Biosynthesis of Chlorogenic Acids in Growing and Ripening Fruits of *Coffea arabica* and *Coffea canephora* Plants

Yukiko Koshiro[a], Mel C. Jackson[b], Riko Katahira[a,c], Ming-Li Wang[b], Chifumi Nagai[b], and Hiroshi Ashihara[a,*]

[a] Graduate School of Humanities and Sciences, Ochanomizu University, Bunkyo-ku, Tokyo, 112-8610, Japan. Fax: +81-3-5978-5358. E-mail: ashihara.hiroshi@ocha.ac.jp
[b] Hawaii Agriculture Research Center, Aiea, Hawaii 96701-3911, USA
[c] Faculty of Home Economics, Tokyo Kasei Gakuin University, Tokyo, 194-0292, Japan

* Author for correspondence and reprint requests

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Chlorogenic acids are major secondary metabolites found in coffee seeds. The accumulation of chlorogenic acids and free quinic acids was studied in *Coffea arabica* cv. Tall Mokka and *Coffea canephora* seeds. Growth stages are specified from I to V, corresponding to rapid expansion and pericarp growth (I), endosperm formation (II), mature (green) (III), ripening (pink) (IV), and fully ripened (red) (V) stages. We detected monocaffeoylquinic acids (3CQA, 4CQA and 5CQA), dicaffeoylquinic acids (3,4diCQA, 3,5diCQA and 4,5diCQA) and a monoferuloylquinic acid (5FQA) in whole fruits (stage I), pericarps and seeds. The most abundant chlorogenic acid was 5CQA, which comprised 50–60% of the total of *C. arabica* and 45–50% of *C. canephora* seeds. The content of dicaffeoylquinic acid, mainly 3,5-diCQA, was high in *C. canephora*. A high content of 5FQA was found in seeds of stages III to V, especially in *C. canephora*. Total chlorogenic acids were accumulated up to 14 mg per fruit in *C. arabica* and 17 mg in *C. canephora*, respectively. In contrast, free quinic acid varied from 0.4–2.0 mg (*C. arabica*) and 0.2–4.0 mg (*C. canephora*) per fruit during growth. High biosynthetic activity of 5CQA, which was estimated via the incorporation of [U-^{14}C]phenylalanine into chlorogenic acids, was found in young fruits (perisperm and pericarp) in stage I, and in developing seeds (endosperm) in stages II and III. The biosynthetic activity of chlorogenic acids was clearly reduced in ripening and ripe seeds, especially in *C. canephora*. Transcripts of *PAL1*, *C3′H* and *CCoAMT*, three genes related to the chlorogenic acid biosynthesis, were detected in every stage of growth, although the amounts were significantly less in stage V. Of these genes, *CCoAMT*, a gene for FQA biosynthesis, was expressed more weakly in stage I. The transcript level of *CCoAMT* was higher in seeds than in pericarp, but the reverse was found in *PAL1*. The pattern of expression of genes for the CQA and FQA synthesis is roughly related to the estimated biosynthetic activity, and to the accumulation pattern of chlorogenic acids.

Key words: Caffeoylquinic Acid, Phenylalanine Metabolism, Gene Expression

Introduction

Chlorogenic acids (esters of certain *trans*-cinnamic acids and quinic acid), caffeine and trigonelline are unique secondary metabolites that accumulate in coffee seeds (Clifford, 1985a). Caffeoylquinic acids (CQA), which are depsides of caffeic acid and quinic acid, are major polyphenolics in fruits of coffee plants (Clifford, 1985b). Chlorogenic acids that have accumulated in coffee seeds seem to be utilized for the deposition of phenolic polymers, such as lignin, in cotyledonary cell walls during germination (Aerts and Baumann, 1994). It has been reported that chlorogenic acids act as antioxidants (Charurin *et al.*, 2002; Yen *et al.*, 2005) and inhibitors of tumour promoting activity (Tavani and Vecchia, 2004; Kishimoto *et al.*, 2005; Lee and Zhu, 2006; Belkaid *et al.*, 2006). A few reports on food chemical analyses of isomers of chlorogenic acids in green and roasted coffee beans exist (Clifford, 2000; Farah and Donangelo, 2006), and the role of coffee chlorogenic acids in

Abbreviations: CQA, caffeoylquinic acids; 3CQA, 3-caffeoylquinic acid; 4CQA, 4-caffeoylquinic acid; 5CQA, 5-caffeoylquinic acid; diCQA, dicaffeoylquinic acids; 3,4diCQA, 3,4-dicaffeoylquinic acid; 3,5diCQA, 3,5-dicaffeoylquinic acid; 4,5diCQA, 4,5-dicaffeoylquinic acid; FQA, feruloylquinic acids; 5FQA, 5-feruloylquinic acid; PAL, phenylalanine ammonia-lyase.

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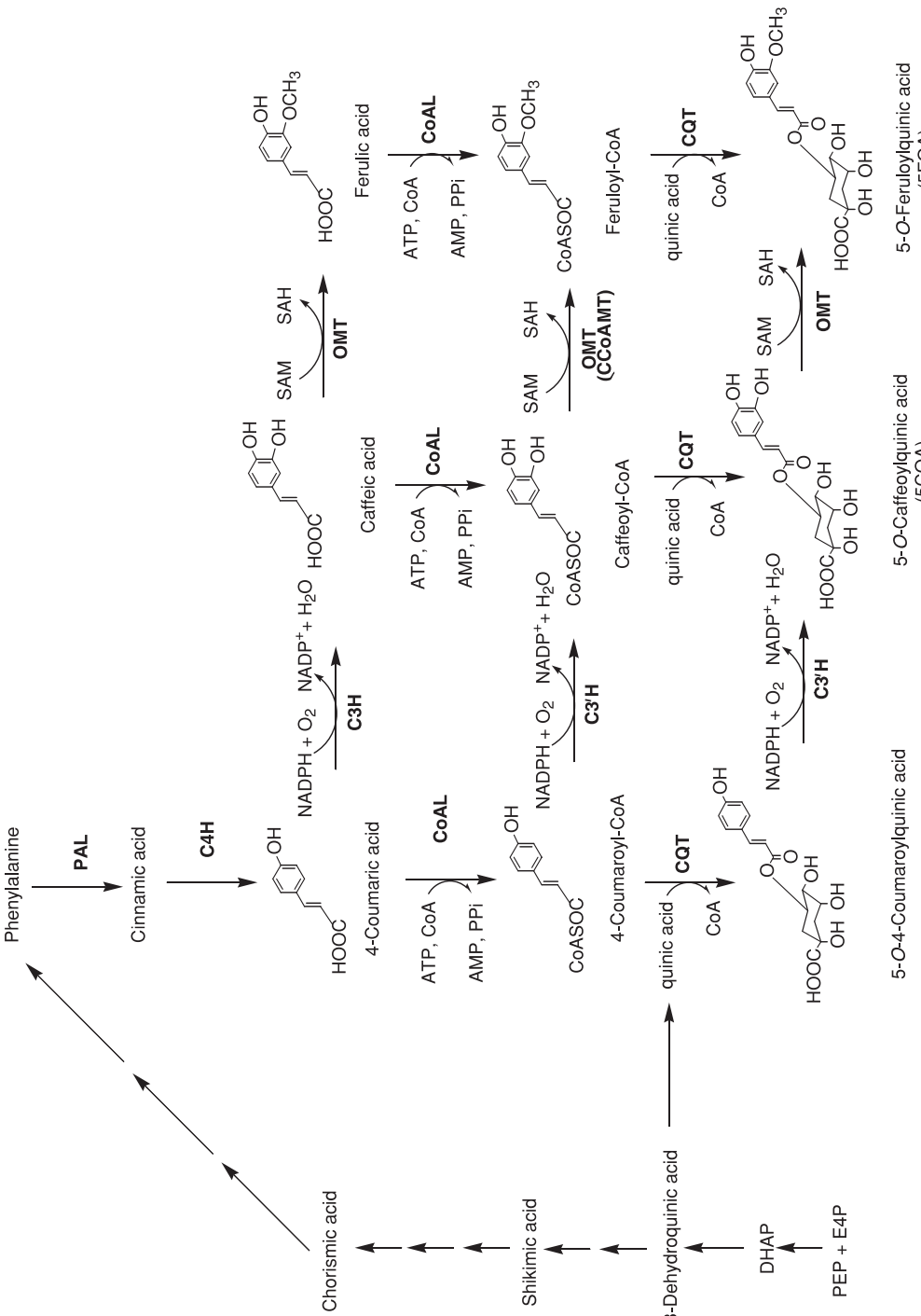

Fig. 1. Possible pathways for 5-caffeoylquinic acid and 5-feruloylquinic acid in coffee plants. Enzyme abbreviations: C3H/C3'H, 4-coumaric acid 3-hydroxylase/4-coumaroylester 3'-hydrolase; C4H, cinnamic acid 4-hydroxylase; CCoAMT, caffeoyl-CoA *O*-methyltransferase; CoAL, CoA ligase; CQT, caffeoyl-CoA: quinic acid caffeoyl transferase (hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase); OMT, *O*-methyltransferase.

human health (Stalmach *et al.*, 2006) has been studied. However, knowledge is lacking on the biochemistry of chlorogenic acid synthesis in coffee fruits. Fragmentary research suggests that chlorogenic acids are formed from the CoA ester of phenylpropanoids and quinic acid in coffee plants, as shown in Fig. 1 (Campa *et al.*, 2003).

C. arabica and *C. canephora* are two types of *Coffea* species used extensively in coffee beverages. They are known commercially as Arabica coffee and as Robusta coffee. Arabica coffee is a higher-priced, milder, fruitier and acidulous beverage (Bertrand *et al.*, 2003), whereas Robusta coffee has been characterized as a neutral coffee, weak-flavoured, and occasionally with a strong and pronounced bitterness (Bertrand *et al.*, 2003; Charrier and Berthand, 1985). In our previous paper (Koshiro *et al.*, 2006), we studied the changing pattern of contents and biosynthetic activities of caffeine and trigonelline during fruit growth and maturation in *Coffea arabica* and *Coffea canephora*. In addition to caffeine and trigonelline, chlorogenic acids are major chemical components of coffee beans, whose content is closely related to the quality of coffee beverages (Farah and Donangelo, 2006; Farah *et al.*, 2006).

In the present study, we monitored changes in the content of various isomers of chlorogenic acids and the biosynthetic activity and expression of some genes involved in chlorogenic acid synthesis, during growth and ripening of *Coffea arabica* and *Coffea canephora* fruits. We use the preferred IUPAC numbering for chlorogenic acids, although the names of genes as reported by others do not always follow this numbering system.

Materials and Methods

Radiochemicals

[U-^{14}C]Phenylalanine (specific activity 14.6 GBq mmol^{-1}) was purchased from Moravek Biochemicals Inc, Brea, CA, USA.

Plant material

Fruits of *Coffea arabica* cv. Tall Mokka (#MA2-7) and of *Coffea canephora* (#6621) were collected at the Experimental Station of the Hawaii Agriculture Research Center, Kunia Station, Oahu Island, Hawaii. These *Coffea* trees were cultivated at the same site at an altitude of *ca.* 95 m above sea level. Fruits were divided into five stages according to growth and maturity.

Analysis of endogenous chlorogenic acids

Chlorogenic acids were analyzed by a method adapted from that of Balyaya and Clifford (1995). Coffee samples were lypholyzed to complete dryness, and then ground to a fine powder using an IKA Universalmühle M20 blade grinder (IKA-Labortechnik, Staufen, Germany). Coffee powder (500 mg) was placed in a round-bottom flask and mixed with 100 ml of 70% (v/v) methanol. The sample was refluxed for 1 h. After cooling to room temperature, 1 ml of each Carrez reagent was added (Carrez 1: 21.9 mg zinc acetate dihydrate and 3 ml acetic acid in 100 ml water; Carrez 2: 10.6 g potassium ferrocyanide trihydrate in 100 ml water). Once the precipitate had settled, samples were filtered using a 0.45 micron nylon filter (Millipore), and were used for HPLC.

The mobile phase consisted of solution A (0.06% TFA) and solution B [0.06% TFA in 45% (v/v) acetonitrile]. Prior to sample injection, the mobile phase was 20% solution B, and the flow rate was 1 ml/min. Immediately after injection of 20 μl of sample, the content of solution B in the mobile phase was increased to 45% within 26 min. The content of solution B then increased to 100% over the next 10 min. The column was then allowed to equilibrate at 20% solution B for 4 min. Chlorogenic acids were detected at 315 nm. They were identified from their relative retention times and molar absorbencies (Balyaya and Clifford, 1995; Clifford *et al.*, 1985).

Analysis of endogenous quinic acids

A powdered sample (1 g) in a 125 ml glass beaker was mixed with 10 ml of 50% (v/v) methanol containing 8 mM sulphuric acid. The sample was sonicated for 15 min and then filtered through a glass microfiber filter (Whatman, Grade GF/A), and a 5 ml-aliquot of filtrate was evaporated to a volume of less than 2.5 ml using a rotary evaporator. Distilled water was added to make up a final volume of 5 ml. The extract (2 ml) was passed through an 1 ml bed volume Sep-Pak® C18 SPE cartridge (Waters), preconditioned with 1 ml of methanol. Residual unbound extract was eluted by addition of 1 ml of distilled water. The eluent was then filtered through a 0.45 μ nylon filter (Millipore), prior to HPLC.

For HPLC analysis of quinic acid a Shimadzu SCL-10A controller and LC-10AS pump (Shimadzu Corporation, Japan) were used. Chromato-

graphic separations were achieved using a Rezex ROA organic acid ion exchange column (300 × 7.5 mm) with a Carbo-H^+ guard column (4 mm × 3 mm; Phenomenex USA, Torrance, CA). The mobile phase consisted of 6.5 mM sulphuric acid in distilled water at a flow rate of 0.4 ml/min. The running time was 24 min followed by a column wash step using a mobile phase of distilled water at a flow rate of 0.52 ml/min for 10 min. The column was re-equilibrated using the original mobile phase at a flow rate of 0.52 ml/min for further 27 min. Quinic acid was detected using a Shimadzu SPD-10A UV-VIS detector, measuring the absorbance at 220 nm.

Metabolism of [U-^{14}C]phenylalanine

Samples (3 mm × 3 mm segments, *ca.* 200 mg fresh wt) and 2.0 ml of 30 mM sodium phosphate buffer (pH 5.6) containing 10 mM sucrose and 0.5% sodium ascorbate were placed in the main compartment of a 30-ml Erlenmeyer flask. The flask was fitted with a small glass tube containing a piece of filter paper, impregnated with 0.1 ml of 20% KOH in the centre well to collect $^{14}CO_2$. Each reaction was started by adding a solution of [U-^{14}C]phenylalanine (10 μl, 37 kBq) to the main compartment. The flask was incubated in an oscillating water bath at 27 °C for 18 h. After incubation, the plant materials were harvested using a stainless steel tea strainer, then washed with distilled water and kept in 80% methanol and stored at −30 °C prior to extraction. Potassium bicarbonate that had been absorbed by the filter paper was allowed to diffuse into distilled water overnight, and aliquots of the resulting solution (usually 0.5 ml) were used for determination of the radioactivity.

The ^{14}C-labelled plant materials were homogenized in 80% methanol and successively extracted with 80% methanol. After centrifuging, the supernatant fractions were combined and fractionated using a Dowex 50W-X8 (H^+-form) column according to the method by Nagaoka and Ashihara (1988). The neutral and anionic compounds passed through the column, which included 5CQA and caffeic acid, and the cationic compounds, including amino acids eluted from the column with 2 M NH_4OH, were collected and the radioactivity was measured. The neutral and anionic fraction was reduced to dryness *in vacuo*. The pellet was dissolved in 50% ethanol and aliquots were analyzed by TLC.

TLC was performed with microcrystalline cellulose plates and silica gel plates (Merck, Darmstadt, Germany). The solvent systems used were *n*-butanol/acetic acid/water (4:1:2, v/v) and ethyl acetate/formic acid/acetic acid/water (100:11:11:27, v/v). The ethanol-insoluble fraction, mainly proteins and lignin, was suspended in 1 ml distilled water, and 25 μl-aliquots were used for determination of radioactivity.

Radioactivity was measured using a multi-purpose scintillation counter (Beckman, Type LS 6500, Fullerton, CA, USA) with scintillation fluid ACS-II (Amersham Pharmacia Biotech). The distribution of radioactivity in metabolites on the TLC sheet was determined using a Bio-Imaging Analyser (Type FLA-2000, Fuji Photo Film Co. Ltd., Tokyo, Japan).

Semi-quantitative reverse transcription (RT)-PCR

For RT-PCR, fruits were frozen immediately at the experimental field with liquid nitrogen, as described previously (Koshiro *et al.*, 2006). Total RNAs extracted from whole fruits, pericarp or seeds were treated with RNase-free DNase I (Promega, Madison, WI, USA). DNA-free total RNA (300 ng) from each sample was used for first strand cDNA synthesis in a reaction volume of 25 μl, containing 50 units of MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and 2.5 μM oligo-d$(T)_{16}$. The PCR reaction mixture (25 μl) contained 0.4 μl of RT reaction mixture and 0.2 μM of each primer in 1× formulation of Go Taq Green Master Mix (Promega).

The gene specific primers used were as follows: *PAL1* (DQ067599), 5′-GGTGATTTGGTTCCACTCTC-3′ and 5′-CTCAAAGAGGACCATGGAAG-3′; *C3′H (p-coumaroyl 3′-hydroxylase, CYP98A-C1)* (DQ269126), 5′-ACAGCCATGGTTGATTCCATC-3′ and 5′-GTTCATCAACCACACCCTCTG-3′; *CCoAMT* (AF534905), 5′-TGATCGAAAAGGCTGGTGTG-3′ and 5′-CCCCAACCTTGACCAACTCG-3′ *α-tubulin* (AF363630), 5′-GCTTTCAACACCTTCTTCAG-3′ and 5′-GCTGCTCAGGGTGGAAGAG-3′.

For PCR, we used a Gene Amp PCR System 2400 thermal cycler programmed at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. Amplification was done for 28–38 cycles, and the reaction tubes were removed every two cycles. The amplicons

Table I. Growth stages, changes in size, and fresh and dry weights of *Coffea arabica* and *Coffea canephora* fruits. The sizes and weights are expressed in mm and mg. Mean values ± SD ($n = 10$) are shown. Stages I to V are defined as follows: I, Rapid expansion and pericarp growth stage; II, endosperm formation stage; III, mature (green) stage; IV, ripening (pink) stage; V, ripe (red) stage.

Sample	Stage	Colour	Size (l × d)	Fresh weight	Dry weight
C. arabica	I	Green	7.0 × 6.5	236 ± 4	39 ± 6
cv. Tall Mokka	II	Green	11.3 × 11.3	800 ± 35	176 ± 9
	III	Green	12.0 × 12.6	849 ± 41	187 ± 9
	IV	Pink	12.4 × 13.3	1,012 ± 39	445 ± 35
	V	Red	13.5 × 14.0	1,288 ± 11	527 ± 43
C. canephora	I	Green	7.0 × 7.0	239 ± 11	37 ± 7
	II	Green	11.0 × 10.0	545 ± 3	213 ± 53
	III	Green	12.1 × 10.1	622 ± 7	259 ± 21
	IV	Pink	12.3 × 11.0	778 ± 73	302 ± 28
	V	Red	13.0 × 11.8	1,112 ± 90	485 ± 34

corresponding to *PAL1*, *C3′H*, *CCoAMT* and α-tubulin were, respectively, 216, 162, 183, and 133 bp in length. The amplification showed a linear curve. The reaction product was visualized by UV light on 2% agarose gels stained with ethidium bromide.

Results

Growth of fruits

In this study, we chose as plant materials *C. arabica* cultivar Mokka and *C. canephora*. Initially, we assumed that fruits from single branches pollinated at the same day and harvested at a set time interval would be the same size. The growth of individual fruits varied widely, however. We therefore defined the growth stages from the sizes of the fruits. Table I shows changes in bean sizes, and fresh and dry weights of fruits from these two plant materials during growth and maturation. The stages of growth correspond to the rapid expansion and pericarp growth stage (I), endosperm formation stage (II), mature (green) stage (III), ripening (pink) stage (IV) and ripe (red) stage (V). The proportion of seeds per fruit in stages III, IV and V, calculated on a dry weight basis, was, respectively, 78%, 65% and 69% in *C. arabica* cv. Mokka and 75%, 60% and 61% in *C. canephora*.

Changing pattern of total content of chlorogenic acids and quinic acid

We determined the accumulation of chlorogenic acids during growth and development of coffee fruits. Total chlorogenic acid contents in fruits of *C. arabica* cv. Mokka and *C. canephora* are shown in Figs. 2a, b. Chlorogenic acid content increased gradually in *C. arabica* fruits (Fig. 2a), but a marked increase in content was found between stages II and III in *C. canephora*. In stage I, coffee fruits consist mainly of perisperm and pericarp, making it difficult to separate seeds and pericarp; but, after stage II, seeds can be isolated from coffee fruits. Most chlorogenic acids were located in seeds in both coffee species (Figs. 2a, b). Contents of total chlorogenic acids in seeds of stage V of *C. arabica* cv. Mokka and *C. canephora* were 13.6 and 16.9 mg per fruit. These values comprise 89.5% and 81.6% of the chlorogenic acid content in the fruits.

Quinic acid, which is a polyol moiety of chlorogenic acid, was found in both pericarp and seed of both coffee species (Figs. 2c, d). The total amount of this compound varies between 0.4 and 2.0 mg (*C. arabica*) and 0.2 and 4.0 mg (*C. canephora*) during growth and maturation.

Content of various chlorogenic acids during growth and maturation

Three monocaffeoylquinic acids (3CQA, 4CQA and 5CQA), three dicaffeoylquinic acids (3,4diCQA, 3,5diCQA and 4,5diCQA) and one monoferuloylquinic acid (5FQA) were detected in pericarps and seeds of *C. arabica* and *C. canephora* (Figs. 3 and 4). In stage I, chlorogenic acids were analyzed in whole fruits, and the data are shown in the percarp section, because seeds have not yet formed. Of the monocaffeoylquinic acids, the most abundant chlorogenic acid was 5CQA, comprising 50–60% of the total chlorogenic acids in seeds of *C. arabica* and 45–50% in *C. canephora*. The content of other monocaffeoylquinic acids, 3CQA and

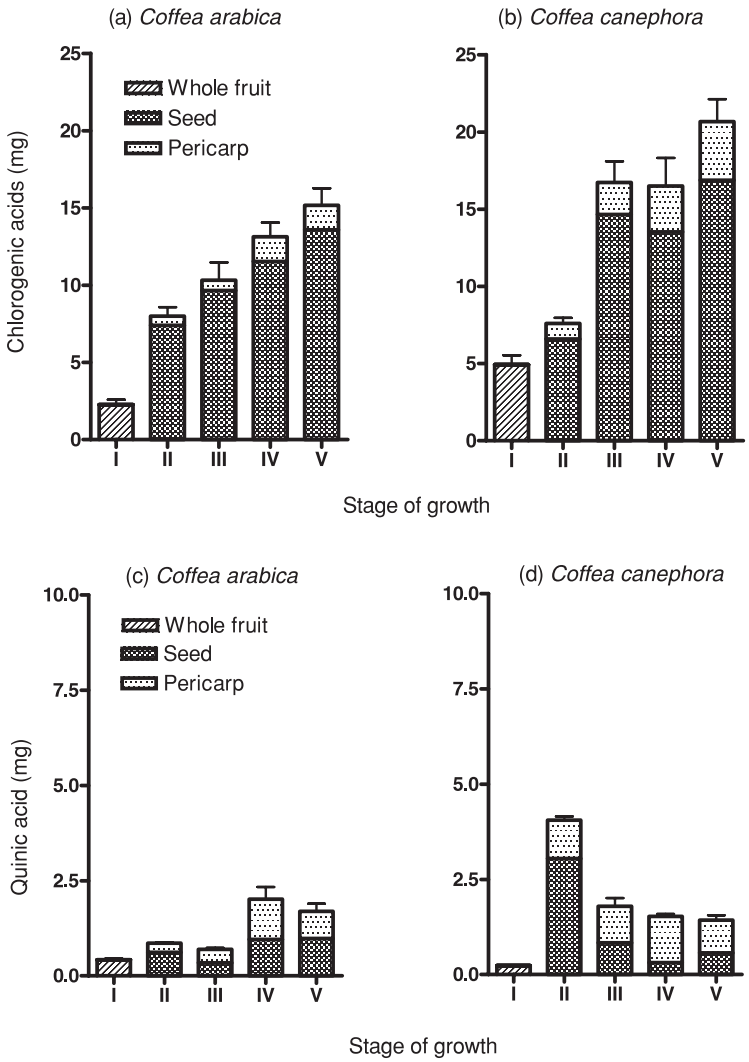

Fig. 2. Changes in total chlorogenic acids (a, b) and quinic acid (c, d) during the development of *Coffea arabica* cv. Tall Mokka (a, c) and *Coffea canephora* (b, d) fruits. Content is expressed as mg per part. Mean values ± SD (n = 6) are shown.

4CQA, was low in pericarps and seeds but the content increased with the growth of coffee seeds of both species. The content of dicaffeoylquinic acids in *C. canephora* was much higher than in *C. arabica*, especially in stages II and III (Figs. 3 and 4). 3,5diCQA was the major dicaffeoylquinic acid, especially in the early stages of fruit growth. Feruloylquinic acid was scarcely found in whole fruits of stage I or in pericarps, but a significant content of 5FQA was detected in seeds. A particularly high relative content of 5FQA was found in *C. canephora* after stage III. 5FQA comprised 22% of the total chlorogenic acids in ripe *C. canephora* seeds. The proportion of 5FQA in ripe seeds of *C. arabica* was rather lower, and its content was less than 10% of total chlorogenic acids.

Metabolism of [U-^{14}C]phenylalanine

The metabolic fate of [U-^{14}C]phenylalanine in various growth stages of fruits of *C. arabica* and *C. canephora* are shown in Table II and Table III, respectively. Approximately half of the radioactivity taken up by the fruit segments was recovered

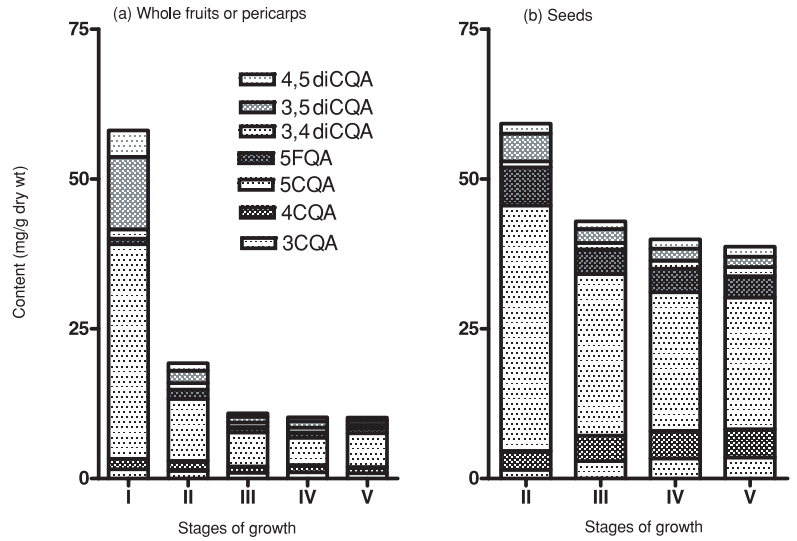

Fig. 3. Changes in chlorogenic acid isomers in whole fruits or pericarp (a) and seeds (b) during the development of *Coffea arabica* cv. Tall Mokka fruits. Content is expressed as mg per dry weight. Mean values (n = 6) are shown.

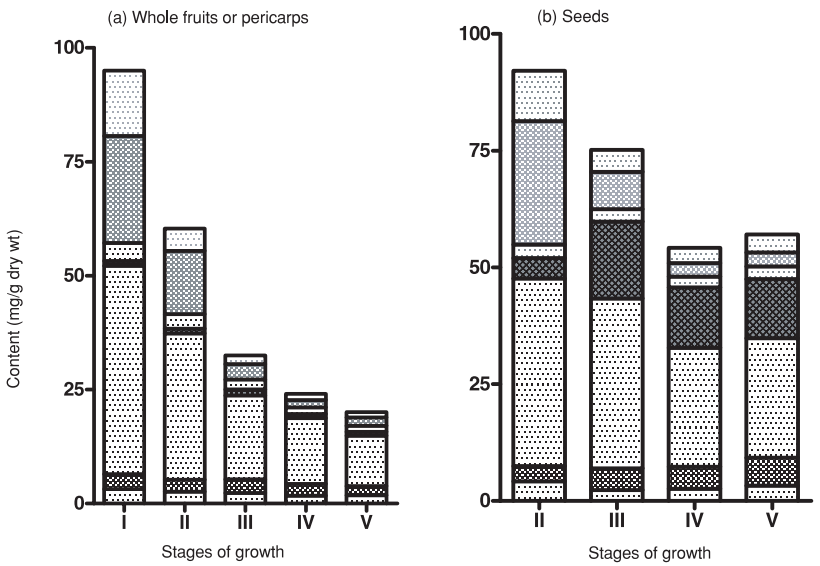

Fig. 4. Changes in chlorogenic acid isomers in whole fruits or pericarp (a) and seeds (b) during the development of *Coffea canephora* fruits. Content is expressed as mg per dry weight. Mean values (n = 6) are shown.

in the 80% methanol-soluble fraction; the rest was found in the methanol-insoluble fraction which consisted of proteins and lignin. The methanol-soluble fraction was further separated into its basic components, which consisted of phenylalanine and other amino acids, and other neutral and acidic components, which contained phenolic acids. Incorporation of radioactivity in 5CQA and caffeic acid was determined by a TLC/radio-imaging system. Up to 12% of total radioactivity was found in 5CQA 18 h after incubation with [U-^{14}C]phenylalanine. In *C. arabica*, greater incorporation of

Table II. Metabolism of [U-^{14}C]phenylalanine (a) in whole fruits (stage I) and pericarps (stages II–V) and (b) in seeds (stages II–V) of *Coffea arabica* cv. Tall Mokka fruits. Total uptake refers to the incorporation of radioactivity into methanol-soluble, methanol-insoluble and CO_2 fractions. Metabolites in the methanol-soluble fraction were separated into basic and other (acidic and neutral) components by ion-exchange chromatography. Caffeic acid and 5CQA were separated from the other components. Values are shown as kBq per 100 mg fresh weight ± SD (n = 3) and percent of total uptake (in parentheses).

(a) Whole fruits or pericarps

	Stage I	Stage II	Stage III	Stage IV	Stage V
Methanol-soluble	8.01 ± 1.26 (50.1)	4.63 ± 1.19 (51.7)	2.63 ± 0.02 (49.0)	1.89 ± 0.34 (39.5)	1.84 ± 0.5 (45.4)
Basic components	1.37 ± 0.15 (18.0)	1.03 ± 0.54 (24.7)	1.67 ± 0.10 (31.1)	0.61 ± 0.09 (24.8)	0.56 ± 0.24 (28.0)
Other components	2.43 ± 0.47 (32.1)	1.13 ± 0.17 (27.0)	0.96 ± 0.07 (17.9)	0.36 ± 0.07 (14.6)	0.35 ± 0.26 (17.5)
Caffeic acid	0.49 ± 0.02 (6.4)	0.35 ± 0.05 (8.3)	0.07 ± 0.01 (1.2)	0.19 ± 0.03 (7.7)	0.12 ± 0.07 (6.0)
5CQA	0.85 ± 0.04 (11.2)	0.19 ± 0.02 (4.5)	0.12 ± 0.01 (2.3)	0.07 ± 0.02 (2.8)	0.12 ± 0.09 (6.0)
Methanol-insoluble	7.84 ± 0.26 (49.0)	4.21 ± 1.49 (47.8)	2.68 ± 0.10 (50.0)	2.82 ± 0.21 (58.9)	2.00 ± 0.22 (49.4)
CO_2	0.15 ± 0.04 (0.9)	0.04 ± 0.01 (0.4)	0.05 ± 0.01 (0.9)	0.08 ± 0.03 (1.7)	0.22 ± 0.01 (5.4)
Total uptake	16.0 ± 1.60 (100)	8.80 ± 2.69 (100)	5.36 ± 0.10 (100)	4.79 ± 0.16 (100)	4.05 ± 0.29 (100)

(b) Seeds

	Stage II	Stage III	Stage IV	Stage V
Methanol-soluble	1.60 ± 0.42 (69.8)	1.54 ± 0.10 (56.9)	0.51 ± 0.01 (37.8)	0.65 ± 0.10 (42.2)
Basic components	0.36 ± 0.11 (39.5)	0.32 ± 0.14 (28.8)	0.24 ± 0.05 (25.2)	0.28 ± 0.03 (28.1)
Other components	0.28 ± 0.12 (30.3)	0.31 ± 0.11 (28.1)	0.12 ± 0.01 (12.9)	0.14 ± 0.02 (14.1)
Caffeic acid	0.07 ± 0.03 (8.1)	0.15 ± 0.04 (13.5)	0.03 ± 0.00 (3.1)	0.04 ± 0.00 (4.0)
5CQA	0.07 ± 0.07 (7.4)	0.09 ± 0.02 (8.1)	0.03 ± 0.01 (3.1)	0.05 ± 0.01 (5.0)
Methanol-insoluble	0.55 ± 0.10 (23.9)	1.01 ± 0.68 (37.3)	0.79 ± 0.13 (58.5)	0.78 ± 0.01 (50.6)
CO_2	0.14 ± 0.09 (6.2)	0.15 ± 0.01 (5.7)	0.06 ± 0.01 (4.4)	0.11 ± 0.03 (7.1)
Total uptake	2.29 ± 0.51 (100)	2.70 ± 0.95 (100)	1.35 ± 0.13 (100)	1.54 ± 0.12 (100)

^{14}C into 5CQA was found in young fruits and seeds of stages II and III. A similar trend was also observed in the fruits of *C. canephora*. Greater incorporation of ^{14}C into 5CQA was found in young fruits (stage I) and seeds of stages II and III. The biosynthetic activity of chlorogenic acids estimated by [U-^{14}C]phenylalanine was clearly reduced in ripening and ripe seeds, especially in *C. canephora*, although radioactivity was still incorporated into 5CQA at the later development stages of *C. arabica*. Radioactivity was also found in caffeic acid in most of the samples.

Expression of PAL1, C3′H and CCoAMT genes

We examined the amount of transcripts of three genes encoding enzymes related to the CQA and FQA biosynthesis. Very limited numbers of genes have been cloned in coffee plants, in contrast to some model plants, such as *Arabidopsis thaliana* and rice. Based on the available information, we chose three genes related to the chlorogenic acid biosynthesis. *PAL1* is a gene for phenylalanine ammonia-lyase (EC 4.3.1.5), which catalyzes the first step of the phenylpropanoid pathway leading to the synthesis of CQA and FQA (Mahesh *et al.*, 2006), and *C3′H* encodes 4-coumaroylester 3′-hydroxylase, which catalyzes the conversion of 4-coumaric acid/4-coumaroylester to caffeic acid/caffeoylester. *CCoAMT* is a gene coding caffeoyl-

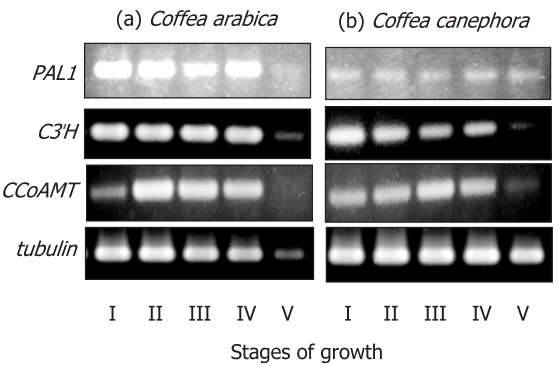

Fig. 5. Expression of *PAL1*, *C3′H*, *CCoAMT* and *α-tubulin* in whole fruits of *Coffea arabica* cv. Tall Mokka (a) and *Coffea canephora* (b) at stages I–V. The results shown were obtained with 28 (*CCoAMT* and *C3′H*) or 35 cycles (*PAL1* and *tubulin*) of PCR amplification.

Table III. Metabolism of [U-^{14}C]phenylalanine (a) in whole fruits (stage I) and pericarps (stages II–V) and (b) in seeds (stages II–V) of *Coffea canephora* fruits. Total uptake refers to the incorporation of radioactivity into methanol-soluble, methanol-insoluble and CO_2 fractions. Metabolites in the methanol-soluble fraction were separated into basic and other (acidic and neutral) components by ion-exchange chromatography. Caffeic acid and 5CQA were separated from the other components. Values are shown as kBq per 100 mg fresh weight ± SD (n = 3) and percent of total uptake (in parentheses).

(a) Whole fruits or pericarps

	Stage I	Stage II	StageIII	Stage IV	Stage V
Methanol-soluble	9.36 ± 0.10 (59.2)	3.72 ± 1.02 (31.1)	1.53 ± 0.02 (33.5)	1.05 ± 0.15 (56.1)	1.96 ± 1.63 (60.5)
Basic components	2.70 ± 0.14 (38.5)	0.67 ± 0.25 (13.0)	1.12 ± 0.17 (24.4)	1.41 ± 0.51 (39.8)	0.94 ± 0.02 (42.1)
Other components	1.45 ± 0.43 (20.7)	0.94 ± 0.27 (18.1)	0.41 ± 0.22 (9.0)	0.58 ± 0.28 (16.4)	0.41 ± 0.05 (18.4)
Caffeic acid	0.33 ± 0.19 (4.7)	0.14 ± 0.02 (2.8)	0.07 ± 0.01 (1.6)	0.05 ± 0.01 (1.4)	0.04 ± 0.01 (1.8)
5CQA	0.26 ± 0.07 (3.6)	0.13 ± 0.01 (2.5)	0.05 ± 0.00 (1.2)	0.06 ± 0.04 (1.7)	0.03 ± 0.02 (1.3)
Methanol-insoluble	6.17 ± 0.35 (39.1)	8.13 ± 0.80 (68.0)	2.96 ± 0.64 (62.9)	0.79 ± 0.19 (42.2)	1.26 ± 0.90 (38.9)
CO_2	0.26 ± 0.07 (1.7)	0.10 ± 0.01 (0.8)	0.16 ± 0.03 (2.0)	0.03 ± 0.01 (1.6)	0.02 ± 0.01 (0.6)
Total uptake	15.8 ± 0.52 (100)	12.0 ± 1.83 (100)	4.65 ± 0.59 (100)	1.87 ± 0.35 (100)	3.24 ± 0.25 (100)

(b) Seeds

	Stage II	Stage III	Stage IV	Stage V
Methanol-soluble	6.55 ± 1.24 (70.0)	1.58 ± 0.22 (56.9)	1.99 ± 0.16 (59.1)	1.67 ± 0.08 (56.0)
Basic components	0.64 ± 0.07 (15.2)	1.09 ± 0.08 (39.3)	1.46 ± 0.23 (47.9)	0.58 ± 0.39 (43.3)
Other components	2.32 ± 0.66 (54.8)	0.49 ± 0.11 (17.6)	0.34 ± 0.10 (11.2)	0.17 ± 0.15 (12.7)
Caffeic acid	0.17 ± 0.01 (3.9)	0.14 ± 0.01 (4.9)	0.03 ± 0.00 (1.0)	0.01 ± 0.01 (0.7)
5CQA	0.17 ± 0.00 (4.1)	0.11 ± 0.00 (3.8)	0.00 ± 0.00 (0.0)	0.01 ± 0.00 (0.7)
Methanol-insoluble	2.73 ± 0.07 (29.2)	0.75 ± 0.14 (26.8)	1.35 ± 0.11 (40.1)	1.29 ± 0.09 (43.3)
CO_2	0.08 ± 0.04 (0.9)	0.46 ± 0.06 (12.0)	0.03 ± 0.00 (0.9)	0.02 ± 0.00 (0.7)
Total uptake	9.36 ± 1.34 (100)	2.79 ± 0.2 (100)	3.37 ± 0.27 (100)	2.98 ± 0.17 (100)

CoA 3-*O*-methyltransferase, which catalyzes the formation of feruloyl-CoA from caffeoyl-CoA. The substrate specificities of 3-/3′hydrolase and *O*-methyltransferase seem to be broad. These enzymes may catalyze other hydrolase and methyltransferase reactions shown in Fig. 1. Fig. 5 shows the expression of these three genes and a gene encoding α-tubulin as control in *C. arabica* cv. Tall Mokka and *C. canephora*. In both *Coffea* species, the transcripts of *PAL1*, *C3′H* and *CCoAMT* were detected in all stages of growth and ripening, although the amounts of the transcripts of *PAL1*, *C3′H* and *CCoAMT* all decreased markedly in stage V. Of these genes, *CCoAMT* was expressed more weakly in stage I in both species. The RT-PCR method is semi-quantitative, so it is difficult to find the absolute amounts of transcripts. Nevertheless, the expression pattern of a gene for FQA synthesis is not always the same as the genes for CQA synthesis: expression of *C3′H* was greater in the early stages, but expression of *CCoAMT* was more intense after stage II than in stage I.

Fig. 6 shows expression patterns of *PAL1*, *C3′H* and *CCoAMT* in pericarps and in seeds from stages III and IV of *C. arabica*. The amounts of transcripts of *PAL1* were much higher in pericarps than in seeds, but were higher in seeds than in pericarps for *CCoAMT* transcripts. Similar expression patterns of *C3′H* were observed in pericarps and seeds.

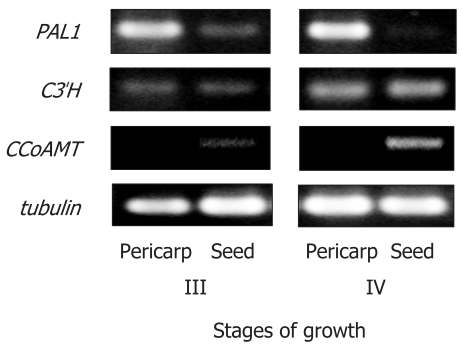

Fig. 6. Expression of *PAL1*, *C3′H*, *CCoAMT* and *α-tubulin* in pericarps and seeds of *Coffea arabica* cv. Tall Mokka (a) and *Coffea canephora* (b) at stages III and IV. The results shown were obtained with 35 cycles of PCR amplification except for *CCoAMT* (38 cycles).

Discussion

Coffee plants produce various isomers of chlorogenic acids, including CQA, diCQA and FQA. The major component is 5CQA. Multiple biosynthetic pathways for 5CQA, which is the most abundant isomer of CQA, have been proposed (Fig. 1). The caffeoyl part of 5CQA is formed via the phenylpropanoid pathway derived from phenylalanine (Hahlbrock and Scheel, 1989). In many plants including coffee plants, the precursors of 5CQA synthesis are hydroxycinnamoyl-CoA derivatives (Stockigt and Zenk, 1974; Ulbrich and Zenk, 1979), although an alternative pathway which involves hydroxycinnamoyl-glucose has also been proposed in sweet potatoes (Villegas and Kojima, 1986; Moriguchi *et al.*, 1988). Caffeoyl-CoA or 4-coumaroyl-CoA is combined with quinic acid, which is derived by the shikimic acid pathway (Gamborg, 1967). We detected: (i) incorporation of radioactivity from [U-^{14}C]phenylalanine into caffeic acid and 5CQA (Tables II and III); (ii) incorporation of [U-^{14}C]caffeic acid into 5CQA (Koshiro, 2006); and (iii) transcripts of *PAL1* and *C3′H* (Figs. 5 and 6). These results suggest that the pathway, phenylalanine → cinnamic acid → 4-coumaric acid → caffeic acid → caffeoyl-CoA → 5CQA is operating in coffee plants. 5FQA appears to be formed from caffeoyl-CoA via feruloyl-CoA, because we detected the transcript of *CCoAMT* (Figs. 5 and 6), which encodes caffeoyl-CoA 3-*O*-methyltransferase (Campa *et al.*, 2003). Participating enzymes in CQA and FQA biosynthesis have broad substrate specificities, so that the operation of alternative pathways is also theoretically possible. Furthermore, reactions generating the formation of 3CQA and 4CQA have not yet been discovered.

The present work shows that the concentrations of chlorogenic acids in the ripe seeds (which are used as raw materials for coffee beverage) of *C. arabica* are lower than in *C. canephora*. This is consistent with previous observations (Farah and Donangelo, 2006; Ky *et al.*, 2001; Clifford and Kazi, 1987). Farah *et al.* (2006) reported that 3,4diCQA levels in green and roasted coffee strongly correlate with high quality. Conversely, higher levels of CQA (predominantly 5CQA), FQA, and their oxidation products are associated with poor cup quality. We found a much higher content of 5FQA in *C. canephora* seeds than in *C. arabica* seeds.

Biosynthesis of chlorogenic acids seems to be controlled by the activity of the phenylpropanoid pathway and the shikimic acid pathway. In the present study, we inferred the 5CQA synthesis from *in situ* tracer experiments using [^{14}C]phenylalanine and expression of *PAL1* and *C3′H*. Quinic acid is derived from 3-dehydroquinic acid, which is the second intermediate of the shikimic acid pathway starting from erythrose-4-phosphate and phosphoenolpyruvate (Stockigt and Zenk, 1974). Since there is a considerable quinic acid pool in *C. arabica* and in *C. canephora* fruits during growth and maturation, supply of this compound may not restrict the biosynthetic activity of chlorogenic acids. Phenylalanine ammonia-lyase (PAL) catalyzes the first step of the general phenylpropanoide biosynthetic pathway, which produces a wide range of secondary compounds, such as flavonoides, coumarins and lignin (Hahlbrock and Scheel, 1989). In earlier papers without any nucleotide sequence information, expression of the *PAL* gene in coffee fruit was reported (Campa *et al.*, 2005; Melo and Mazzafera, 2005). Campa *et al.* (2005) used two *PAL* gene sequences (*PAL1* and unregistered *PAL2*). Semi-quantitative RT-PCR on *C. canephora* suggested that *PAL1* is expressed in green, yellow and red stages; these are probably identical to stages III to V in our study, but *PAL2* is expressed only at the red stage. Melo and Mazzafera (2005) used unpublished EST sequences of *PAL*, but the changing expression pattern was similar to ours, and the level of transcript also decreased at the mature stage. Our results show that the level of *PAL1* transcript is much higher in pericarps than in seeds. The reason is not known, but PAL genes are generally organized as a small multi-gene family in plants (Mahesh *et al.*, 2006); the other *PAL* gene(s) may be expressed in coffee seeds. *C3′H* was expressed similarly in pericarps and seeds, and the level of *C3′H* transcript decreased in stage V. This observation is consistent with the report of Melo and Mazzafera (2005). High expression of the *CCoAMT* gene was found in stages II to IV and in seeds. These findings support the hypothesis that high accumulation of 5FQA in coffee seeds after stage II is due to the gene expression of *CCoAMT* in these tissues.

The present results provide the biochemical outline of chlorogenic acid synthesis in *C. arabica* and *C. canephora*. These data can help in selecting good quality coffee by breeding, and also in preparing genetically/metabolically modified coffee beans having high cup quality.

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