a]	6.50[a] d	2.1	101.0[a]
7			nm			161.5[c]
8	6.78[a] d	2.2	95.9[a]	6.78[a] d	2.1	96.0[a]
8a			nm			160.2[c]
1′			nm			122.7
2′	8.09 d	8.9	132.5	8.09 d	8.8	132.5
3′	6.89 d	8.9	116.1	6.89 d	8.8	116.2
4′			nm			158.1[c]
5′	–	–	116.1	–	–	116.2
6′	–	–	132.5	–	–	132.5
			3-*O*-Rutinoside			
G1	5.31 d	7.4	103.7	5.17 d	7.2	103.9
G2	3.43 m	nm	75.8	3.41 m	nm	75.7
G3	3.42 m	nm	78.0	3.40 m	nm	78.0
G4	3.30 t	ca. 8	71.6	3.22 t	9.0	71.6
G5	3.20 m	nm	78.5	3.32 m	nm	77.2
G6	3.70 m	nm	62.7	3.80 d	9.6	68.7
G6	3.52 m	nm		3.36 m	nm	
R1	–	–	–	4.49 d	1.6	102.4
R2	–	–	–	3.56 dd	1.8, 3.5	72.0
R3	–	–	–	3.48 m	nm	72.2
R4	–	–	–	3.25 t	9.4	73.8
R5	–	–	–	3.42 m	nm	69.7
R6	–	–	–	1.10 d	6.2	17.9
			7-*O*-Glucoside			
G′1	5.06[b] m	7.1	101.6	5.07[b] m	7.1	101.4
G′2	3.48 m	nm	74.8	3.49 m	nm	74.7
G′3	3.49 m	nm	77.9	3.49 m	nm	77.7
G′4	3.20 t	ca. 8	71.4	3.38 m	nm	71.2
G′5	3.54 m	nm	78.3	3.54 m	nm	78.3
G′6	3.92 m	nm	62.5	3.93 dd	12.3, 2.5	62.4
G′6′	3.71 m	nm		3.70 dd	12.2, 6.0	

[a] Assigned by analogy to rutin (data not shown) via the ^{13}C chemical shift. [b] The 7-*O*-glc anomeric has extra lines (in addition to the expected doublet) due to the second order effect of δH-2 being almost equal to δH-3. [c] Assignment interchangeable. [d] Assignments of the sugar signals were confirmed by TOCSY and HMQC experiments; nm, not measured; d, doublet. Spectra were referenced to internal TMS in methanol-d_4.

experiments allowed assignment of the ^{13}C shifts of the sugars, unequivocally identifying them both as terminal glucose. The large coupling (>7 Hz) on the anomeric protons identified the β-D-glucoside in each case. Once again, the downfield shift of both the H-6 and H-8 protons relative to rutin and quercetin-3-*O*-glucoside strongly suggested that one of the glucose moieties was *O*-linked to the 5- or 7-position. In a ROESY experiment, through-space correlations observed from the glucose anomeric signal at 5.06 ppm to both the H-6 and H-8 resonances identified the point of attachment as C-7. No ROESY signals were observed from the other glucose anomeric resonance to aglycon protons; furthermore, the measurable B-ring ^{13}C shifts were almost identical to those of **2**. Therefore, the remaining glucose sugar was *O*-linked to the 3-position of the aglycon and **1** was identified as kaempferol-3,7-di-O-β-D-glucoside. The molecular weight of **1** (deduced from the mass spectrum) was 610, which is consistent with the proposed structure.

Scheme 1. CID (Collision-Induced Dissociation) of $[M]^+$ Ions of Dihydrokaempferol



Identification of 3 and 4. The two other main peaks in **Figure 1** at 13.37 and 21.36 min were initially assumed to be naringenin-like compounds, as the UV spectra were similar to that of the naringenin standard. However, LC/MS analysis revealed that those two compounds have similar features in their mass spectra: two major ions at *m*/*z* 473 and 289 (**Figure 4**A). The only common flavonoids to have an aglycon mass of 288 are eriodictyol and dihydrokaempferol. The spectra suggested that compounds **3** and **4** were hexosides (the mass difference between 473 and 289 is 184, that is a hexose unit of *m*/*z* 162 after subtracting 22 for the sodium adduct). A small ion at *m*/*z* 451 was also present (MH^+). A standard of eriodictyol-7-*O*-glucoside was analyzed by LC/MS, but the retention time did not match that of compounds **3** or **4**. Standards of eriodictyol and dihydrokaempferol were analyzed by LC/MS/MS, and the comparison of the mass spectra strongly suggested that both compounds were dihydrokaempferol hexosides (**Figure 4B**). The main fragmentations of $MH^{+\cdot}$ ions of dihydrokaempferol that account for the observed pattern are illustrated in **Scheme 1**.

Effects of Genetic Modification and Maturation on Flavonoid Content and Quantification. There are eight main flavonoid peaks in the red and turning transgenic tomatoes from line 2059 observable at 270 nm (**Figure 5**). Naringenin chalcone elutes at 41.63 min, but is not displayed here because of the monitoring wavelength chosen. Red and turning controls have as main flavonoids only rutin and naringenin chalcone and a small amount of kaempferol-3-*O*-rutinoside (**6**). Green transgenic and control chromatograms are very similar to each other (**Figure 5**). Both types of green tomato contain rutin (**5**), as well as small amounts of kaempferol-3-*O*-rutinoside (**6**) and a quercetin glycoside (at 21.07 min). In these two samples some minor peaks could be assigned to cinnamic compounds (e.g. chlorogenic acid) on the basis of their UV spectra; others remain unknown. Rutin and naringenin chalcone levels are similar for both transgenic and control (green to red), but the other seven compounds are present at much higher levels in transgenic red and turning tomatoes. Inspection of the UV characteristics of minor peaks indicated that, apart from a small amount of **6**, the other six compounds were not detected in red and turning control tomatoes.

The amounts of the individual flavonoid conjugates, expressed as the concentration of the aglycon fragment of each compound, are shown in **Table 2**. These values were calculated from calibration plots of the appropriate standard (see Materials and Methods). There was not sufficient dihydrokaempferol available to construct a calibration plot, so the dihydrokaempferol hexosides (**3** and **4**) were quantified using the response factor

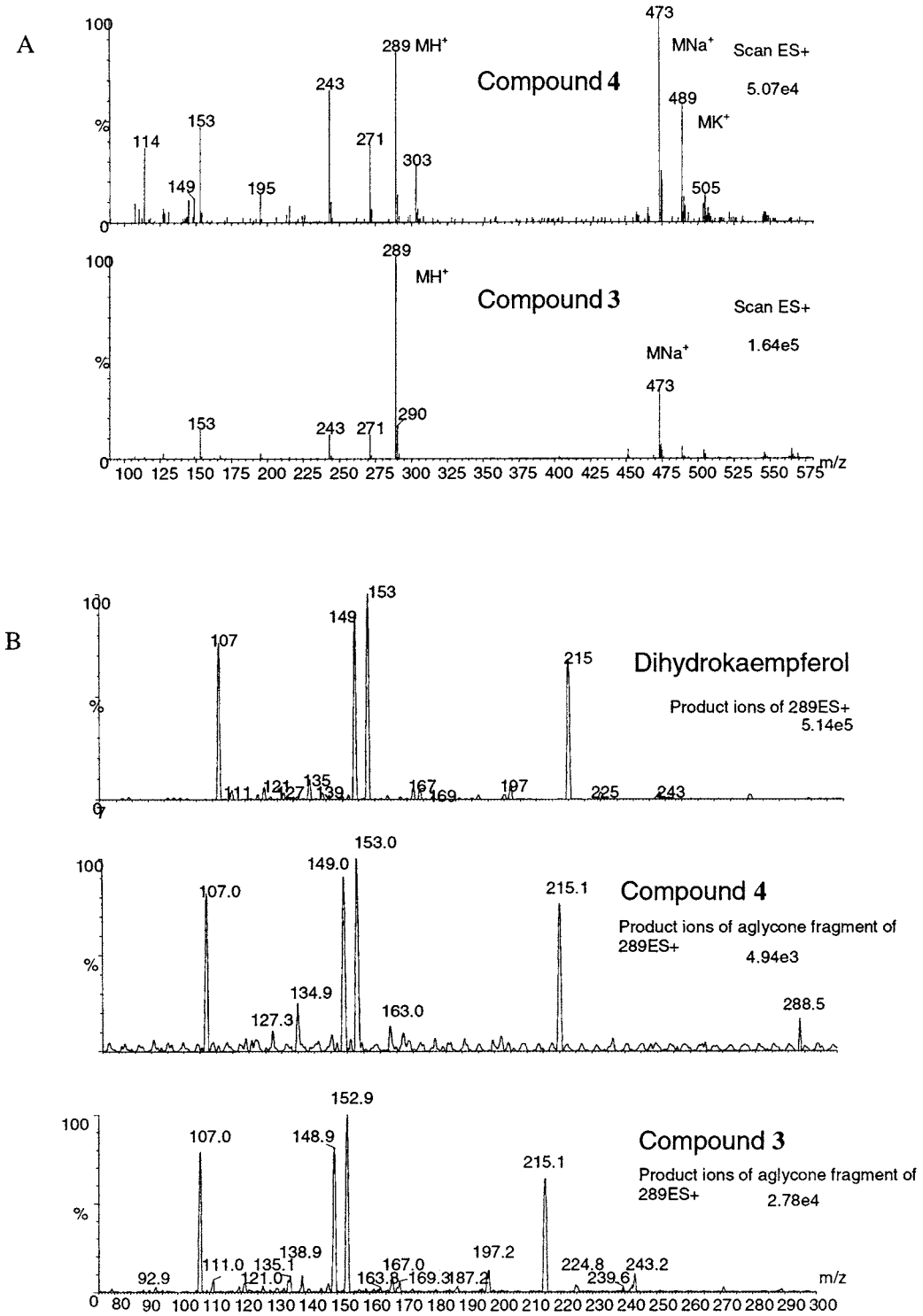


Figure 4. Positive-ion ESI mass spectra of **3** and **4** (from line 2059+) and (below) MS/MS spectra of a dihydrokaempferol standard and equivalent aglycon fragment ion of **3** and **4**.

for naringenin (the UV spectra were very similar). The kaempferol glycosides account for 60% of the total flavonoid glycosides for both red and turning transgenic tomato extracts. The flavonoids in turning and red controls are mainly rutin and naringenin chalcone, while control and transgenic green extracts contain 90% of rutin and hardly any naringenin chalcone. Naringenin chalcone increases markedly in turning tomatoes but decreases at the red stage (4-fold), as found by Muir et al. (*13*) and suggested by Bovy et al. (*12*). However, the level of naringenin chalcone in modified tomatoes is quite similar to that of controls at both these stages (**Table 2**). The level of rutin is almost constant from green to red tomatoes, regardless of whether they are modified or not. However, the levels of the seven other compounds are much higher in transgenic extracts. As mentioned above it was not possible to detect kaempferol-3,7-di-*O*-glucoside (**1**), kaempferol-3-*O*-rutinoside-7-*O*-glucoside (**2**), the dihydrokaempferol-hexosides (**3** and **4**), kaempferol-3-*O*-glucoside (**7**), and naringenin-7-*O*-glucoside (**8**) in any of the control extracts. There is a 60-fold difference in levels of kaempferol-3-*O*-rutinoside (**6**) between the transgenic and control red tomato extracts, a 20-fold difference between the turning transgenic and control, and no significant difference

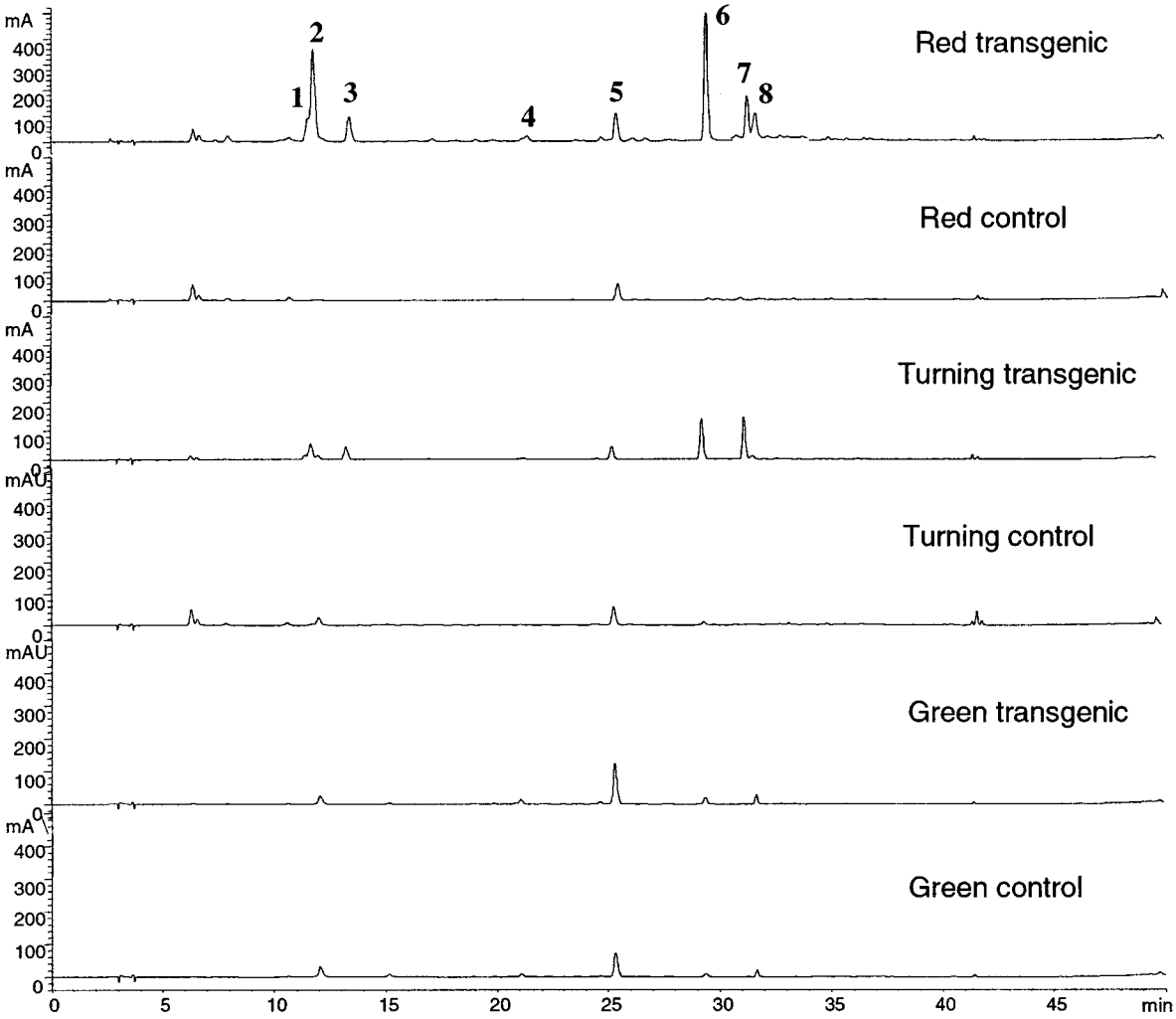


Figure 5. HPLC analysis of transgenic and control tomato extracts (line 2059+) at three stages of maturity (5 μL injection): Signal wavelengths at 270 nm. See **Figure 1** for key assignment.

Table 2. Content of Individual Flavonoid Conjugates in the Six Pooled Whole Fruit Tomato Extracts (Line 2059)[a]

	K-3,7-di-glc, **1**	K-3-rut-7-glc, **2**	DHK-hex, **3**	DHK-hex, **4**	Q-3-rut, **5**	K-3-rut, **6**	K-3-glc, **7**	Nar-7-glc, **8**	Nar chalc, **9**
red transgenic	44.2 ± 9.4	233.8 ± 62.5	182.6 ± 43.5	76.3 ± 15.3	130.4 ± 28.3	364.6 ± 74.1	107.9 ± 20.2	173.8 ± 36.9	226.5 ± 11.3
red control	nd[b]	nd	nd	nd	87.2 ± 3.9	5.4 ± 0.3	nd	nd	345.6 ± 106.8
turning transgenic	17.7 ± 13.6	53.0 ± 30.4	131.6 ± 35.7	39.4 ± 14.1	88.4 ± 26.8	165.9 ± 52.7	156.2 ± 52.2	33.8 ± 9.8	1397.4 ± 639.1
turning control	nd	nd	nd	nd	75.1 ± 12.4	6.4 ± 1.3	nd	nd	1894.7 ± 290.6
green transgenic	nd	nd	nd	nd	154.9 ± 30.8	12.5 ± 2.3	nd	nd	1.7 ± 0.2
green control	nd	nd	nd	nd	122.7 ± 17.6	7.5 ± 0.9	nd	nd	1.4 ± 0.2

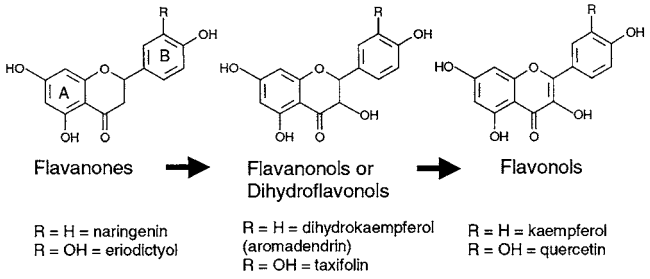
[a] Micrograms per gram of freeze-dried powder expressed as aglycon, with **3** and **4** expressed as naringenin (see text) (four replicates). [b] nd, not detected

between the two green tomato extracts. As pointed out by Bovy et al. (*12*), the increase in flavonoid levels seems to be established by the turning stage, which correlates with the activity profile of the tomato E8 promoter, which was used to drive expression of the *LC* gene. During subsequent ripening, the largest increases in concentration are found for **1**, **2**, and **8**, where around 4-fold differences are observed between transgenic red and turning, and for **4** and **6**, where 2-fold differences were noted. Differences for the other compounds are less than 2-fold.

DISCUSSION

The current work has shown that kaempferol-3,7-di-*O*-glucoside (**1**), kaempferol-3-*O*-rutinoside-7-*O*-glucoside (**2**), kaempferol-3-*O*-rutinoside (**6**), kaempferol-3-*O*-glucoside (**7**), naringenin-7-*O*-glucoside (**8**), and two dihydrokaempferol-hexosides (**3** and **4**) constitute the major compounds responsible for the increase in flavonoids seen in *LC*/*C1* transformed tomato flesh. The compounds are already present in significant amounts at the turning stage, but the highest flavonoid levels are found only once the tomato fruit has ripened. The maize transcription factors *LC* and *C1* appear to have induced increased levels of flavonoid glycosides containing only one hydroxyl (at position 4′) on the B ring. The levels of the flavonoids with hydroxyl substitutions at positions 3′ and 4′ (e.g. quercetin derivatives) remain unchanged, while flavonoids with a B ring substitution

Scheme 2. Schematic Overview of a Part of the Flavonoid Biosynthesis Pathway



different from those shown in **Scheme 2** were not detected. This is comparable to the findings of Bovy et al. (*12*), who worked on the previous generations of *LC*/*C1* tomato lines. The results of this study contrast with those of Muir et al. (*13*), where a *Petunia* gene encoding chalcone isomerase was overexpressed in tomato. Whereas this study has found increased levels of mainly kaempferol glycosides in the flesh, they found increased levels of flavonoids (quercetin glycosides) restricted to the peel.

All seven compounds have been described before, although only kaempferol-3-*O*-rutinoside (**6**) has previously been reported in tomato (*19*). Both kaempferol-3-*O*-rutinoside (**6**) and kaempferol-3-*O*-glucoside (**7**) are commonly found in food plants. Kaempferol-3-*O*-rutinoside is found in green beans (*17*); kaempferol-3-*O*-glucoside in broccoli (*20*), leek, onion (*19*) and endive (*18*); and both compounds are present in grape (*21*) and tea (*22*). The two remaining kaempferol glycosides are less common and most references cite their appearance in nonfood plants [only kaempferol-3,7-di-*O*-glucoside (**1**) was found in the leaves of broad bean plant (*19*)]. Kaempferol-3,7-di-*O*-glucoside has been detected in some types of fern (*23*), one of which is traditionally used by the natives in Mexico to treat kidney diseases (*24*). Kaempferol-3-*O*-rutinoside-7-*O*-glucoside (**2**) has also been identified in a type of fern (*23*), a garden plant from the *Liliacee* family (*25*), a species of mint used as a medicinal plant (*26*) and a tobacco plant (*27*).

Flavanone glycosides are usually found in citrus fruits, but naringenin-7-*O*-glucoside (**8**), also called prunin, has only been detected in the immature fruits of grapefruit and sour orange (*28*). The authors suggested that prunin was actually an intermediate compound in the formation of naringin during the ripening of the fruit. Choi et al. (*29*) concluded that prunin could significantly reduce the glucose and lipid levels in the blood of diabetic rats after having isolated the compound from an extract of *Prunus-Davidiana*, a Korean folk medicine plant used as a hypolipidemic drug.

The large proportion of the MH^+ ion *m*/*z* 289 in **Figure 4** suggests that the sugar moieties of **3** and **4** are easily cleaved, and therefore, those two compounds are most likely *O*-hexosides [dihydrokaempferol *C*-glycosides are extremely rare; only three have been identified so far in the plant kingdom (*30*)]. It was not possible to obtain definitive proof of the structures of **3** and **4**, but NMR evidence presented in the accompanying paper (*16*) was consistent with the presence of a dihydrokaempferol-7-*O*-glycoside. Dihydrokaempferol-*O*-glycosides are minor flavonoids but not uncommon (*31*). Most reports refer to the glucosides: dihydrokaempferol-7-*O*-glucoside has been detected in the powdered stem bark of *Afzelia bella* Harms (Leguminose), used as a treatment for suppurative skin diseases (*32*), and in the flowers of two types of *Crocus* (*33*), while dihydrokaempferol-3-*O*-glucoside had been found in Riesling wine (*34*). Dihydrokaempferol as aglycon (also called aromadendrin) and as 7-*O*-glucoside has demonstrated anti-HIV activity (*35*).

The identification of flavonol glucosides in the *LC*/*C1* transformed tomatoes is of potential significance. The attachment of a glucose group to the quercetin aglycon has been shown to greatly enhance its absorption (*14*), possibly through interaction with the sodium-dependent glucose transporter in the small intestine (*15*). Furthermore, a recent report indicates that absorption of both quercetin-3-*O*-glucoside and 4′-*O*-glucoside is very much more rapid than absorption of quercetin-3-*O*-rutinoside (*36*). This suggests that the sugar moiety is an important determinant of the absorption and bioavailibility of flavonoids, with glucosides being superior to other sugars. If this hypothesis is applicable to kaempferol glycosides, then it is possible that kaempferol-3-*O*-glucoside (**7**) and kaempferol-3,7-di-*O*-glucoside (**1**) may be rapidly absorbed. At this stage, insufficient research is available to assess the impact of more complex glycosylation patterns e.g. kaempferol-3-*O*-rutinoside-7-*O*-*β*-D-glucoside (**2**), although it has been suggested that rutinosides might transit the small intestine and be absorbed in the colon (*14*, *20*).

The overexpression of the transcription factors *LC* and *C1* in the tomato fruits studied here has led to production of a number of flavonoids that can be found in significant amounts in the flesh of ripe fruits. These flavonoids have the potential to exhibit a range of beneficial biological activities including antioxidant behavior and possible treatment and prevention of cancer and other pathologies. However, the use of GMOs as foods raises important safety issues. The safety testing of GMOs is now a priority for regulation authorities, and there is a need to characterize any novel foods as fully as possible. This paper has dealt with the "intended effects" of the genetic modification, concentrating on products of the targeted metabolic pathway. High-resolution ^{1}H NMR has been used in a "nontargeted" screening approach on the same tomato material in order to detect any additional effects on metabolite composition following the overexpression of *LC* and *C1* genes. Results are discussed in the accompanying paper (*16*).

ABBREVIATIONS

LC, liquid chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry; HPLC/DAD, high-performance liquid chromatography/diode array; *LC* and *C1*, *leaf color* and *colorless-1*; rha, rhamnose; glc, glucose or glucoside; ROESY, rotating-frame Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; HMQC, heteronuclear multiple-quantum correlation; K, kaempferol; Q, quercetin; nar chalc, naringenin chalcone; DHK, dihydrokaempferol; rut, rutinoside; hex, hexoside.

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