Metabolism of Phenolic Compounds during Loquat Fruit Development

Chang-Kui Ding,*,† Kazuo Chachin,† Yoshinori Ueda,† Yoshihiro Imahori,† and Chien Y. Wang‡

College of Agriculture, Osaka Prefecture University, Gakuen-cho, Sakai, Osaka 599-8531, Japan, and Produce Quality and Safety Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

Phenolic compounds in loquat fruit were identified as 5-caffeoylquinic acid (chlorogenic acid), neochlorogenic acid, hydroxybenzoic acid, 5-*p*-feruloylquinic acid, protocatechuic acid, 4-caffeoylquinic acid, epicatechin, *o*-coumaric acid, ferulic acid, and *p*-coumaric acid. Neochlorogenic acid was found to be dominant in the early stages of loquat fruit development. Both the concentrations and types of phenolic compounds were high in young fruit but then decreased steadily during growth. However, the concentration of chlorogenic acid increased during ripening and became predominant in ripe fruit. The large rise in chlorogenic acid concentration appears to be a characteristic of loquat fruit ripening. In all of the cultivars tested, the types of phenolic compounds were similar but the total phenolic content varied from 81.8 to 173.8 mg/100 g of fresh pulp. In the biosynthetic pathway of chlorogenic acid, the enzyme activities of phenylalanine ammonia-lyase (PAL), 4-coumarate:CoA ligase (CL), and hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase (CQT) were high at the early stage of growth, diminished to low levels ∼3 weeks prior to harvest, but then rose to a peak at 1 week before harvest. The changes of these enzyme activities seemed to be associated with variations in chlorogenic acid concentration during development, maturation, and ripening of loquat fruit.

Keywords: *Phenolic compounds; loquat fruit; chlorogenic acid; neochlorogenic acid; phenolic metabolism*

INTRODUCTION

Phenolic compounds are widely distributed in plants. They are particularly important in fruits and vegetables, to which they contribute color and flavor. Polyphenols are involved in astringent and bitter tastes, which contribute to "the overall mouthfeel" of fresh fruits (*1*). Moreover, phenolic compounds of fruit may contribute to antioxidant intake, presumed to have a health protective action (*2*). Free radicals and reactive oxygen species may give rise to initiating events in cancer, atherosclerosis, and cataracts in humans (*3*). Dietary antioxidants may help in protecting the body from these reactive species (*4*). Polyphenol consumption as flavonoids has been shown to decrease the risk of heart disease in a cross-cultural epidemiological study (*5*). Several studies concerning phenolic compounds in fruits have been carried out in relation to the technical problems posed in the storage and processing of the fruits (*1*, *6*). Hydroxycinnamic derivatives are particularly interesting because of their relatively high concentration in fruits. Their contribution during food processing, particularly in browning phenomena (enzymatic or nonenzymatic), has been demonstrated by several authors (*7*). Recent research indicated that benzoic and cinnamic acid derivatives have been recognized as potent antioxidants (*8*).

* Address correspondence to this author at the Produce Quality and Safety Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Bldg. 002, BARC-W, Beltsville, MD 20705 [fax (301) 504-5107; e-mail dingc@ba.ars.usda.gov].

† Osaka Prefecture University.

‡ U.S. Department of Agriculture.

Changes in phenolic compounds during fruit maturation have been reported for apple (*9*, *10*), grape (*11*), peach (*12*), pear (*13*), and plum (*14*). However, most researchers have approached the problem by studying changes only in the total phenolic compounds (anthocyanins, tannins, etc.). Evolution of techniques, particularly the progressive introduction of high-performance liquid chromatography (HPLC), has now made it possible to monitor the individual evolution of specific phenolic compounds. Because individual phenolic compounds have been shown to vary in their browning rates (*15*) and the composition of phenolic compounds varies greatly with cultivar, stage of maturity, and postharvest storage conditions (*16*), it is important to know the concentration of individual phenolic compounds in fruit and their changes during maturation and in different cultivars.

Loquat (*Eriobotrya japonica* Lindl.) is widely cultivated in the subtropical regions of southern China, Japan, northern India, Israel, and the Mediterranean. Loquats are round or oval in shape, weighing ∼20−80 g. The fruit has a thin tough skin. Ripe fruit flesh is soft and juicy, varying in color from white to deep orange or salmon (*17*). Loquats are consumed mainly as fresh fruit, which contain nearly all of the essential nutrients, particularly minerals and carotenoids (vitamin A) (*18*). Because loquats have relatively high rates of polyphenols (*19*) and polyphenol oxidase activity (*20*), it is not desirable to use loquat for processing such as for jams, juices, jellies, or canned products. The changes of phenolic compounds in loquat during storage at high CO_2 concentration atmospheres have already been

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investigated (*19*), but no information is available on phenolic metabolism in developing fruit and among cultivars. Knowledge of the activity of enzymes responsible for phenolic biosynthesis is necessary to understand the mechanism of phenolic variation during loquat ripening. Therefore, changes in individual phenolic compounds and the enzymes responsible for their biosynthesis were studied during loquat fruit development.

MATERIALS AND METHODS

Plant Materials. Loquat cultivars Mogi and Tanaka were used for experiments of different maturity stages during 1995−1997. Loquat trees were grown at the farm of the College of Agriculture, Osaka Prefecture University, Osaka, Japan. Recommended herbicide and pesticide programs were followed, and all trees received uniform annual fertilizer application. Loquat fruit grow rapidly ~1 month prior to harvest and were sampled at 7-day intervals during the interval. Thirty loquats showing typical coloration were collected at each sampling point from the outside of five trees. The fruits used for experiments of various cultivars were obtained from Nagasaki Fruit Tree Experiment Station, Japan. Approximately 5 kg of each cultivar was hand-picked from 10 trees at the ripe stage (suitable for fresh consumption). The samples were brought to the laboratory, then peeled, and analyzed immediately. Samples were frozen in liquid N_2 and, if not used immediately, stored at −80 °C.

Extraction and Separation of Phenolic Compounds. Twenty grams of freshly prepared pulp was homogenized in 80 mL of cold methanol (95%) for 1 min, shaken for 10 min, and filtered. The pellet was extracted twice again with cold methanol (80%). The combined extracts were evaporated under vacuum at 35 °C until the MeOH was removed. The concentrate was then extracted with hexane three times to remove lipids, carotenoids, and chlorophyll. The aqueous phase was evaporated again to remove the hexane. Finally, water was added to the extract to constitute a total of 50 mL. Total phenolics were determined with the Folin−Ciocalteu phenol reagent using this extract solution (*21*). For HPLC analysis, a 5-mL aliquot of the extract was adjusted to pH 2.5 with 1 N HCl and passed through a C18 Sep-Pak cartridge (Waters, Milford, MA) to remove sugars (*22*). The absorbed fraction was eluted with methanol from the cartridge, and the first 2 mL of the eluate was collected and analyzed.

The polyphenols were separated by an HPLC, equipped with a Hitachi L-6200 intelligent pump, a Hitachi D-2000 chromatointegrator, and two detectors. A photodiode array UV−vis detector (Shimadzu, SPD-M6A) was used for the characterization of each peak at 280 and 325 nm, and a Hitachi L-4200 UV−vis detector was used for quantification at 280 nm. After a known amount of extract had been injected into a GL Sciences Inertsil ODS-2 (4 × 250 mm) column, solvent A, acetic acid/water (5:95, v/v), and solvent B, acetic acid/acetonitrile/water (5:80:15, v/v) were used to elute the column. During analysis, the solvent gradient was programmed from 0 to 50% B in A for 50 min, at a flow rate of 1.0 mL/min at room temperature (23 °C). Peaks were detected at 280 nm after the injection of 20 μL of the sample. Identification of the phenolic compounds was done by comparing the UV spectra and HPLC retention times with those of authentic standards, and quantification was performed by reporting the measured integration area in the calibration equation of the corresponding standards as described previously (*19*). Cresol served as an internal standard.

Extraction of Crude Enzymes: Phenylalanine Ammonia-lyase (PAL), 4-Coumarate:CoA ligase (CL), and Hydroxycinnamoyl CoA:Quinate Hydroxycinnamoyl Transferase (CQT). Fifty grams of loquat pulp was homogenized at 0 °C in 200 mL of 0.1 M Tris buffer (pH 8.0) containing 0.25 M sucrose, 1 mM ethylenediaminetetracetate (EDTA), 1 mM dithiothreitol (DTT), 5 mM mercaptoethanol, and 5 g of Polyclar CB-100. The homogenate was filtered and centrifuged at 12000*g* for 20 min. The supernatant was made to 85% saturation with $(NH_4)_2SO_4$, and the resultant protein precipitate was obtained by centrifugation at 20000*g* for 30 min. The precipitate was resuspended in 0.1 M Tris buffer (pH 8.0) and desalted. This desalted extraction was used to determine PAL, CL, and CQT activities. Protein concentration in a crude extract was determined according to the method of Bradford (*23*) using bovine serum albumin as a standard.

PAL activity was determined using the method described by Zucker (*24*). The assay medium contained 0.2 mM borate buffer (pH 8.8), 60 μM phenylalanine, and the enzyme extract in a total volume of 6 mL. The mixture was incubated at 40 °C for 30−60 min. The reaction was stopped by adding 0.1 mL of concentrated HCl. PAL activity was determined by measuring the absorbance of the assay mixture at 290 nm for the production of cinnamic acid. An increase in absorbance at 290 nm of 0.01 in 1 h under the described specified conditions was defined as one unit of enzyme activity.

CL activity was determined using the method described by Rhodes and Wooltorton (*25*). A mixture of cinnamic acid (0.5M), phosphate buffer (50 mM, pH 7.5), $MgCl_2$ (100 M), ATP (0.5 M), DTT (1.0 M), and crude enzyme was incubated at 30 °C. CoA (0.2 M) was added to the test cell, and the increase in absorbance at 333 nm was determined for 2 min. One unit of CL activity was defined as the increase in absorbance of 0.001 per minute under the specified conditions described above.

CQT activity was determined using the method described by Rhodes and Wooltorton (*26*). A mixture of chlorogenic acid (0.2 M), phosphate buffer (0.1 M, pH 7.0), and EDTA (1 M) was incubated at 30 °C. CoA (0.2 M) was added to the test cell, and the increase in absorbance at 360 nm was determined for 2 min. One unit of CQT activity was defined as the increase in absorbance of 0.01 per minute under the specified conditions described above.

Determination of Color, Total Soluble Solids, and Acidity. Twenty loquats from each sample were weighed; their skin color was determined using a color difference meter (100 DP, Nippon Denshoku Kogyo Co., Ltd.), and total soluble solids was determined by a refractometer (PIKA Tokyo). A 50-g pulp sample was homogenized with an equal weight of cold distilled water for 1 min and the mixture filtered. The titratable acidity of the filtrate was determined with 0.1 N NaOH, expressed as grams of malic acid per 100 g of fresh weight (FW).

RESULTS AND DISCUSSION

Changes of Phenolic Compounds during Fruit Development. During the rapid development of fruit within 1 month before harvest, the weight of the loquat (Mogi) increased 4-fold while total soluble solids increased from 4.0 to 12.1% (Figure 1). These results were similar to those of the early investigates in cv. Mogi fruit (*27*, *28*). Loquat fruit began to turn yellowish green ($a = 1.3$) at 2 weeks before harvest and became orange when the fruits were ready to be harvested for consumption. Uchino et al. (*29*) had compared the skin color with the fruit quality in Mogi and indicated that the *a* value was an important item for determining the ripeness. Cv. Tanaka loquats followed patterns of changes similar to those of the Mogi fruit but took 1 week longer to mature (Figure 1B). Growth of the loquat fruit can be divided into two phases: the growth phase before the color-change point and the ripening phase from color turnover to harvest, which is characterized by changes in skin color and total soluble solids (Figure 1).

Typical HPLC chromatograms of the Mogi loquat fruit at three typical stages are shown in Figure 2. The HPLC analysis of methanolic extracts from loquats revealed the presence of 3-, 4-, and 5-caffeoylquinic acids and 5-*p*-feruloylquinic acid as well as protocatechuic acid, hydroxybenzoic acid, epicatechin, *o*-coumaric acid, ferulic

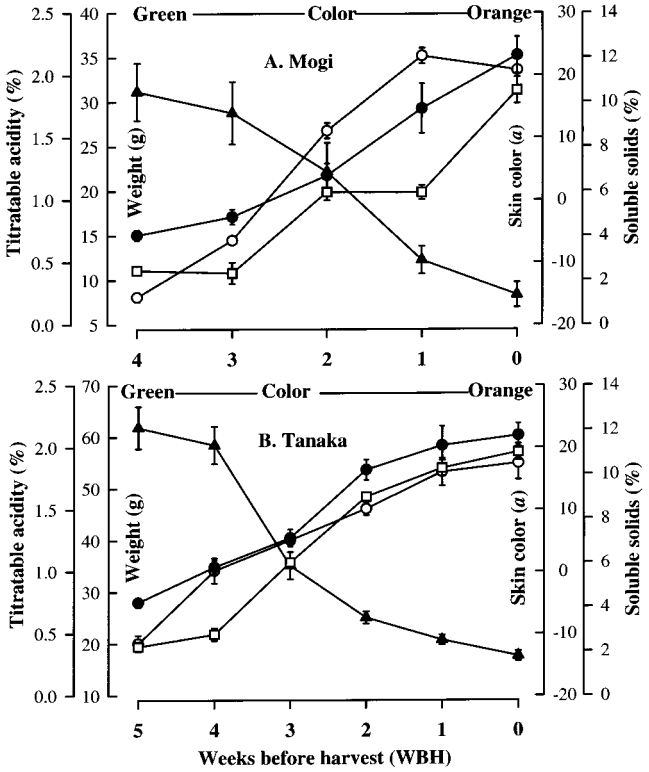

Figure 1. Changes in weight (grams, ○), soluble solids (°Brix, ●), titratable acidity (grams/100 g of FW, ▲), and skin color (*a* value, □) of loquat fruit during development: (A) Mogi; (B) Tanaka. Data of soluble solids and titratable acidity shown are the means (±SD) of three replicates. Data of fruit weight and skin color shown are the means (±SD) of 20 determinations.

acid, and *p*-coumaric acid. In ripe fruit, the most prominent compounds were chlorogenic acid, neochlorogenic acid, hydroxybenzoic acid, and 5-feruloylquinic acid. These phenolics are of interest to food technologists because they contribute to enzymatic browning. The presence of chlorogenic acid, neochlorogenic acid, and hydroxybenzoic acid has also been reported in plum, pear, and apple fruits (*1*). Relatively less information is reported on 5-feruloylquinic acid in fruits.

In young fruit (cv. Mogi), total phenolics were high both in their concentration (352 mg/100 g of FW) (Figure 3) and in the types of compounds (Figure 2). Total phenolics and individual compounds decreased steadily during growth between 4 and 2 weeks prior to harvest. Such decreases have been reported in apple (*30*), peach (*12*), and grapes (*31*). Interestingly, in loquat fruit, total phenolics and chlorogenic acid rose again within the last 2 weeks prior to harvest (Figure 3). During this ripening period, total phenolics and chlorogenic acid per 100 g of fresh pulp in loquat fruits increased 2.2- and 8.2-fold, respectively, while the fruit weight increased from 26.8 to 34.6 g (Figure 1). The concentrations of the other three phenolic compounds varied little during fruit ripening (Figure 3). Thus, the percentage of chlorogenic acid in total phenolics increased significantly from 13.7 to 52.0% during fruit ripening. This result indicated that the increase of chlorogenic acid contributed to the increase of total phenolics during fruit ripening.

The temporal changes of phenolic compounds seen in cv. Tanaka fruit were similar to those in Mogi loquats. The concentration of chlorogenic acid increased during fruit ripening, and it became the predominant phenolic.

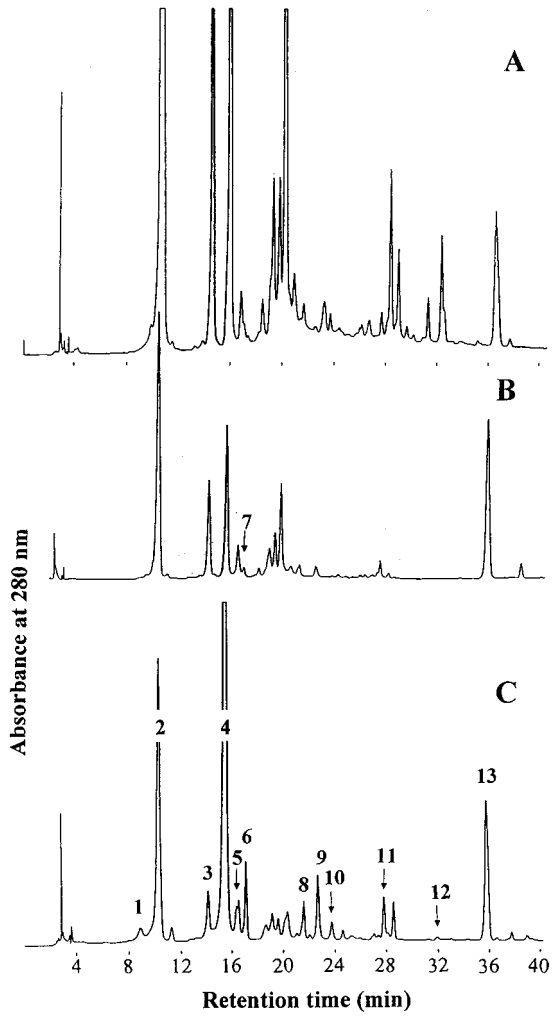

Figure 2. HPLC chromatograms of phenolic compounds from loquat fruit (cv. Mogi) during development, maturation, and ripening: (A) 4 weeks before harvest; (B) 2 weeks before harvest; (C) at harvest. Phenolic compounds from loquat: (peak 1) protocatechuic acid; (peak 2) 3-caffeoylquinic acid (neochlorogenic acid, 3-CQA); (peak 3) hydroxybenzoic acid; (peak 4) 5-caffeoylquinic acid (chlorogenic acid, 5-CQA); (peak 5) 4-caffeoylquinic acid (cryptochlorogenic acid, 4-CQA); (peak 6) unidentified; (peak 7) caffeic acid; (peak 8) epicatechin; (peak 9) 5-feruloylquinic acid (5-FQA); (peak 10) *o*-coumaric acid; (peak 11) ferulic acid; (peak 12) *p*-coumaric acid; (peak 13) internal standard (cresol).

However, the concentrations of chlorogenic acid and total phenolics leveled off in the last week before harvest (Figure 4).

CoSeteng and Lee (*9*) reported that the changes in total phenolics and chlorogenic acid in apple showed a decrease from the early stages of fruit development until harvest. Macheix et al. (*1*) have stated that in many fruit species, the maximum concentration of hydroxycinnamic acid and catechin occurs during early growth. There is then a rapid decrease in concentration during maturation. The increase of chlorogenic acid during fruit ripening has not been reported. Therefore, the large rise in chlorogenic acid concentration appears to be a characteristic of loquat fruit ripening. This phenomenon shows the existence of active phenolic synthesis during loquat ripening, which led us to explore the metabolism of chlorogenic acid during loquat ripening.

Variations of Phenolic Compounds in Different Loquat Cultivars. The individual phenolics of seven

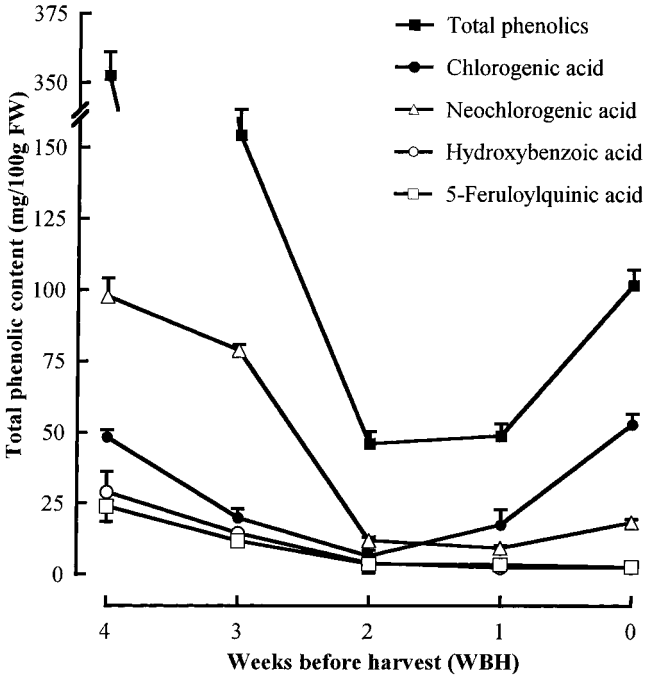

Figure 3. Changes in phenolic compounds of loquat (cv. Mogi) fruit during development. 4 weeks before harvest (WBH), small green fruit; 2 WBH, color turning stage; and 0 WBH, at harvest (ripening stage). Data shown are the means (±SD) of three replicates.

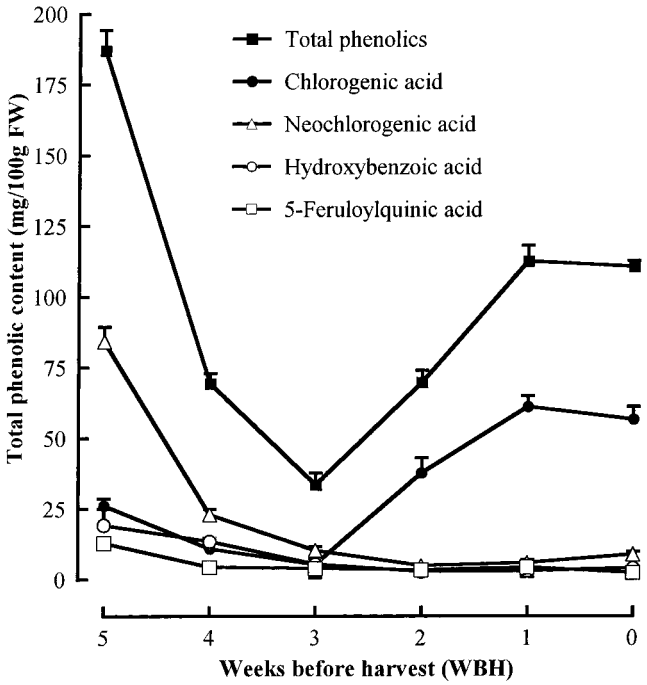

Figure 4. Changes in phenolic compounds of loquat (cv. Tanaka) fruit during development. 5 WBH, small green fruit; 3 WBH, color turning stage; and 0 WBH, at harvest (ripening stage). Data shown are the means (±SD) of three replicates.

loquat cultivars were analyzed by HPLC. The fruit color of cv. Toi and Shiromogi is white, whereas others vary in color from orange to dark orange. The types of phenolic compounds were similar for all seven cultivars, but the total phenolics varied from 81.8 to 173.8 mg/100 g of fresh pulp (Table 1). The cultivars could be divided into two groups according to the total phenolics. Cv. Yukawa and Toi had the highest phenolic content and belonged to the higher level group, whereas the other five cultivars fell into the lower level group of total phenolics.

Variations in the five major phenolic compounds among various cultivars are shown in Table 1. In all of the cultivars tested, the concentration of chlorogenic acid was highest, ranging from 33.1 to 90.7 mg/100 g of pulp, and contributed between 35.3% (Shiromogi) and 54% (Tanaka) to total phenolics. The second highest was neochlorogenic acid, which represented 9.2−16.9% in total phenolics. Cv. Shiromogi had the lowest rate of chlorogenic acid and the highest rate of neochlorogenic acid among all of the cultivars tested.

Changes in the Activity of Enzymes of Phenolic Metabolism during Fruit Growth and Ripening. Chlorogenic acid increased significantly during the ripening of loquat fruit and became a predominant polyphenol in ripe fruit. The possible pathways of chlorogenic acid biosynthesis are shown in Figure 5. PAL (EC 4.3.1.5), a key enzyme of phenylpropanoid biosynthesis, catalyzes the deamination of L-phenylalanine to *trans*-cinnamic acid. This is the first step in the transformation of phenylalanine into the caffeoyl moiety of chlorogenic acid (*24*). Two enzymes, CL (EC 6.2.1.12) and CQT, acting together, catalyze the conversion of *p*-coumaric acid to 5-*p*-coumarylquinic acid and of caffeic acid to chlorogenic acid. The second step that was catalyzed by CQT is reversible (*26*) (Figure 5).

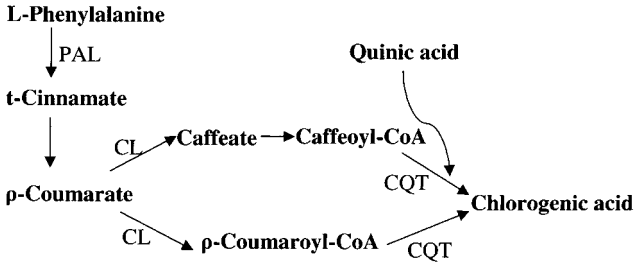

Figure 5. Possible biosynthetic pathways of chlorogenic acid from L-phenylalanine through *trans*-cinnamic acid, *p*-coumaric acid, and *p*-coumaroyl CoA or caffeoyl CoA.

Table 1. Phenolic Compounds (Milligrams per 100 g of Fresh Weight) in Different Varieties of Loquat Fruit at Ripe Stage[a]

cultivar	total phenolics	5-caffeoylquinic acid	3-caffeoylquinic acid	4-caffeoylquinic acid	5-feruloylquinic acid	hydroxybenzoic acid
Yukawa	173.8 ± 12.3	90.70 ± 6.5	20.70 ± 3.1	2.15 ± 0.21	14.52 ± 2.5	7.82 ± 0.8
Toi	158.3 ± 25.2	76.05 ± 8.2	19.21 ± 2.8	3.00 ± 0.24	8.51 ± 1.8	8.15 ± 0.7
Tsukumo	110.0 ± 9.8	52.13 ± 5.8	16.60 ± 3.5	4.33 ± 0.34	2.81 ± 2.1	3.64 ± 0.5
Tanaka	103.4 ± 11.2	55.82 ± 3.5	9.55 ± 3.5	0.81 ± 0.13	5.42 ± 1.7	2.65 ± 0.4
Shiromogi	93.9 ± 8.6	33.11 ± 3.2	15.87 ± 3.1	1.14 ± 0.12	9.25 ± 1.5	4.08 ± 0.5
Mogi	91.1 ± 7.9	42.54 ± 3.5	14.30 ± 2.4	2.00 ± 0.15	7.01 ± 1.3	4.01 ± 0.4
Nagasakiwase	81.8 ± 8.3	32.92 ± 4.2	10.76 ± 2.2	0.52 ± 0.08	4.47 ± 0.7	2.24 ± 0.3

[a] Means ± SD of three determinations.

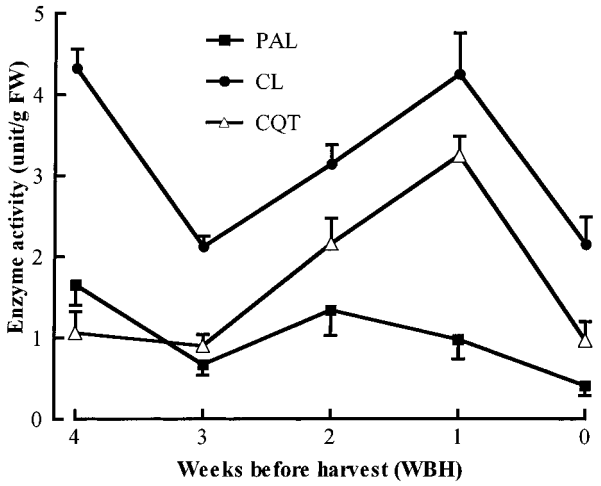

Figure 6. Changes in activities of PAL, CL, and CQT of loquat (cv. Mogi) fruits during growth and ripening. 4 WBH, small green fruit; 2 WBH, color turning stage; and 0 WBH, at harvest (ripening stage). Data shown are the means (±SD) of three replicates.

Figure 6 shows the variations in activities of PAL, CL, and CQT in loquat fruits (cv. Mogi) during growth and ripening. PAL activity was greatest at the early stage of growth, diminished to the lowest activity of 0.67 unit/g of FW ~3 weeks prior to harvest, and then rose to a peak of 1.34 units/g of FW ~2 weeks before harvest. The change pattern of CL activity was similar to that of PAL. During fruit growth, the activity of CL showed a marked decrease from the early stage of growth to ~3 weeks prior to harvest and then increased to a peak of 4.25 units/g of FW at ~1 week before harvest. The enzyme CQT catalyzes the reversible exchange of CoA thioester and quinate groups in the synthesis and breakdown of chlorogenic acid (*26*). The activity of the enzyme has been shown to be stimulated with advanced ripening. The activity was 1.06 units/g of FW at the early stage of growth and then increased significantly to reach a maximum of 3.25 units/g of FW 1 week before harvest. Due to these characteristic increases in enzyme activity, the biosynthesis of chlorogenic acid during loquat ripening may be sustained, and the increase in enzyme activity leads to an accumulation of this phenolic compound. The metabolism of chlorogenic acid may be considered to be a biochemical marker for ripening of loquat fruit.

Application of exogenous abscisic acid (ABA) stimulates accumulation of anthocyanins in grapes without having an effect on the total soluble solids or titratable acidity (*32*). It has been suggested that anthocyanins in grape may be controlled by ABA accumulation. Ding and Zhang (*33*) reported that the concentration of endogenous ABA in loquat fruit increased significantly during ripening. Therefore, it can be postulated that the increases of total phenolics and chlorogenic acid in loquat fruit may be related to ABA accumulation during fruit ripening. There is little information on how ABA stimulates phenolic production in fruit. Walton and Sondheimer (*34*) suggested that ABA might activate enzymes involved in the biosynthesis of phenolic compounds, particularly PAL. The mechanisms that lead to the large increases of chlorogenic acid and total phenolic compounds in loquat during ripening need further study.

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